Universitat de Girona

STRATEGIES FOR THE PRODUCTION OF CATIONIC α-HELICAL ANTIMICROBIAL PEPTIDES IN PLANT BIOFACTORIES

Núria Company Casadevall

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Universitat de Girona

PhD Thesis

Strategies for the production of cationic α-helical antimicrobial peptides in plant biofactories

Núria Company Casadevall

2014

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Strategies for the production of cationic α-helical antimicrobial peptides in plant biofactories

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PhD Program: Technology

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That they have supervised the work carried out by Núria Company Casadevall in the Food Technology Area at the University of Girona entitled *Strategies for the production of cationic α-helical antimicrobial peptides in plant biofactories*, which is submitted as a paper-compendium format in this dissertation to apply for the Doctor degree by the University of Girona. All the requirements to be submitted as a paper-compendium format and to get the International mention are complied.

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Girona, May 2014

Als de casa (tant als que hi són com als que ja no hi són),

i a en David.

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LIST OF ABBREVIATIONS

2DE	Two-dimensional gel electrophoresis
ABA	Abscisic acid
AFP	*Aspergillus giganteus* antifungal protein
AMP	Antimicrobial Peptide
APD2	Antimicrobial Peptide Database
BY-2	Bright Yellow-2
CaMV35S	*Cauliflower mosaic virus* 35S promoter
CecA	Cecropin A
Ct	Cicle threshold
DNA	Deoxyribonucleic acid
DsRed	Discosoma red fluorescent protein
Ea	*Erwinia amylovora*
EFSA	European Food Safety Authority
ELP	Elastin-like polypeptides
EPSPS	5-enolpyruvylshikimate-3-phophate synthase
ER	Endoplasmic reticulum
EU	European Union
FAO	Food and Agricultural Organisation
FDA	US Food and Drug Administration
GFP	Green fluorescent protein
GM	Genetically modified
GMO	Genetically modified organism
GMP	Genetically modified plant
GRAS	Generally recognized as safe
GS	Glutamine synthetase
GUS	*β*-glucuronidase
Hgh	Human growth hormone
HGT	Horizontal gene transfer
hpt	Hygromycin phosphotransferase
Hsp	Heat shock protein
ISAAA	International Service for the Acquisition of Agri-biotech Applications
Kan	Kanamycin
Kb	Kilobases
KDa	KiloDalton
LUC	Luciferase
MIC	Minimum inhibitory concentration
mRNA	Messenger RNA
MW	Molecular weight
nos	Nopaline synthase
npt II	Neomicyin phosphotransferase II
NT-1	*Nicotiana tabacum*-1
ocs	Octopine synthase

OECD	Organisation for Economic Co-ordination and Development
Os	*Oryza sativa*
PB	Protein bodies
PCR	Polymerase chain reaction
pI	Isoelectric point
PMF	Plant Molecular Farming
Pss	*Pseudomonas syringae* pv. *syringae*
PSV	Protein storage vacuole
qPCR	Real-time PCR
Ri	Root-inducing
RNA	Rybonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T-DNA	Transfer DNA
TFB	Transcription binding site
Ti plasmid	Tumor-inducing plasmid
TMV	Tobacco mosaic virus
TSP	Total soluble proteins
TSS	Transcriptional start site
Ub	Ubiquitin
UTR	Untranslated regions
vir	Virulence gene
WHO	United Nations World Health Organisation
Xav	*Xanthomonas axonopodis* pv. *vesicatoria*

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LIST OF PUBLICATIONS

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BMC Plant Biology has an impact factor of 4.354 and it is in the first quartile (Q1) in the “Plant Sciences” category (2012 Journal Citation Reports Science Edition, published by Thompson Reuters).

Company N, Nadal A, La Paz J-L, Martínez S, Rasche S, Schillberg S, Montesinos E, and Pla M (2014a) “The production of recombinant cationic α-helical antimicrobial peptides in plant cells induces the formation of protein bodies derived from the endoplasmic reticulum” Plant Biotechnology Journal **12**, 81–92.

Plant Biotechnology Journal has an impact factor of 6.279 and it is in the first quartile (Q1) in the “Plant Sciences” and “Biotechnology & Applied Microbiology” categories (2012 Journal Citation Reports Science Edition, published by Thompson Reuters).

Company N, Nadal A, Ruiz C, and Pla M (2014b) “Production of phytotoxic cationic α-helical antimicrobial peptides in plant cells by use of inducible promoters” PLOS ONE. Submitted.

PLOS ONE has an impact factor of 3.730 and it is in the first quartile (Q1) in the “Multidisciplinary Sciences” category (2012 Journal Citation Reports Science Edition, published by Thompson Reuters).

SUMMARY

Antimicrobial agents allow controlling many infectious diseases. However, abuse of antimicrobial agents has accelerated the emergence of multidrug-resistant microorganisms. In the phytosanitary field, disease control is also based on preventive application of compounds that are toxic to the environment, e.g. copper derivatives for fire blight in pear and apple trees. Thus, there is an urgent need for novel sustainable antimicrobial agents. Antimicrobial Peptides (AMPs) have attracted attention as a novel class of antimicrobial agents because AMPs efficiently kill a wide range of species, including bacteria, fungi, and viruses, via a novel mechanism of action that makes the appearance of resistance extremely unlikely. This Thesis focuses on production of synthetic AMPs with phytosanitary applications in genetically modified (GM) plant biofactories.

In the last years, new peptides have been designed based of the natural structure of AMPs to optimize their activity against selected pathogens. In particular, researchers at the University of Girona developed a series of cationic amphypatic α-helical undecapeptides based on hybrids of the Cecropin A and Melittin by means of rational design assisted by combinatorial chemistry, among which BP100 (KKLFKKILKYL-NH2) outstands by its high bactericidal activity against three pathogens with phytosanitary interest, biocompatibility and optimal stability.

Specific challenges for production of recombinant BP100 in plants were the possible peptide degradation by plant proteases and its putative toxicity to the host plant. We designed BP100 derivatives (BP100ders) of 15 to 48 amino acids that included the KDEL sequence to accumulate recombinant peptides into the endoplasmic reticulum (ER) of the host plant. This should protect the host plant against the recombinant peptide and at the same time, shelter BP100ders from potential degradation by proteolytic activities *in planta*.

BP100ders were designed on the basis of (i) tandem repeats of the BP100 unit (two or three copies in sense or in antisense orientation, linked by the structurally flexible AGPA sequence), and (ii) BP100 fusion to sequences from natural AMPs such as Melittin, Cecropin A or Magainin, using the same linker. Massive production of BP100ders using a strong constitutive promoter could be toxic to the host plant and, in some cases, was incompatible with its viability. Phytotoxicity was not directly linked to antimicrobial activity but to the haemolytic activity of BP100ders. Therefore, it is possible to design highly antimicrobial BP100ders that can be constitutively produced in plants as recombinant peptides, using the haemolytic activity as a phytotoxicity marker. Among nine BP100ders studied in this Thesis, those encompassing BP100 fused to portions of Magainin and Cecropin A had the best combination of potent antibacterial activity and minimal toxicity to the

host, and could be produced in transgenic plants with yields up to 0.5% total soluble protein. We proved that these recombinant peptides were active against the target microbial species.

BP100 related peptides are difficult to detect and purify due to the extreme physicochemical properties of BP100: strong cationic character (pI = 11), low extinction coefficient and lack of immunogenicity. The DsRed fluorescent tag and the immunogenic tag54 allowed tracing recombinant BP100ders in plants without affecting their activity. Upon transient transformation of *Nicotiana benthamiana* leaves, it accumulated in large vesicles derived from the ER, along with typical ER luminal proteins. Interestingly, the BP100 sequence caused formation of these vesicles. This type of vesicular pattern was also observed in stably GM *Arabidopsis thaliana* and rice seedlings expressing transgenes encoding tagged BP100ders, and could potentially facilitate purification of recombinant proteins from plant tissues.

Certain BP100ders may have activities with high interest as therapeutics or preservatives, but at the same time they may be highly toxic to plant cells upon accumulation at high levels. We approached its production as recombinant peptides in plants by using heat-shock inducible promoters. We showed that recombinant phytotoxic peptides can be produced in GM plants through promoters that have minimal activity during the transformation and regeneration of GM plant process. On the basis of whole-genome transcriptomic data available online, the promoter of *Os.Hsp82* was identified that fulfilled this requirement and was strongly induced in response to heat shock. Using this promoter and the ER retention, transgenic rice events were obtained that produced moderate yields of severely phytotoxic BP100ders upon exposure to high temperature. Moreover, a threshold for gene expression in selected tissues and stages could be experimentally established, above which the corresponding promoters would not allow the survival of plants for production of toxic compounds. In view of the growing transcriptomics data available, this approach can be of interest to assist promoter selection for specific purposes.

RESUM

Els agents antimicrobians permeten controlar moltes malalties infeccioses. No obstant, l'abús dels agents antimicrobians ha fet accelerar l'aparició de microorganismes amb múltiples resistències. En el camp fitosanitari, el control de malalties es basa també en l'aplicació preventiva de compostos que són tòxics per al medi ambient, per exemple, derivats de coure utilitzats pel control del foc bacterià en pereres i pomeres. Per tant, hi ha una necessitat urgent de nous agents antimicrobians sostenibles. Els pèptids antimicrobians (PAMs) han atret l'atenció com una nova classe d'agents antimicrobians perquè són efectius contra una àmplia gamma d'espècies, incloent bacteris, fongs, i virus, a través d'un mecanisme d'acció únic que fa que sigui molt improbable que els microorganismes puguin desenvolupar mecanismes de resistència. Aquesta Tesi se centra en la producció de PAMs sintètics amb aplicacions fitosanitàries en plantes modificades genèticament (MG), utilitzades com a biofactoria.

En els últims anys s'han dissenyat nous pèptids en base a l'estructura natural dels PAMs per optimitzar la seva activitat contra patògens d'interès. En concret, investigadors de la Universitat de Girona han desenvolupat una sèrie d'undecapèptids catiònics amb estructura α-helicoidal amfipàtica, basats en híbrids de Cecropina A i Melitina, mitjançant disseny racional assistit per química combinatòria. El pèptid BP100 (KKLFKKILKYL-NH2) destaca per la seva elevada activitat bactericida contra tres patògens d'interès fitosanitari, a més de ser biocompatible i presentar una estabilitat òptima.

La producció de BP100 recombinant en plantes plantejava uns reptes específics: la possible degradació del pèptid per les proteases vegetals, i la seva possible toxicitat per a la planta hoste. Es van dissenyar derivats del BP100 (BP100ders) de 15 a 48 aminoàcids, que incloïen la seqüència KDEL per acumular els pèptids recombinants al reticle endoplasmàtic (RE) de la planta hoste. Aquesta estratègia hauria de protegir la planta contra el pèptid recombinant i, a la vegada, protegir els BP100ders de la degradació provocada per activitats proteolítiques de la planta.

Els BP100ders van ser dissenyats en base a (i) repeticions en tàndem de la unitat BP100 (dos o tres còpies en orientació sentit o antisentit, unides mitjançant la seqüència flexible AGPA), i (ii) fusió del BP100 amb seqüències de PAMs naturals, com Melitina, Cecropina A o Magainina, utilitzant la mateixa seqüència d'unió. La producció massiva d'alguns BP100ders recombinants sota el control d'un promotor constitutiu fort, va ser, en alguns casos, tòxic per la planta hoste i, fins i tot, incompatible amb la seva viabilitat. Vam observar que la fitotoxicitat no estava directament relacionada amb l'activitat antimicrobiana dels BP100ders, però si que ho estava amb la seva

activitat hemolítica. Per tant, l'activitat hemolítica es pot considerar un marcador per assistir el disseny de BP100ders fortament antimicrobians i que, alhora, puguin produir-se de forma constitutiva en plantes com a pèptids recombinants. Dels nou BP100ders estudiats en aquesta Tesi, els fusionats amb fragments de Magainina i Cecropina A van presentar la combinació més idònia d'activitat antibacteriana i toxicitat per a la planta, i es van poder obtenir plantes MG que els produeixen amb uns rendiments de fins a 0,5% de proteïna soluble total (TSP). També es va poder demostrar que aquests pèptids recombinants són actius contra les espècies microbianes seleccionades.

Els pèptids que contenen el BP100 són difícils de detectar i purificar degut a les propietats fisicoquímiques extremes del BP100: caràcter catiònic fort (pI = 11), coeficient d'extinció baix i immunogenicitat molt baixa. Mitjançant fusió amb la proteïna fluorescent DsRed i la seqüència immunogènica tag54 es van poder detectar els BP100ders recombinants, sense comprometre la seva activitat antimicrobiana. Els BP100ders recombinants sintetitzats en fulles de *Nicotiana benthamiana* agroinfiltrades, s'acumulaven en grans vesícules derivades del RE, juntament amb altres proteïnes pròpies del RE. Va ser interessant comprovar que l'undecapèptid BP100 és responsable de la formació d'aquestes vesícules. Aquest tipus de patró vesicular també es va observar en plantes MG d'*Arabidopsis thaliana* i arròs que expressen transgens que codifiquen BP100ders. La formació de vesícules podria facilitar la purificació de les proteïnes recombinants a partir de teixits de plantes.

Alguns BP100ders podrien tenir activitats de gran interès com a agents terapèutics o conservants, però al mateix temps, ser altament fitotòxics per a les cèl·lules hoste quan s'hi acumulen en elevades concentracions. Per això es va dissenyar una estratègia d'expressió alternativa, basada en l'ús de promotors induïbles per elevades temperatures. Es va demostrar que els pèptids fitotòxics recombinants poden ser produïts en plantes MG si els transgens que els codifiquen estan regulats per promotors que tenen una activitat mínima durant el procés de transformació i regeneració d'una planta transgènica. Mitjançant les bases de dades de transcriptoma d'arròs disponibles a la xarxa, es va poder identificar el promotor del gen *Os.Hsp82*, que compleix aquest requisit i és induït en resposta a xoc tèrmic. Aquest promotor va permetre obtenir plantes transgèniques d'arròs que produeixen, en resposta a alta temperatura, BP100ders altament fitotòxics amb rendiments moderats. A més, es va establir experimentalment un llindar per a l'expressió gènica en teixits i etapes del procés de transformació, que permet preveure si un determinat promotor serà apte o no per a dirigir l'expressió de transgens que codifiquen compostos tòxics per a la planta biofactoria. L'augment de dades transcriptòmiques disponibles a la xarxa permet considerar aquesta aproximació d'interès per a la selecció de promotors per a finalitats específiques.

RESUMEN

Los agentes antimicrobianos permiten controlar muchas enfermedades infecciosas. Sin embargo, el abuso de agentes antimicrobianos ha acelerado la aparición de microorganismos con múltiples resistencias. En el campo fitosanitario, el control de enfermedades se basa también en la aplicación preventiva de compuestos que son tóxicos para el medio ambiente, por ejemplo, derivados de cobre utilizados para el control del fuego bacteriano en perales y manzanos. Por lo tanto, existe una necesidad urgente de nuevos agentes antimicrobianos sostenibles. Los péptidos antimicrobianos (PAMs) han atraído la atención como una nueva clase de agentes antimicrobianos por ser efectivos frente a una amplia gama de especies, incluyendo bacterias, hongos, y virus, a través de un mecanismo de acción único que hace muy improbable el desarrollo de mecanismos de resistencia. Esta Tesis se centra en la producción de PAMs sintéticos con aplicaciones fitosanitarias en plantas modificadas genéticamente (MG), utilizadas como biofactoría.

En los últimos años se han diseñado nuevos péptidos en base a la estructura natural de los PAMs para optimizar su actividad contra patógenos de interés. En particular, investigadores de la Universitat de Girona desarrollaron una serie de undecapéptidos catiónicos con estrucutra α-helicoidal anfipática, basados en un híbrido de la Cecropina A y Melitina, mediante diseño racional asistido por química combinatoria. El péptido BP100 (KKLFKKILKYL-NH2) destaca por su elevada actividad bactericida contra tres patógenos de interés fitosanitario, además de ser biocompatible y presentar una estabilidad óptima.

La producción de BP100 recombinante en plantas planteaba unos retos específicos: la posible degradación del péptido por proteasas vegetales, y su posible toxicidad para la planta huésped. Se diseñaron derivados del BP100 (BP100ders) de 15 a 48 aminoácidos, que incluyen la secuencia KDEL para acumular los péptidos recombinantes en el retículo endoplasmático (RE) de la planta huésped. Ello debería proteger a la planta contra el péptido recombinante y, al mismo tiempo, proteger al BP100der recombinante frente a la actividad proteolítica de la planta.

Se diseñaron BP100ders en base a (i) repeticiones en tándem de la unidad BP100 (dos o tres copias en orientación sentido o antisentido, unidas por la secuencia flexible AGPA), y (ii) fusión del BP100 con secuencias de PAMs naturales tales como Melitina, Cecropina A o Magainina, utilizando la misma secuencia de unión. La producción masiva de BP100ders recombinantes bajo el control de un promotor constitutivo resultó, para algunos BP100ders, tóxico para la planta huésped, llegando incluso a ser incompatible con la viabilidad de la misma. Observamos que la fitotoxicidad no está directamente relacionada con la actividad antimicrobiana de los BP100ders pero sí con su actividad hemolítica. Por lo tanto, la actividad hemolítica puede considerarse un marcador adecuado para

asistir el diseño de BP100ders fuertemente antimicrobianos y que, a su vez, puedan producirse de forma constitutiva en plantas como péptidos recombinantes. De los nueve BP100ders estudiados en esta Tesis, aquellos fusionados con fragmentos de Magainina y Cecropina A presentaron la combinación más idónea de actividad antibacteriana y toxicidad para la planta, y se pudieron obtener plantas MG que los producen con rendimientos de hasta 0,5% de proteína soluble total (TSP). Se demostró asimismo que los péptidos recombinantes obtenidos son activos contra las especies microbianas seleccionadas.

Los péptidos que contienen BP100 son difíciles de detectar y purificar debido a las propiedades físico-químicas extremas del BP100: fuerte carácter catiónico (pI = 11), bajo coeficiente de extinción y la falta de inmunogenicidad. Mediante fusión con la proteína fluorescente DsRed y la secuencia inmunogénica tag54 pudieron detectarse los BP100ders recombinantes, sin que ello afectara su actividad antimicrobiana. Los BP100ders recombinantes sintetizados en hojas de *Nicotiana benthamiana* agroinfiltradas, acumularon en grandes vesículas derivadas del RE, junto a otras proteínas típicas del RE. Resultó interesante comprobar que el undecapéptido BP100 es responsable de la formación de estas vesículas. Este tipo de patrón vesicular también se observó en plantas MG de *Arabidopsis thaliana* y arroz que expresan transgenes que codifican BP100ders. La formación de vesículas podría facilitar la purificación de las proteínas recombinantes a partir de tejidos de plantas.

Algunos BP100ders podrían tener actividades de gran interés como agentes terapéuticos o conservantes, pero al mismo tiempo ser altamente fitotóxicos para la célula huésped, cuando se acumulan a elevadas concentraciones. Por ello se diseñó una estrategia de expresión alternativa, basada en el uso de promotores inducibles por elevadas temperaturas. Se demostró que los péptidos fitotóxicos recombinantes pueden ser producidos en plantas MG si los transgenes que los codifican están regulados por promotores que tienen una actividad mínima durante el proceso de transformación y regeneración de una planta transgénica. A través de las bases de datos del trascriptoma de arroz disponibles en la red, fue posible identificar el promotor del gen *Os.Hsp82* que cumplía con este requisito y se inducía en respuesta a choque térmico. Este promotor permitió obtener plantas transgénicas de arroz que producen, en respuesta a elevadas temperaturas, BP100ders altamente fitotóxicos con rendimientos moderados. Además, se estableció experimentalmente un umbral de expresión génica en una selección de tejidos y estadios del desarrollo, que permiten prever si un determinado promotor será apto o no para dirigir la expresión de transgenes que codifican compuestos tóxicos para la planta biofactoría. El aumento de datos transcriptómicos disponibles en la red permite considerar esta aproximación de interés en la selección de promotores con fines específicos.

CHAPTER I

GENERAL INTRODUCTION

1. Antimicrobial peptides

Microorganisms are the most abundant life forms in the planet and they play important roles in the biosphere, including inorganic matter fixation and organic matter decomposition and mineralization. However, some microorganisms are potential pathogens for many eukaryotic organisms and are capable of causing harmful diseases. In order to overcome infections, living organisms have developed host-defense mechanisms, which have different levels of interacting systems. In plants, these include preexisting factors e.g. structural barriers, antimicrobial and inhibiting compounds, recognition factors and receptors; and induced biochemical defense reactions such as hypersensitivity. During the initial stages of infection, the nonspecific innate immune system is started. In the past few years, a wide range of molecules have been described to be involved in this response, such as Antimicrobial Peptides (AMPs), sometimes referred as host defense peptides (HDPs) (Hancock and Diamond, 2000; Boman, 2003; Diamond et al., 2009). These peptides usually display a broad activity as they act on Gram negative and Gram positive bacteria, fungi, insects, metazoans and other parasites, viruses and even cancer cells (Zasloff, 2002; Hoskin and Ramamoorthy, 2008) and they may be constitutively expressed or induced in response to exposure to foreign microorganisms or cells.

AMPs are peptide sequences typically having 12-50 amino acids, molecular mass below 10kDa and often sharing a cationic nature, given by the abundance of cationic amino acids, especially lysine and arginine, which confer an overall positive net charge at physiological pH. Only a small number of anionic peptides exist and mostly are isolated from mammals (Sarika et al., 2012). Moreover, the AMPs usually have up to 50% hydrophobic amino acids (isoleucine, leucine, valine, phenylalanine and tryptophan), contributing to the common amphipatic conformation that they tend to assume at the lipid membrane interface upon interaction with the target cell (Papo and Shai, 2003; Yeaman and Yount, 2003; Yount et al., 2006).

Over 2000 AMPs have been described to date (2343 are listed in the "Antimicrobial Peptide Database [APD2]" [Wang et al., 2009], http://aps.unmc.edu/AP/main.php, 25th of February, 2014) in a great variety of organisms such as bacteria (Jack and Jung, 2000; Cooter et al., 2010) fungi (Degenkolb et al., 2003; Ng, 2004), insects (Hancock, 2001; Bulet and Stocklin, 2005) amphibian, mammals (Zasloff, 2002; Tincu and Taylor, 2004; Toke, 2005) and plants (Lay and Anderson, 2005). Animals other than humans gather most known AMPs (74.5%), followed by plants (13.6%) and humans (3.1%). In contrast to the large amount of data currently available, only 500 AMPs had been described in 2003. This progress reflects the increasing interest raised by AMPs and has led to the development of AMPs databases accessible online, including the above-mentioned APD2, AMSdb

(Tossi and Sandri, 2002), Peptaibol (Whitmore and Wallace, 2004), CAMP (Thomas et al., 2010), ANTIMIC (Brahmachary et al., 2004), Penbase (Gueguen et al., 2006), AMPer (Fjell et al., 2007), etc. (for more details, see [Sarika et al., 2012]). Several thousand AMPs have also been *de novo* designed and synthetically produced. They are gathered in specific databases such as SAPD (Wade and Englund, 2002).

The diversity of AMPs makes its classification difficult. AMPs can be categorized according to their source, biosynthetic pathway, structural elements or predominant amino acid residues. One of the better accepted classifications is based on the secondary structure and distinguishes four groups (Epand and Vogel, 1999; van 't Hof et al., 2001):

i) **Linear α-helical peptides:** Most known AMPs belong to this class. They tend to be highly flexible in aqueous solution whereas at least part of the peptide sequence folds into an amphiphatic α-helical conformation upon interaction with membranes and membrane-mimicking environments. The Cecropin family of AMPs is among the better studied peptides in the group. Cecropins A, B and D are 35-40 amino acids long and share close homology. They were initially isolated from the pupae of the cecropia moth. The Magainin family, comprising 23-residue α-helical membrane-active peptides that are expressed in the skin and intestine of frogs, have been well-characterized as well. Melittin is a 26-residue peptide found in bee venom that exhibits potent and broad-spectrum antibacterial activity and high haemolytic activity. Several *de novo* designed peptides belong as well to this group.

ii) **Peptides with β-sheet structure:** β-sheet antimicrobial peptides fold in a well-defined number of β-strands organized in the common amphipathic pattern, with relatively few or no helical domains. Most peptides are constrained either by disulfide bonds or by cyclization of the peptide backbone. Cysteine-containing β-sheet peptides are a highly diverse group of molecules, mainly represented by defensins.

iii) **Linear peptides with an extended structure with predominance of one or more amino acids:** This type of peptides is defined by predominance of particular amino acid(s) that impose specific constraints to their structure (Yeaman and Yount, 2003). Some examples are indolicin, with many tryptophan residues (Falla et al., 1996), and histatins with histidine (Broekaert et al., 1995).

iv) **Peptides with loop structures**: They display a looped structure, such as lantibiotics and peptides with a single disulfide bridge. Lantibiotics are cyclic peptides formed by thioether bridges due to posttranslational dehydratation of serine or threonine side chains, followed

by reaction with cysteine resulting in formation of the unusual amino acids lanthionine and methyllanthionine, respectively. Nisin and mersacidin are examples of lantibiotic peptides. Examples of peptides formed by a disulfide bridge are thanatin, lactoferricin B and bactenecin-1.

1.1. Mechanisms of action of antimicrobial peptides

Many AMPs have no fixed structure in aqueous environments but adopt amphipathic structures in membranous surroundings. They most commonly adopt α-helix or β-sheet secondary structure (Boman HG, 1995), although they can also be found in more extended structures, as stated above. These features give AMPs the possibility to interact with negatively charged surfaces such as those of many viral, bacterial or fungal membranes by electrostatic interactions (van der Biezen, 2001). Several mechanisms of action of AMPs have been proposed, and they include membrane permeabilization through formation of stable or transitory pores. This only takes place above a concentration threshold that is characteristic for each peptide and membrane composition (Huang, 2000; Melo et al., 2009). Target membrane disruption can involve different mechanisms in different peptide types. Models such as the barrel-stave, toroidal pore and carpet (Figure 1) have received most attention, although other models like molecular electroporation (Miteva et al., 1999) or sinking raft (Pokorny and Almeida, 2004; Pokorny and Almeida, 2005) may prove to be important as well. Upon peptide accession into the intracellular space, cell death is promoted by a sequential leakage of ions and other metabolites, loss of cytoplasmic components and dissipation of electrochemical potentials (Brogden, 2005). On the other hand, it has also been shown that membrane permeabilization is not the only mechanism of microbial eradication. Several studies suggest that internalized peptides can interfere with DNA synthesis or certain metabolic pathways by inhibiting the synthesis of nucleic acids, proteins, and cell-wall components or essential enzymatic activities (Nguyen et al., 2011). Nevertheless, the exact mode of action of these peptides is poorly understood.

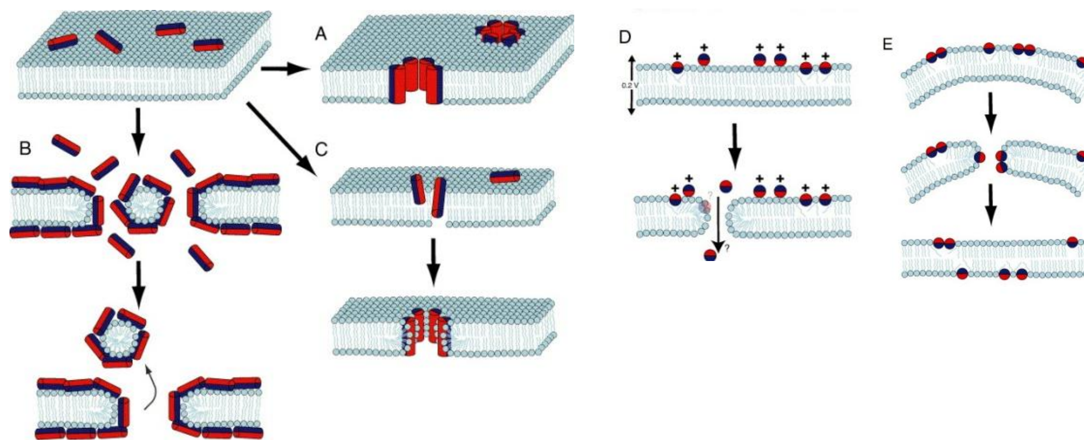

Figure 1. Antimicrobial peptide mechanisms of action. In this figure, various models are shown, illustrating the advances made in proposed mechanisms of antimicrobial peptide action. The red part of the peptide represents a hydrophilic surface, while blue is hydrophobic. A, B, and C all start from the same conformation, with the peptides associating with the bacterial membrane (top left). (A) In the barrel-stave model, the peptides span the membrane and form a pore with the hydrophilic portion lining the pore. (B) The carpet model is characterized by the spanning of the membrane by the peptide followed by a detergent-like action that disrupts the membrane structure. (C) The toroidal model differs from the barrel–stave mechanism in that the hydrophilic portion of the peptide (in its amphipathic conformation) is associated with the lipid headgroup. (D) In the molecular electroporation model, the interaction of the cationic peptide with the pathogen membrane promotes an electrical potential difference across the membrane. When this potential reaches about 0.2 V, a pore is believed to be created by molecular electroporation. (E) The sinking raft mechanism proposes a mass imbalance between the two leaflets of the membrane induced by the peptide. By creating a curvature gradient along the membrane and by self-association, peptides sink into the membrane and form transient pores that are thought to promote a transitory increase on membrane's permeability and leakage of intracellular contents. After membrane relaxation, peptides will reside on both leaflets of the membrane. (Adapted from [Chan et al., 2006; Teixeira et al., 2012]).

1.2. Applications

AMPs are highly biocompatible, moderately biodegradable, exhibit a rapid antimicrobial effect, often within minutes *in vitro*, and can have potent activity against a large spectrum of microorganisms and tumor cells. In addition, development of resistance by target microorganisms is unlikely. For these reasons, AMPs are particularly attractive to different industrial sectors including therapeutics, biopreservatives in food, cosmetics and biomaterials, and as antifoulings (Scholz et al., 2006).

AMPs are gaining attention in the pharmaceutical industry as substitutes to antibiotics, which have serious problems due to the appearance of resistant bacterial strains. Even if the haemolytic / cytotoxic character of some AMPs at high concentrations has to be taken into account, examples of AMP medical applications exist. Magainin, Dermaseptin and other AMPs isolated from the skin of frogs have been applied in skin treatments (Mangoni, 2006). Analogs of Cecropin A proved to be effective against eye infections (Mannis, 2002); and certain AMPs are considered as promising anticancer therapeutics. Lee SJ and colleagues (Lee et al., 2011c) demonstrated that AMPs can lyse

liposomes and induce apoptosis *in vitro*, and significantly reduce tumor size in mouse studies carried out *in vivo*.

AMPs are also used as alternatives to chemical food preservatives. There is an increased demand for natural food preservatives, which has motivated food scientists to investigate the effectiveness of inhibitory compounds such as organic acids, essential oils, bacteriocins or plant extracts. As a promising example, the *Staphylococcus hyicus* 2.1 KDa bioactive peptide Hyicin 3682 can efficiently inhibit growth of pathogenic bacterial species such as *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus* in pig milk (Cotter et al., 2005; Fagundes et al., 2011) and has been used to prevent the proliferations of these undesirable bacteria in non-processed food.

Plant disease control can benefit as well from AMPs. A potential market has been postulated for AMPs as novel plant protective and therapeutic agents. Traditional crop protection against phytopathogens is based on highly effective chemical pesticides (Agrios, 2005). However, their use has a negative impact on the environment due to accumulation in soils and water, affecting as well consumer's health (Margni et al., 2002; Karabelas et al., 2009); and resistance in some plant pathogens has been found (Sundin and Bender, 1993). Consequently, new regulations have strongly limited the use of a number of pesticides in several countries, which difficults plant disease management due to the lack of sustainable and effective compounds. Therefore, research in the plant protection field has been progressively reoriented towards the rational use of pesticides and the development of improved compounds with minimal harmful effects against the host plant, the environment and public health. In this frame, AMPs can play an important role as new phytosanitary tools (Moreno et al., 2006; Marcos et al., 2008). Many studies report the activity of different AMPs against a range of plant pathogens. As an example, inoculation of mustard Defensin in tobacco and peanut plants resulted in resistance against fungal infection (Swathi Anuradha et al., 2008). Ferré and co-workers (Ferré et al., 2006) evaluated synthetic short peptides of 11 residues against the economically important plant pathogenic bacteria *Erwinia amylovora (Ea),* the deceased of the fireblight disease, which mainly affects pears, apples and ornamental plants of the Rosacea family, *Pseudomonas syringae (Ps),* capable of attacking a great variety of vegetable species and *Xanthomonas vesicatoria (Xv)* that causes bacterial leaf spot on peppers and tomatoes.

1.3. Improvement of AMPs

Although natural AMPs can have interesting activities, they often are poorly selective, which entails certain toxicity against non-target organisms. Moreover, they tend to display low stability due to

rapid proteolytic cleavage; and sometimes they show relatively high minimum inhibitory concentration (MIC), thus high dosages are needed (Liu et al., 2010). In the last decade, novel AMPs have been rationally designed with improved properties in terms of increased specificity, reduced toxicity against nontarget cells and better stability. This has been achieved through different strategies, including AMPs congeners, AMPs mimetics, cyclotide AMPs, AMPs conjugates and hybrid AMPs (Brogden and Brogden, 2011). Regardless of the basic strategy, small modifications in the peptide length, structural flexibility and charge can have a strong impact on its properties.

i) **AMPs congeners**. A congener is a chemical compound wich composition is closely related to another, and exerts either similar or antagonistic effects. Congener AMPs are designed through modification of natural peptides by amino acid addition, deletion or replacement. Numerous congeners of defensins, CAP18, human CAP18/LL-37, SMAP29 and enterocin have been described with interesting properties (Larrick et al., 1996; Travis et al., 2000; Ciornei et al., 2005; Xiong et al., 2006; Saravanan et al., 2010; Dawson and Liu, 2011). Similarly, congeners of Cecropin B such as SB-37 and Shiva-1 showed reduced toxicity against plant protoplasts while maintaining the amphipathic properties of the parental molecule and potent activity against specific plant pathogenic bacteria (Nordeen et al., 1992; Jaynes et al., 1993). D4E1, also based on Cecropin B, proved to be effective to confer transgenic tobacco (Cary et al., 2000) and cotton (Rajasekaran et al., 2005) broad-spectrum control of fungal and bacterial pytopathogens. Similarly, the Magainin 2 analog MSI-99 has been expressed as recombinant protein in different plant species including tobacco (*N. tabacum*), grapevine (*V. vinifera*), banana (*Musa* sp.) and tomato (*L. esculentum*), conferring protection against distinct microbal pathogens (DeGray et al., 2001; Chakrabarti et al., 2003; Alan et al., 2004; Vidal et al., 2006).

ii) **Mimetic AMPs** are synthetic, non-peptidic molecules that mimic the properties and activities of naturally occurring AMPs. They are constructed from peptoids, β-peptides, arylamides, oligomers or phenylene ethynylenes. These molecules are designed to capture the central physicochemical features of their natural AMP archetypes thereby mimicking peptide activity and function (Lienkamp and Tew, 2009; Rotem and Mor, 2009). The use of mimetic AMPs overcomes some physical limitations of natural peptides.

iii) **Cyclotides** are cyclopeptides with a head-to-tail cyclic backbone. Such peptide modification can have a dual effect, i.e. increased stability and selectivity against target microorganisms due to conformational constraints that facilitate the amphipathic spatial organization of residues within the molecule. This approach was initially applied to cationic

tryptophan-rich peptides (Rozek et al., 2003; Dathe et al., 2004), but it has been successfully extended to other types of peptides (Monroc et al., 2006a; Monroc et al., 2006b).

iv) **AMPs conjugates**. In a few examples, AMP sequences have been coupled to certain ligands to narrow down their activity spectrum and/or improve their MIC. Peschen and co-workers (Peschen et al., 2004) generated a fusion protein comprising a *Fusarium*-specific antibody linked to the AFP antifungal peptide and proved that expression of this recombinant molecule resulted in better protection of host plants against a *Fusarium oxysporum* f. sp. *matthiolae* infection. AMPs have also been successfully conjugated to micelles and liposomes equipped with uptake-mediating mechanisms (Leupold et al., 2009); and with fatty acids or steroids achieving increased antimicrobial activity (Ding et al., 2004; Chu-Kung et al., 2010).

v) **Hybrid AMP**s are constructed by combining the active regions of two to three naturally occurring peptides with the aim of capturing the potential benefits of each individual fragment. Cecropin A-Magainin and Cecropin-Melittin hybrids have been designed that retain high antibacterial activities while reducing the haemolytic activity, which is especially potent in Melittin (Lee et al., 2004; Marcos et al., 2008). The BioPeptide 100 (BP100) (Montesinos and Bardaji, 2008) and its derivatives (this Thesis [Nadal et al., 2012; Company et al., 2014]; [Badosa et al., 2013]) are successful examples of hybrid peptides.

The natural AMPs Cecropins were firstly isolated from the hemolymph of the giant silk moth *Hyalophora cecropia* (Hultmark et al., 1980; Steiner et al., 1981; Hultmark et al., 1982). They are typically 31-39 amino acids in length and display powerful antibacterial activity against virtually all Gram negative bacteria and some Gram positive bacteria. They do not have significant cytotoxic effects against human erythrocytes and other eukaryotic cells. However, their use as phytosanitary agents is impaired by their low stablility in plant extracts due to their susceptibility to protease degradation (Steiner et al., 1981; Hultmark et al., 1982; Andreu et al., 1983; Mills et al., 1994). On the other hand, the natural AMP Melittin, found in the venom of the honey bee *Apis mellifera*, is a 26 amino acids peptide that displays a powerful and broad-spectrum antimicrobial activity, and a high toxicity against eukaryotic cells (Boman et al., 1989; Andreu et al., 1992; Raghuraman and Chattopadhyay, 2007). In an effort to improve their properties, a number of short Cecropin-Melittin hibrid peptides were designed on the basis of their α-helical active domains. Upon chemical synthesis, some were found to display similar antimicrobial activities as the parental peptides, haemolytic activity below that of Melittin, and decreased susceptibility to protease degradation. Examples are the peptides CEMA and its derivative MsrA1 (Piers et al., 1994; Piers and Hancock,

1994; Osusky et al., 2000); CAMEL, with activity against different species of *Pectobacterium* (Kamysz et al., 2005); Pep1, active against distinct phytopathogenic fungi (Cavallarín et al., 1998); and Pep3, the WKLFKKILKVL-NH_2 undecapeptide derived from CAMEL, that exhibits an interesting profile of antifungal and antibacterial activities against plant pathogens, displaying aslo low cytotoxicity (Cavallarín et al., 1998; Ali and Reddy, 2000). Based on the ideal α-helical wheel diagram of Pep3, the LIPPSO and the PV groups at the University of Girona designed and chemically synthesized 22 undecapeptides derived from the hybrid Pep3. Upon experimental evaluation against the plant pathogenic bacteria *Erwinia amylovora (Ea)*, *Xanthomonas axonopodis* pv. *vesicatoria (Xav)* and *Pseudomonas syringae* pv. *syringae (Pss)* (Ferré et al., 2006), BP76 was selected to further improve its antibacterial activity, cytotoxicity and susceptibility to protease degradation. A new 125-member linear undecapeptide library (CECMEL11) was designed using a combinatorial chemistry approach by incorporating amino acids possessing various degrees of hydrophobicity and hydrophilicity at positions 1 and 10 and varying the N-terminus (Badosa et al., 2007). The antibacterial activity, antifungal activity, haemolytic activity and proteolytic susceptibility of syntetic CECMEL11 peptides were *in vitro* evaluated and up to 7 BP76 analogues were selected on the basis of the balance of the analysed characteristics. Additional assays were subsequently carried out *ex vivo* to test their activity against *Ea* in detached apple and pear flowers. The peptide H-KKLFKKILKYL-NH2 (BP100) combined 63-76% inhibition of growth of the pathogenic bacteria at 100 μM (i.e. more potent than BP76 and similar as the streptomycin antibiotic currently used to control fire blight) (Badosa et al., 2007). Whole plant tests were carried out in pear infected with *Ea* and *Pss*, and in pepper exposed to *Xav*. BP100 proved capable to control fire blight in pear and bacterial blight in pear and pepper, albeit to varying degrees (Güell et al., 2011). BP100 is also highly biocompatible as assessed by acute oral toxicity tests in mice (Montesinos and Bardaji, 2008). Thus, BP100 is an excellent candidate phytosanitary tool, which might be envisaged as a substitute for the currently used antibiotics and toxic chemicals as copper (http://ec.europa.eu/sanco_pesticides).

2. Genetically modified plants and their applications

According to the European Union (EU) (Directive, 2001), genetically modified organisms (GMOs) are those (with the exception of human beings) which genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. Only organisms obtained by recombinant nucleic acid techniques, methods for direct introduction of DNA into a cell such as micro-injection, macro-injection and micro-encapsulation, and cell or protoplast fusion techniques (where the fusion takes place by means of methods that do not occur naturally) are considered below this definition. In contrast, *in vitro* fertilization techniques and natural processes such as conjugation, transduction, transformation and polyploidy induction are specially excluded from the definition. Even mutagenesis and cell (or protoplast) fusions of organisms which exchange genetic material through traditional breeding methods are exempt from GMOs legislation.

Since the first genetically modified plants (GMPs) were produced in the early 1980s by two different research teams (Herrera-Estrella et al., 1983; Barton et al., 1983) and the first GM plant was commercialized in 1995, a large variety of genes have been introduced into plants with different purposes. Recently, not only sequences from species other than the host have been introduced into GM plants (transgenic plants) but also sequences from the same species (cisgenic plants). In this section, different applications of GMPs are presented.

2.1. GMPs and research

Plant transformation has allowed huge advances in plant basic research. GMPs are unvaluable tools to assist understanding the functions of certain genes (e.g. those related to metabolism and certain biological processes) and their relationship with physiological characters; unraveling the mechanisms of response to exogenous stimuli; or e.g. characterizing promoter activity. Some examples, in which different transformation technologies have been applied, are briefly outlined below.

Insertion mutagenesis was initially used in *Arabidospis thaliana* (Szabados et al., 2002) to generate a series of mutants derived from random insertion of exogenous DNA into the host plant genome through transposable elements or T-DNA. On the basis of the known inserted element, the sequence of the disrupted genes (flanking the exogenous DNA) has been assessed and introduced into specific databases. This tool facilitates association of a particular sequence with a specific

phenotype. More recently, the approach has been extended to other plant species such as rice (Sallaud et al., 2004) and tomato (Gidoni et al., 2003).

Genetic engineering tools have been used to modify the expression of a particular gene in order to investigate its function and the role of specific molecules. Specially, technologies such as *knock-down* or gene silencing have been used to reduce specific mRNA levels. Different tools have been developed to that end, including cosuppression (Napoli et al., 1990), antisense mediated gene silencing (Bourque, 1995), virus-induced gene silencing (VIGS) (Fire et al., 1998) or interference RNA (iRNA) (Baulcombe, 2000). Double stranded RNA (dsRNA) structures are generated (Fire et al., 1998), which are cleaved by an enzyme known as DICER (Bernstein et al., 2001) in 21–23 nucleotides fragments of small interfering RNAs (siRNAs). Then, one strand of a siRNA (or microRNA, miRNA) is integrated into an active RNA-induced silencing complex (RISC) and guided to the homologous target messenger RNA (mRNA), activating RNAse and knocking down the expression of the gene. The siRNA methodology proved to be a powerful tool to reduce gene expression in various plant species (Nunes et al., 2006). However, Xu and co-workers (Xu et al., 2006a) demonstrated silencing occurred as well in some non-specific target genes. More recently, miRNA has been used as a more specific gene silencing tool, because its activity depends not only on the sequence but also on the secondary structure of RNA. Examples of the miRNA silencing approach have been reported in *Arabidopsis* (Alvarez et al., 2006; Schwab et al., 2006), tobacco (Alvarez et al., 2006), tomato (Alvarez et al., 2006) and rice (Warthmann et al., 2008).

Both stable and transient transformations are potent tools for promoter characterization. Promoters are DNA regions upstream a gene's coding region that contain specific sequences recognized by proteins involved in the initiation of transcription (Buchanan et al., 2002). Thus, promoter activity is directly linked to the expression pattern of a given gene, which is in turn linked to the function of the corresponding protein. Well-characterized promoters with activity patterns of interest can be used to regulate the expression of transgenes in heterologous systems. In consequence, the activity of numerous promoters has been studied both in the context of their original genome and in different plant species. Most of these studies use transgenic plants in which a reporter transgene is placed under the control of the promoter of interest. In a recent example the maize Wip1 promoter, and a number of proximal fragments, were placed upstream the sequence encoding the GUS reporter and used to generate transgenic *Arabidopsis*, tobacco and rice plants (Zhang et al., 2013). The Wip1 promoter displayed different activity in dicotiledoneous and monocotiledoneous plants; and some sequence *cis*-acting elements, responsible for the specific expression pattern, were identified on the basis of the activity of truncated promoters. Recently, 3 heat-inducible promoters (OsHsfB2cp, PM19p, and Hsp90p) were used to drive *gus* gene expression

in rice. Combination of *gus* mRNA and GUS histochemical staining showed that OsHsfB2cp and PM19p were heat induced to higher levels in panicles and flag leaves than Hsp90p (Rerksiri et al., 2013).

2.2. GMPs as a tool for plant breeding

Classical plant breeding has traditionally been used to improve a variety of agronomic traits in cultivated crops. It is based on selection of superior individuals from each generation to cross and obtain some improved individuals in the next generation, thus gradually promoting the development of crops with more desirable traits (e.g., larger fruits, higher yield, disease resistance, etc.), at the same time minimizing undesirable traits. This breeding method is very laborious and time-consuming. In addition to these drawbacks of classical plant breeding, it is limited to traits that are present within crossable varieties. As an alternative, mutagenesis approaches based on radiation and chemicals are widely used to broaden the genetic diversity of a given species (Ahloowalia and Maluszynski, 2001). As it can be expected, large numbers of mutations are introduced this way, which can lead to non-desired and non-controlled traits (Batista et al., 2008). In this context, genetic engineering can be considered as an interesting tool, allowing transfer of a precise DNA construct from the same or a different species.

The specific traits introduced into GM varieties have been classified into two groups; those related to improved farming characteristics (e.g. higher yields, easier crop management) and those addressed to consumers (e.g. improved nutritional value).

2.2.1. First generation GMPs

The first generation of commercialized GMPs displays improved farming characteristics (e.g. herbicide tolerance, insect resistance, virus resistance, disease tolerance and delayed ripening), which result in enhanced crop yields and reduced production costs and thus, they are primarily beneficial to growers. The majority of commercial GMPs carry this type of transgenes. In the 1996 to 2013 period, culture of herbicide tolerant GMPs has covered the largest surface sown with biotech crops. In 2013 alone, herbicide tolerant crops occupied 59% of the global biotech area. Herbicide tolerance is a single-gene trait that confers the transgenic plant a very specific mechanism to overcome the effects of a given herbicide. Thus, the specific herbicide can be applied along the growing season of such transgenic crops, which is an effective and inexpensive way to control weed

growth. Indeed, weeds are generally regarded as a very serious problem for farmers since they compete with crop plants for water, light and nutrients, thus resulting in yield losses (Ahmad et al., 2012; Brookes and Barfoot, 2013). The most common and effective herbicides are glyphosate and glufosinate (James, 2013). Glyphosate kills plants by blocking the 5-enolpyruvylshikimate-3-phophate synthase (EPSPS), an enzyme involved in the biosynthesis of aromatic amino acids, vitamins and many secondary plant metabolites. This compound is the active ingredient in herbicides such as RoundUp™. Glyophosate-tolerant soybean was the first GM herbicide-tolerant plant commercially grown (Padgette et al., 1995) and carries a bacterial form of the EPSPS enzyme that is not affected by glyophosate. Nowadays, glyophosate tolerant events of various plant species are in the market (e.g. sugar beet, canola, cotton, alfalfa, wheat and maize). Glufosinate is the active ingredient in commercial herbicides such as Libery™. Its mode of action is based on inhibition of the plant enzyme glutamine synthetase (GS), which is essential to synthesize glutamine. This amino acid biosynthetic pathway uses the excess nitrogen in the cell, as ammonium. Upon inhibition of GS by glufosinate, ammonium concentrations rise to toxic levels.

About 16% biotech surface was devoted to insect resistant crops in 2013 (James, 2013), which minimizes the risk of significant pest damage. The *cry* genes, expressed in the spores of the soil bacterium *Bacillus thuringiensis* (Bt) produce a series of Cry toxins and are highly effective againsts various insect pests (de Maagd et al., 1999). This trait has been successfully introduced into many commercialised crop species, amongst which cotton and maize were the most used in 2013, the latter also authorized for commercialization in the EU (event MON810). There are also some commercial GM crops which confer resistance to virus, such as papaya resistant to papaya ring spot virus (PRSV). Besides, there is a very intensive research in obtaining GM cereals tolerant to pathogens, with promising results at the level of laboratory and field tests. Among the genes that have been shown to provide resistance to pathogens, genes encoding e.g. bovine lactoferrin (Han et al., 2012; Lakshman et al., 2013), the *Arabidopsis thaliana* NPR1 (Gao et al., 2013) or the antimicrobial peptides MsrA2 and 10R (Badea et al., 2013) have been studied in detail. Coca and co-workers (Coca et al., 2004) demonstrated that overexpression of the *Aspergillus giganteus* gene encoding the antifungal protein AFP makes transgenic rice resistant against various economically important fungal pathogens, including the rice blast fungus *Magnaporthe grisea*. Other genes have been described conferring resistance to rice blast (Peng et al., 2012; Kouzai et al., 2013).

During the last decade GM crops containing more than one trait have been developed by crossing single trait GM lines. These GM *stacked events* simultaneously display e.g. insect resistance and herbicide tolerance, or resistance to different types of insects. They are becoming increasingly

important in the global biotech area, occupying 47 million hectares or 27% of the 175 milion hectares (James, 2013), being maize with stacked traits the second biotech dominant crop.

Environmental factors are essential components which affect crop yield to a great extent. Abiotic stresses such as salinity, drought, heavy metals, extreme temperatures and oxidative stress reduce plant growth and the final yields. Crops resistant to abiotic stress conditions have the potential of significantly increasing the overall agricultural productivity (Ahmad et al., 2012), and this is a priority in the agricultural research. The first commercial crop of this type is the GM maize DroughtGard™, which expresses *cspB*, a *Bacillus subtilis* RNA chaperone that increases yield under water-limited conditions (Castiglioni et al., 2008).

2.2.2. Second generation GMPs

Second generation genetically modified plants provide beneficial traits to consumers such us high nutritional value, premium quality and/or low allergenicity. Numerous genetically engineered staple crops accumulating modified proteins (Wenefrida et al., 2013), carbohydrates (Sestili et al., 2012; Jiang et al., 2013), oils (Liu et al., 2012; Zaplin et al., 2013) and other compounds such as vitamins and minerals have been reported (Table 1). These enhanced varieties are mostly at the laboratory testing phase or in early field trials, but at least one approaches broad release: Golden Rice. It contains three transgenes that encode different enzymes required to synthesize pro-vitamin A (β-carotene): phytoene synthase (*psy*) and lycopene β-cyclase from *Narcisus pseudonarcissus*, and phytoene desaturase from *Erwinia uredovora*. Golden Rice accumulates high levels of pro-vitamin A in the grain, giving the endosperm a yellow colour (Ye et al., 2000b), hence the given trade name. The Golden Rice was further improved to Golden Rice2 by replacing the daffodil with the maize *psy* transgene, which increased β-carotene accumulation over 23-fold (Paine et al., 2005). Other plant species such as potato or maize have been subsequently transformed to increase β-carotene levels (Lopez et al., 2008; Zhu et al., 2008; Naqvi et al., 2009).

In a complementary approach, staple crops have been developed with simultaneously enhanced levels of different nutrients, providing a balanced and nutritionally complete meal in a manageable portion. Since it simultaneously affects three different traits, such crops would be extremely difficult to generate by conventional breeding even if sufficient genetic diversity was available (Naqvi et al., 2010). Arjó and co-workers (Arjó et al., 2012) developed a prototype multivitamin corn engineered to concurrently accumulate high levels of β-carotene, ascorbate and folate, therefore addressing vitamins A, B9 and C deficiencies (Zhu et al., 2008; Naqvi et al., 2009). Trangenic kernels achieved up

to 57 µg/g dry weight β-carotene (>169-fold increase), 106.94 µg/g dry weight ascorbate (>six-fold increase) and 200 µg/g dry weight folate (two-fold increase) and contained no known allergens. In addition, diets prepared with this multivitamin corn had no adverse effects on mice in feeding trials.

Table 1. Examples of transgenic crops with enhanced mineral and vitamin contents (Extracted from Pérez-Massot et al., 2012).

Nutrient	Species	Genes used	Total increase (fold increase over wild type)	References
Vitamin A	Maize (*Zea mays*)	*PacrtB* and *PacrtI*	33.6 µg/g Dry Weight (DW) (34)	(Aluru et al., 2008)
	Maize (*Z. mays*)	*Zmpsy1, Pacrtl, PcrtW, Gllycb*	146.7 µg/g DW (133)	(Zhu et al., 2008)
	Maize (*Z. mays*)	*Zmpsy1, Pacrtl*	163.2 µg/g DW (112)	(Naqvi et al., 2009)
	Wheat (*Triticum aestivum*)	*Zmpsy1, PacrtI*	4.96 µg/g DW (10.8)	(Cong et al., 2009)
	Potato (*Solanum tuberosum*)	*EuCrtB, EuCrtI, EuCrtY*	114 µg/g DW (20)	(Diretto et al., 2007)
	Potato (*S. tuberosum*)	*BoOr*	28.22 µg/g DW (6)	(Lopez et al., 2008)
	Potato (*S. tuberosum*)	*AtZEP*	60.8 µg/g DW (5.7)	(Römer et al., 2002)
	Potato (*S. tuberosum*)	*PacrtB*	35.5 µg/g DW (6.3)	(Ducreux et al., 2005)
	Cassava (*Manihot esculenta*)	*PacrtB*	21.84 µg/g DW (33.6)	(Welsch et al., 2010)
	Rice (*Oryza sativa*)	*Nppsy1, EucrtI*	1.6 µg/g	(Ye et al., 2000a)
	Rice (*O. sativa*)	*Zmppsy1, EucrtI*	37 µg/g (23)	(Paine et al., 2005)
Vitamin C	Corn (*Z. mays*)	*Osdhar*	110 µg/g DW (6)	(Naqvi et al., 2009)
	Tomato (*Solanum lycopersicum*)	*Acggp*	46–111 mg/100 g Fresh Weight (FW) (3–6)	(Bulley et al., 2011)
	Potato (*S. tuberosum*)	*StVTC2A*	1.65 mg/g FW (3)	(Bulley et al., 2011)
Folic acid	Rice (*O. sativa*)	*Atgtpchi, Atadcs*	38.3 nmol/g (100)	(Storozhenko et al., 2007)
	Tomato (*S. lycopersicum*)	*Atgch, Atadcs1*	25 nmol/g (25)	(Storozhenko et al., 2007)
Iron (Fe)	Rice (*O. sativa*)	*Osnas2*	19 µg/g DW in polished seeds (4.2)	(Johnson et al., 2011)
	Rice (*O. sativa*)	*Gm ferritin, Af phytase, Osnas1*	7 µg/g DW in polished seeds (4–6.3)	(Wirth et al., 2009)
	Rice (*O. sativa*)	*Activation tagging of Osnas3*	32 in µg/g DW in dehusked (2.9)	(Lee et al., 2009)
	Corn (*Z. mays*)	*Gm ferritin and Af phytase*	30 µg/g DW in whole seed (2)	(Drakakaki et al., 2005)
	Cassava (*M. esculenta*)	*Crfea1*	40 µg/g DW in tuber	(Sayre et al., 2011)
Zinc (Zn)	Rice (*O. sativa*)	Activation tagging of *Osnas2*	40–45 µg/g DW in polished seeds (2.9)	(Lee et al., 2011a)
	Rice (*O. sativa*)	*Osnas2*	52–76 µg/g DW in polished seeds (2.2)	(Johnson et al., 2011)
	Rice (*O. sativa*)	*Gm ferritin, Af phytase, Osnas1*	35 µg/g DW in polished seeds (1.6)	(Wirth et al., 2009)
	Cassava (*M. esculenta*)	*Atzat1*	Tuber (4)	(Sayre et al., 2011)
	Cassava (*M. esculenta*)	*Atzip*	Tuber (2–10)	(Sayre et al., 2011)
Selenium (Se)	Indian Mustard (*Brassica juncea*)	*Ataps1*	(2–3)	(Pilon-Smits et al., 1999)
	Indian mustard (*B. juncea*)	*Absmt1*	(2–4)	(LeDuc et al., 2004)
Calcium (Ca)	Carrot (*Daucus carota*)	*scax1*	3.9 mg/g DW (1.6)	(Park et al., 2004)
	Lettuce (*Lactuca sativa*)	*scax1*	18.9 mg/g DW (1.3)	(Park et al., 2009)
	Potato (*S. tuberosum*)	*scax1*	1.7 mg/g DW (in tuber) (3)	(Park et al., 2005)
		cax2b chimeric	2.5 mg/g DW (3)	(Kim et al., 2006a)

2.3. Molecular farming in plants

Plant genetic modification has been extensively used in recent years to accumulate products of interest in the industrial, therapeutic and agricultural fields. This is known as "plant molecular farming (PMF)" or "third generation genetically modified plants". Alternative recombinant production systems such as bacterial and mammalian cell cultures can be limited in some aspects such as scalability and production cost because they require specific fermentation equipment and expensive downstream processing. Production of recombinant proteins in GM plants can be considered a complementary tool to meet the rising market demand (Boehm, 2007), at the same time providing options not viable in other systems (Table 2). Some of the specific advantages of plant biofactories are summarised below:

i) Cheaper production platforms. It is estimated that recombinant proteins can be produced in plants at 2-10% of the cost of microbial fermentation systems and at 0.1% of the cost of mammalian cell cultures, although this depends on the product yield (Giddings, 2001; Chen et al., 2005). Yields of 0.1-1% total soluble proteins (TSP), the typical levels observed for the production of pharmaceutical proteins such as recombinant antibodies are sufficiently competitive with other expression systems to make plants economically viable (Hood et al., 2002).

ii) Different crop plant tissues like leaves, seeds, tubers and fruits can be used as biofactories. Moreover, tissues such as seeds provide a highly stable method for storage and transport of recombinant products, without the need of refrigeration (Twyman et al., 2003).

iii) Culture of plant biofactories is easily scalable; in consequence its production can be rapidly modulated in response to the market demand by simply using more or less land.

iv) In contrast to mammalian cell cultures, plants are not hosts for human pathogens and in consequence, contamination of plant-produced recombinant proteins with human infections agents is not considered a safety issue (Giddings et al., 2000).

v) Several types of recombinant proteins produced in plants can be consumed raw or partially processed, thus diminishing the purification costs. Moreover, in case purification is required, recombinant protein accumulation in tissues such as seeds makes it easier and less expensive than using other production platforms (Lau and Sun, 2009).

vi) Plants can produce complex proteins and carry out post-translational modifications similarly to animals, except for some differences in the glycosylation patterns. The activity of a number of proteins is directly linked to their adequate glycosliation (Rothman et al., 1989; Sethuraman and Stadheim, 2006). Glycosylation takes place into the endoplasmic reticulum (ER) and Golgi apparatus. While *N*-glycosylation occurring in the ER does not change in the different species (Sturm et al., 1987; Chen et al., 2005; Brodzik et al., 2006), mammal and plant glycosylation patterns occurring in the Golgi apparatus are dissimilar (Gomord et al., 2005). Plant glycosylation is based on β(1,2)-xylose and α(1,3)-fucose, whereas mammals tend to include α(1,6)-fucose, galactose and sialic acid (Gomord et al., 2004). These minor differences in glycan structure could potentially modify the activity, biodistribution and longevity of recombinant proteins. Therefore, various strategies have been developed to 'humanize' the glycan patterns in transgenic plants, including modification of the enzymatic machinery of the plant Golgi apparatus. This has been achieved through knock-down of genes involved in β(1,2)-xylosylation and α(1,3)-fucosylation (Strasser et al., 2004; Cox et al., 2006; Strasser et al., 2008); and addition of new glycosyltransferase genes (Frey et al., 2009).

Table 2. Comparison of available hosts for production of heterologous proteins (extracted from Desai et al., 2010).

System	Production cost	Production time scale	Scale- up capacity	Expression level	Glycosylation*	Contamination risks	Storage cost
Bacteria	Low	Short	High	High	Absense	Endotoxins	Moderate
Yeast	Medium	Medium	High	Low-High	Higher manosylation	Low risk	Moderate
Insect cell culture	High	Medium	Medium	Low-High	Higher manosylation	High	Expensive
Mammalian cell culture	High	Long	Very low	Low-moderate	Similar to human	Viruses, Prions	Expensive
Transgenic animal	High	Very long	Low	Moderate-high	Similar to human	Viruses, Prions	Expensive
Plant cell culture	Low	Short	High	Moderate-high	Minor differences	Low risk	Inexpensive
Transgenic Plants	Very low	Long	Very high	Moderate-high	Minor differences	Low risk	Inexpensive

* Glycosylation pattern is given in comparison with that in humans.

The human growth hormone (hGH) was the first recombinant protein produced in plants (Barta et al., 1986). Nine years later, Hood and co-workers (Hood et al., 1997) reported the production of the first protein in GM plants for the specific purpose of purification and commercialization: the egg protein avidin. Since then, a wide variety of biomolecules such as antibodies, vaccines, hormones, cytokines, enzymes or polymers have been commercially produced in plants.

Recombinant proteins with industrial interest are increasingly on demand. The potential of this technology is here illustrated with a few examples. Fibrous proteins such as silk and collagen have

been produced in *Arabidopsis* (Ruggiero et al., 2000; Yang et al., 2005). Polyhydroxyalkanoates (PHAs), biodegradable polymers which offer an environmentally sustainable alternative to polypropylene (PP) and other petroleum-based plastics, have been synthesized in a variety of plant species such as oilseed rape (Houmiel et al., 1999) and sugarcane (Petrasovits et al., 2007). Enzymes such as cellulase and xylanase have been produced in plants for industrial purposes. Examples are cellobiohydrolase (Devaiah et al., 2012) in maize grain, thermostable xylanase in maize stover (Shen et al., 2012), glycoside hydrolases (Brunecky et al., 2012) and *Acidothermus cellulolyticus* endoglucanases in rice seeds (Zhang et al., 2012).

Plant-based oral vaccines are a promising emergent technology that could help alleviate disease burden worldwide by providing a low-cost, heat-stable, oral alternative to parenterally administered commercial vaccines. Examples are the hepatitis B surface antigen (HBsAg) in maize seeds, which proved to be highly immunogenic upon bioencapsulation (Hayden et al., 2012a; Hayden et al., 2012b); the H1N1 vaccine produced in *Nicotiana benthamiana* plants to combat serious respiratory diseases caused by influenza viruses (Shoji et al., 2008; Shoji et al., 2011; Cummings et al., 2013); and a VP2 protein produced in *Nicotiana benthamiana*, effective against Infectious Bursal Disease Virus (IBDV), a highly contagious disease that affects young birds (Gómez et al., 2013). Similarly, large amounts of recombinant interleukin-10 (an anti-inflammatory cytokine) have been produced in tobacco BY-2 suspension cells (Bortesi et al., 2012) (for a review see Ahmad et al., 2012; Xu et al., 2012; Dunwell, 2013).

Due to the low cost, use of plants to produce recombinant proteins for application in the food and agricultural fields has a potential market. Specifically, production of recombinant AMPs, which are also relevant in the pharmaceutical and cosmetic fields, is under robust development.

2.3.1. AMP production

AMPs can be directly purified from their natural sources, although these peptides are often accumulated at low concentrations and thus, purification processes are costly and time consuming (Li et al., 2008). Alternatively, AMPs can be chemically synthesized. This strategy allows production of both natural and synthetic AMPs. However, extensive peptide synthesis is economically viable only for short peptides (ideally around six amino acids) with high added-value applications. Finally, recombinant AMP production systems are considered as highly effective in terms of both, time and production costs (Rao et al., 2005; Xu et al., 2007). AMPs have been produced in different prokaryotic and eukaryotic hosts (Oard and Enright, 2006; Stotz et al., 2009).

Li and Chen, 2008 reported that 97.4% of developed recombinant AMP production systems used bacterial and yeast hosts. Bacterial cultures are advantageous from an economic viewpoint (Baneyx, 1999; Sørensen and Mortensen, 2005a). Nevertheless, some important limitations need to be overcome to achieve effective AMP production in bacteria. The possible AMP activity against the host strain needs to be prevented in order to allow bacterial growth. Bacterial proteases tend to result in instability of the recombinant AMP (Li, 2011). AMPs produced in bacteria are often expressed as fusion proteins, a strategy that has proved to be effective in masking these peptides lethal effect towards the host and protecting them from the proteolytic degradation (Sørensen and Mortensen, 2005b; Xu et al., 2006b; Li et al., 2011; Li, 2011). In an alternative approach, natural AMPs sequences have been altered both to increase the yields and to alter the antimicrobial spectrum (Cao et al., 2005; Kang et al., 2008), although only moderate yields have been attained. Even so, many AMPs have been obtained through recombinant production in various heterologous bacteria, among which *Escherichia coli* has been the most widely used one. Examples are bovine Lactoferricin (Kim et al., 2006b; Luo et al., 2007), *Musca domestica* Cecropin (Liang et al., 2006; Xu et al., 2007), and human and flounder (*Paralichthys olivaceus*) Hepcidins (Gagliardo et al., 2008; Srinivasulu et al., 2008) (for a review, see Parachin et al., 2012). Heterologous AMPs have also been produced in yeast systems such as *Saccharomyces cerevisiae* and *Pichia pastoris* (Parachin et al., 2012). Being eukaryotic organisms, yeast hosts present some advantages over prokaryotic cells, especially concerning post-translational modifications, such as glycosylation patterns (Cregg et al., 2009).

Expression of recombinant AMPs in transgenic plants has been recently reported with the aim of conferring the host plant resistance against pathogen attack (Montesinos, 2007; Marcos et al., 2008). As an example, constitutive expression of the insect Cecropin A resulted in transgenic rice resistant to fungal pathogens (Coca et al., 2006). Similarly, expression of Magainin, the defensins Heliomycin and Drosomycin, and Sarcotoxin in tobacco; and Tachyplesin in potato resulted in resistant phyenotypes. Expression of the radish Rs-AFP2 defensin protects tomato and tobacco plants from *Alternaria longipes* (Terras et al., 1995) and the recombinant alfalfa antifungal peptide (Alf-APF) reduced *Verticillium dahliae* infection of potato plants (Gao et al., 2000).

More recently, plants have arisen as promising expression system for the production of heterologous proteins, including AMPs (Desai et al., 2010) with therapeutic and phytosanitary purposes. A gene encoding the anti-HIV C4V3 synthetic peptide was recently introduced into tobacco chloroplasts achieving yields up to 25 µg recombinant protein per g (Rubio-Infante et al., 2012). Similarly, genes encoding Retrocyclin-101 and Protegrin-1, used as therapeutic agents against bacterial and/or viral infections, were introduced into the tobacco plastome, yielding

around 20% of TSP (Lee et al., 2011b). Certain fusion AMPs have been produced in plants with high yields (Table 3). However, in general, plant production of single peptides proved to be difficult and result in moderate or low yields, probably because of the unstability of short heterologous peptides in the plant cell environment, rich in endogenous peptidases (Florack et al., 1995). Increasing the length of these peptides has been proposed to limit degradation while maintaining their biological properties (Streatfield, 2007; Lico et al., 2012). Due to their particular characteristics, production of AMPs in plants is still a challenge and research in this field is currently very active. The Food Technology and the Plant Pathology groups at the University of Girona, in the frame of the MICINN AGL2010-17181 and the PLANT-KBBE (EUI2008-03769) projects, have established a research line focusing on the production of active AMPs derived from BP100 in plant systems. Specifically, two approaches were pursued: constitutive expression and accumulation in the ER (Montero, 2012); and accumulation in rice embryo and endosperm tissues e.g. by fusion to Oleosin (Montesinos, 2014). The presence in seeds of protein bodies and oil bodies, specialized storage organelles, facilitate recombinant protein accumulation. However, major hurdles still exist for using seed-based systems as recombinant protein bioreactors. For example, there is a strong reluctance among scientists, regulators, and the general public to use seeds of major crops (that is, maize, rice and wheat) for biopharmaceutical production, given the possibility of contaminating the food chain (Vitale and Pedrazzini, 2005). On the other hand, transgenes encoding two and three tandem copies of BP100 under the regulation of the constitutive maize Ubiquitin promoter were transformed into rice and either no GM plants or an unusually low number of transgenic plants (only for BP100.2i and BP100.2mi) were obtained, which had very reduced transgene mRNA levels. These plants were not useful as biofactories, although they had pathogen resistant phenotypes (Montero, 2012). This Thesis develops the later approach and further explores alternative strategies.

Table 3. Examples of plant biofactories producing high yields of heterologous AMPs.

AMP	Source	Host plant	Transformed genome	Activity (target pathogen)	Promoter type	Yield	Subcellular localization	References
C4V3	Synthetic	*Nicotiana tabacum*	Chioroplast	Anti-HIV activity, induction of mammalian immune response	Organelle-specific promoter - Chloroplast 16S ribosomal RNA gene promoter (Prrn)	25 μg/g fresh weight (FW) tobacco leaves	Chloroplast	(Rubio-Infante et al., 2012)
EtMIC2	*Eimeria tenella*	*Nicotiana tabacum*	Transient expression	*Eimeria tenella* (chicken coccidiosis)	Constitutive promoter - Cauliflower mosaic virus 35S promoter (CaMV eP35S)	Not quantified, but immunoblot demonstrated plant expressed EtMIC2 protein	Cytosol	(Sathish et al., 2011)
Retrocyclin-101 and Protegrin-1	Artificial AMP based on rhesus monkey (*Macaca mulatta*) circular minidefensins	*Nicotiana tabacum*	Chioroplast	*Erwinia carotovora*, tobacco mosaic virus	Organelle-specific promoter - tobacco plastid psbA promoter	RC101 and PG1 accumulated up to 32%–38% and 17%-26% respectively of TSP	Chloroplast	(Lee et al., 2011b)
Atlantic salmon IFN (SasaIFN-α1)	*Salmo salar*	Rice and potato	Nuclear	Pancreatic necrosis virus (fish cells treated with plant produced AMP)	Constitutive promoter - CaMV 35S promoter	Not quantified, but Western blot analyles showed that the SasaIFN-α1 protein was barely detectable in the insoluble fractions and was not found in the soluble fractions from the transgenic plants	Cytosol	(Fukuzawa et al., 2010)
Pal and Cpl-1	Phages infecting *S. pneumoniae*	*Nicotiana tabacum*	Chloroplast	*Streptococcus pneumoniae*	Organelle-specific promoter - Chloroplast 16S ribosomal RNA gene promoter (Prrn)	Pal accumulated to ~30% of TSP and Cpl-1 accumulated to ~10% of TSP	Chloroplast	(Oey et al., 2009)
Chicken alpha Interferon (ChIFN-apha)	*Gallus gallus*	Lettuce	Transient expression	Vesicular stomatitis virus (VSV)	Constitutive promoter - CaMV 35S promoter	0.393 μg protein/kg tissue (0.0004% of the TSP)	Protein storage vacuoles by fusion to *Brassica napus* napin signal peptide (*tp*)	(Song et al., 2008)
MsrA2	Synthetic derivative of dermaseptin B1	Potato	Nuclear	Fungal phytopathogens (*Alternaria, Cercospora, Fusarium, Phytophthora, Pythium, Rhizoctonia and Verticillium species*) and bacterial pathogen *Erwinia carotovora*	Constitutive promoter - CaMV 35S promoter	1–5 μg/g of fresh tissue	Cytosol	(Osusky et al., 2005)
Synthetic DPT polypeptide	Synthetic	Tomato	Nuclear	*Corynebacterium diphteriae, Bordetella pertussis and Clostridium tetani*	Constitutive promoter - CaMV 35S promoter	1–2.1 μg/g leaf and 2.25–6.8 μg/g fruit	Endoplasmic reticulum (ER)	(Soria-Guerra et al., 2007; Soria-Guerra et al., 2011)

In summary, the majority of commercial recombinant proteins are currently produced in mammalian cell lines or bacteria, in a few cases in insects, yeast and fungi. However, these expression systems have limitations in terms of suitability, cost, scalability, purification and post-translational modifications. Transgenic plants could provide an attractive alternative in terms of low production cost and lower capital investment in infrastructure, and with appropriate post-translational modifications. However, the yields in plants need to be improved by optimizing a few limiting steps of plant expression systems.

3. Genetic transformation

Plant transformation refers to the introduction of a DNA sequence of choice into the genome (nuclear or plasmidic) of plant cells. For production of transgenic plants, the experimental procedures involve the introduction of foreign gene(s) into plant cells (transformation), and tissue culture technologies aiming at selection of transformed cells and regeneration of transgenic plants.

3.1. DNA delivery systems

Although various methods have been developed to introduce DNA into plant cells, the concept of using *Agrobacterium tumefaciens* to genetically transform many agronomically and horticulturally important species has become increasingly used in the last decades. In the seventies, transfer of specific DNA segments from *A. tumefaciens* to plant cells was first used (Chilton et al., 1977; Chilton et al., 1978; Depicker et al., 1978). *Agrobacterium* are Gram-negative bacterial species described by Smith and Townsend, 1907 and Conn, 1942, subsequently divided into a number of species reflecting the specific disease symptomatology and host type. Specifically, *A. tumefaciens* provokes crown gall disease, *A.rhizogenes* causes hairy root disease, *A. rubi* origins cane gall disease, *A. vitis* has been related to grape gall and *A. radiobacter* is an avirulent species (Otten et al., 1984).

Crown gall disease caused by *A. tumefaciens* was considered a major problem in horticultural production during the first decades of the last century. It caused significant loss of crop yield in many perennial horticultural crops (Kennedy and Alcorn, 1980) such as cherry (Lopatin, 1939), apple (Ricker et al., 1959) and grape (Schroth et al., 1988). However, nowadays the perception of this bacterium has changed because *A. tumefaciens* is widely used for plant genetic transformation (Veluthambi et al., 2003; Gelvin, 2003; Lacroix et al., 2006; Tzfira and Citovsky, 2006; Vasil, 2008). *A. tumefaciens* pathogenicity is associated with the natural presence of the tumor-inducing (Ti) plasmid, which contains a T-DNA (transferred DNA) sequence that contains two types of genes: oncogenic genes, encoding enzymes involved in the synthesis of auxins and cytokinins (causing plant cell proliferation), and genes involved in opine production (opines can be used by *A. tumefaciens* as a source of carbon and energy). The T-DNA is transferred into the host cell and randomly integrated into the plant genome. Native T-DNA is 10 to 30 kbp in size (Barker et al., 1983; Byrne et al., 1983; Suzuki et al., 2000), representing less than 10% of the whole Ti plasmid; and it is delimited by 25-bp directly repeated sequences, left border (LB) and right border (RB). Small phenolic and sugar compounds produced by wounded plant cells trigger *A.tumefaciens* infection (Loake et al., 1988), permitting recognition and bacteria-plant attachment, establishing complex biofilms at colonization

sites. This induces coordinated expression of virulence genes in the Ti-plasmid. The *vir* region (approximaly 30 kb) includes at least six essential operons (*virA*, *virB*, *virC*, *virD*, *virE* and *virG*) encoding proteins that are required to sense plant signal molecules, T-DNA transfer to the plant cell, protect T-DNA against cellular nucleases (Citovsky et al., 1989) and integrate the T-DNA into the plant genome (Tzfira and Citovsky, 2000; Gelvin, 2000; Ziemienowicz et al., 2001).

Numerous studies demonstrate that the native T-DNA sequence can be replaced by any other DNA sequence without inhibiting its transfer to the host plant. This property has been used to transfer exogenous DNA into plants. The Ti plasmid was modified into a binary vector system (de Framond et al., 1983; Hoekema et al., 1983) in which the *vir* and the T-DNA regions of Ti-plasmids were split onto two separate plasmids that coexisted in the same *Agrobacterium* cell. This way, virulence proteins would act in *trans* to mediate processing and exportation of the T-DNA to the plant. The plasmid containing the T-DNA region is relatively small, which makes its manipulation easier and facilitates the cloning steps.

In the early 1980s the first transgenic plant was achieved; and since then significant progress has been made in understanding the *Agrobacterium*-mediated gene transfer to plant cells. This has allowed optimizing the protocols for transformation of dicotyledonous plants, the natural *Agrobacterium* hosts, and expanding the range of target species to monocotyledonous plants such as rice (Hiei et al., 1994; Cheng et al., 1998), corn (Ishida et al., 1996), sugarcane (Enríquez-Obregón et al., 1997; Arencibia et al., 1998; Enriquez-Obregon et al., 1998) or wheat (Cheng et al., 1997). *Agrobacterium*-mediated gene transfer is currently among the most common methods for plant transformation because it tends to generate transgenic plants with low transgene copy numbers, intact foreign genes, and stable inheritance and transgene expression over generations (Dai et al., 2001), which is usually desirable e.g. to minimise transgene silencing associated with multiple copy integration events (Schubert et al., 2004; Chawla et al., 2006; Meng et al., 2006). Recently, improved *in planta* agrotransformation methods have been developed to minimize the labour-intensive *in vitro* culture steps normally required to prepare the adequate starting material (e.g. callus) and regenerate transgenic plants. Examples are pollen (Wang et al., 2001), pistil (Chumakov et al., 2006; Mamontova et al., 2010), ovary dip (Yang et al., 2009) and floral dip transformation methods (Clough and Bent, 1998). Floral dip is currently the method of choice for *Arabidopsis* transformation. *Arabidopsis* developing floral tissues, which contain female gametes, are diped into a solution containing *A. tumefaciens* carrying the gene(s) to be transferred and allowed to develop normally. Seeds are subsequently collected and those harboring the transgene are then selected, usually by germination in the presence of a specific antibiotic or herbicide which action the product of the selection transgene is capable to overcome (Bent, 2006).

Alternative DNA transfer methods have been developed such as polyethyleneglycol-mediated transfer, protoplast and intact cell electroporation, or microparticle bombardment (biolistics or gene gun). Microparticle bombardment (Sandford, 1988) is based on shooting gold or tungsten microcarriers, coated with the DNA of interest, against virtually any tissue and organism that is to be transformed. On impact against plant cells, foreign DNA can elute off the particles, reach the cytoplasm or organelles and be functional. In some cases, it will incorporate into the host genome and give rise to a stably transformed cell. Some of the presently commercialized GM plants, such as the insect resistant MON810 maize, were generated using this approach. However, it often leads to integration of multiple copies of the transgene and superfluous DNA sequences, which has been associated to gene silencing and poor transgene stability. This method has successfully been used to simultaneously transform plant cells with multiple sequences simply by using plasmids harboring various genes in the T-DNA (Campbell et al., 2000; Schmidt et al., 2005).

Stable integration of foreign genes into the plant genome confers transgenic plants stably inheritable traits. However, generation of a transgenic plant is not always necessary but transient expression, which can be achieved in a substantially shorter time, can be sufficient for certain applications. Foreing DNA introduced in a cell can function for a short time in the nucleus as an extra chromosomal entity, but integration into the genetic material of the target cell is necessary for long-term functionality and expression. Transient expression is exactly what the phrase suggests: a short-term expression of the introduced DNA(s). It has been largely used to optimize transformation and DNA introduction methods; and in addition, this type of rapid transgene expression can also be used to facilitate speedy analysis of factors that influence the strength and stability of transgene expression and the functionality of expressed products. In a recent example, transient expression allowed identifying stress-responsible elements in different promoters (Gao et al., 2014); and Patro and colleagues (Patro et al., 2013) used the same approach to demonstrate the functionality of novel promoters. Transient expression has been used as well as a plant molecular farming platform (Rybicki, 2010), achieving considerable yields.

Transient transformation has been typically carried out using particle bombardment of e.g. epidermal cells; and more recently, using virus-based tools that are capable to autonomously replicate in plant cells and move from cell to cell, resulting in high transient expression of transgenes in plant cells (Chung et al., 2006). However, nowadays *Agrobacterium*-mediated transient assays and in particular leaf agroinfiltration has become the technique of choice since it is an easy, fast and non-invasive tool (Wroblewski et al., 2005). This method was developed by Kapila and co-workers (Kapila et al., 1997) and involves the injection or infiltration of recombinant *Agrobacterium tumefaciens* into tobacco leaf tissue, resulting in transfer of T-DNA to a high

percentage of cells and strong transgene expression. Agroinfiltration is carried out by forcing an *Agrobacterium* suspension into the internal leaf airspace by tightly holding a syringe (without the needle) to the leaf and pushing the plunger. A variation of this method requires dipping the plant leaves into an *Agrobacterium* suspension to subsequently force the bacteria into the internal leaf airspace by vacuum application. *Agrobacterium* mediated transient transformation assays usually lead to production of recombinant proteins in the 24 h to several days span.

3.2. Transformed cells selection, regeneration and acclimatization

The most stable transformation protocols for obtaining a whole plant from the cell or tissue expressing the gene of interest require an *in vitro* regeneration step. Successful production of transgenic plants requires transformation of plant cells whith good capacity to divide and differentiate into whole plants. This property is known as totipotency. Although it is probably true that all plant cells are totipotent, this capability decreases in highly differentiated cells. *In vitro* manipulation of nutritional, hormonal and physical conditions can lead to controlled dedifferentiation of plant cells and formation of new organs or regeneration of whole plants. Plants can be produced from a cultured tissue (e.g. callus) by organogenesis (caulogenesis and rhizogenesis, for shoot and root development, respectively) and somatic (nonzigotic) embryogenesis (Shewry et al., 2008). Tissue culture protocols are rigorous procedures that require a number of chemical, physical and environmental factors and work under aseptic conditions. Plant tissues are grown on artificial media containing macronutrients, micronutrients, plant growth regulators, vitamins, amino acids, other nitrogen supplements and sugars. These elements supply all necessary nutrients to develop *in vitro* raised plantlets (Staden et al., 2008).

Transformation methods are inefficient, as only a very small proportion of cells becomes stably transformed (Rakoczy-Trojanowska, 2002). As a result, a selection system is necessary to obtain GM plants from regeneration of the few transformed cells. To implement this system, a selection agent or a selection marker gene incorporated into the transformation *cassete* confers transformed cells an advantage under particular media conditions (Shewry et al., 2008). Since the first transgenic plants were reported in 1983, a wide number of marker genes have been devised. They are classified into two main categories: selectable marker genes and non-selectable or reporter genes (Table 4).

Table 4. Categories of marker genes used in plants with selected examples.

Classes of marker gene		Examples	Source of genes	Selective agent
Selectable marker gene				
Positive	Conditional - toxic	*npt II*	*Escheriichia coli* Tn5 (bacteria)	Kanamycin
		hpt	*Escheriichia coli* (bacteria)	Hygromycin B
		pat	*Streptomyces viridochromogenes* (bacteria)	Phosphinothricin
		bar	*Streptomyces hygroscopicus* (bacteria)	Phosphinothricin
	Conditional-promotive	*manA*	*Escheriichia coli* (bacteria)	Mannose
		xylA	*Streptomyces rubiginosus* and *Thermo-anaerobacterium thermosulfurogenes (bacteria)*	Xylose
		galactose	*Escheriichia coli* (bacteria)	Galactose-1-phosphate uridyltransferase
	Non-conditional	*ipt*	*Agrobacterium tumefaciens* (bacteria)	N/A*
Negative	Conditional	*codA*	*Escheriichia coli* (bacteria)	5-Fluorocytosine
	Non-conditional	*barnase*	*Bacillus amyloliquefaciens* (bacteria)	N/A*
Non-selectable marker gene				
	Conditional	*gusA*	*Escheriichia coli* (bacteria)	X-gluc
		luciferase	*Photinus pyralis* (insect)	luciferin
	Non-conditional	*gfp*	*Aepuorea victoria* (jellyfish)	N/A*

*Nonapplicable

Regarding selectable marker genes, Miki and McHugh, 2004 suggested to discriminate the selection systems according to (i) the selection strategy (*positive* when transgenic cells are promoted to grow and *negative* when transgenic cells die), (ii) the presence or absence of a selective agent (*conditional* in the presence of the selective agent and *non-conditional* in the absence of it); and (iii) the effect of the selective agent on transgenic and non-transgenic cells (Joersbo, 2011), being *conditional-toxic* when growth of transgenic cells is possible and the effect on non-transgenic cells is death, and *conditional-promotive* when there is an enhanced growth of transgenic cells while non-transgenic cells suffer growth arrest. As a function of their characteristics, selection systems are classified into four groups (positive conditional, positive non-conditional, negative conditional and negative non-conditional), being the positive ones the most widely used:

i) **Positive conditional selection systems.** This group is classified into two subgroups:

i.i) **toxic selective agents**. The first generation markers are based on toxic selective agents, mainly antibiotics and herbicides that kill untransformed cells while those carrying the transgene are resistant. They are highly efficient and applicable to many different plant species. In the most common case, the selectable marker gene encodes an enzyme that derivatizes or degrades the specific selective agent. Examples are:

- Neomicyin phosphotransferase II (*npt II*) (Herrera-Estrella et al., 1983; Fraley et al., 1983), which inactivates aminoglycoside antibiotics, usually kanamycin.
- Hygromycin phosphotransferase (*hpt*) (Waldron et al., 1985), which confers resistance to the antibiotic hygromycin B.
- Phosphinotgricin N-aceyltransferase (*pat* [Wohlleben et al., 1988] and *bar* [Thompson et al., 1987]). They have been used in plants to convert PPT (ammonium gufosianate or phosphinothricin), an active ingredient of several commercial broad-spectrum herbicide formulations (e.g. Basta™, Ignite™, Liberty™) to a non-toxic acetylated form (White et al., 1990). As a result, only cells which have incorporated the *pat* or *bar* gene will grow in the presence of PPT.

i.ii) **promotive selective agents**. They were originally developed in response to the growing concern on the risk for transfer of transgenes, including antibiotic and herbicide resistance genes, to mircoorganisms, and the ecological and medical implications of GM crop cultivation and GM food consumption (EFSA, 2004; Darbani et al., 2007) (see below). Examples are phosphomanose isomerase (PMI) (*manA* [Miles and Guest, 1984]), xylose isomerase (*xylA*), and *galactose,* used because some crops are sensitive to low levels of this sugar.

ii) **Positive non-conditional selection systems.** They are based on the use of genes that confer a growth advantage, distinct morphology or that selectively induce differentiation of transformed tissues, without any specific selection agent. Cytokinin has been used as a positive non-conditional selection marker.

Optionally, non-selectable reporter genes can be used. They mostly encode proteins involved in the production of an easily scorable response, normally visual but also detectable through taste (e.g. ThaumatinII [Witty, 1989]) or smell. Upon detection, transformed tissues are manually selected. They include:

i) **Conditional selection**. Detection of the reporter requires an external substrate. β-glucuronidase (*uidA* or *gusA*) is the most widely used. This enzyme hydrolyses 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) to form a bluish precipitate. The firefly enzyme luciferase catalyzes the ATP-dependent oxidative decarboxylation of luciferin and produces light.

ii) **Non-conditional selection**. The pacific jellyfish green fluorescent protein (*gfp*) is becoming the most important reporter gene system in plants. GFP emits green fluorescent light upon exposure to ultraviolet light (Ormo et al., 1996), thus it does not require any substrate. Moreover, this type of molecules allows direct visualization in living tissues and is not invasive or destructive.

On the whole, it should be considered that different selection systems could be suitable to different purposes. Concerns have been expressed on the possible medical implications of GM crop culture and consumption, including the possibility that the transgenic DNA may be horizontally transferred to bacteria such as those living in the gastrointestinal tract. However, no evidence has been given to date indicating that the antibiotic resistance marker genes presently in use pose risks to human or animal health (ISAAA, 2009). Moreover, numerous experiments strongly suggest that HGT from plants to gastrointestinal bacteria is an extremely rare event (Keese, 2008; Rizzi et al., 2012). In consequence, the European Food Safety Authority (EFSA) issued a statement concluding that the use of the *nptII* and *hpt* genes as selectable markers in GM plants and derived food or feed does not pose a risk to human or animal health or to the environment (EFSA, 2009). On the other hand, the possible effects of marker genes on GM plants have been studied using profiling thechnologies, and often only minimal effects could be detected. In particular, selection genes such as *hpt* proved not to be associated with pleiotropic effects on the transcriptomes of transgenic plants (Miki et al., 2009). Even though, production of GM plants not carrying any selectable marker has also been considered, either by using marker-free transformation methods or through strategies to eliminate selectable markers. Examples are co-transformation (the simplest marker removal approach developed so far [Depicker et al., 1985; McKnight et al., 1987; De Block and Debrouwer, 1991]), site-specific recombination (Dale and Ow, 1991; Gleave et al., 1999), use of multi-autotransformation vectors (Ebinuma et al., 1997), transposition systems (Goldsbrough et al., 1993) and homologous recombination (Puchta, 2000; Zubko et al., 2000).

Upon regeneration, transgenic plantlets need to be transferred to the greenhouse. Especially for certain species this is a critical step, because there is a dramatic change between *in vitro* conditions and those in the greenhouse that results in morphological and physiological differences in the

plants (Kozai, 1991; Pospisilova et al., 1992; Buddendorf-Joosten and Woltering, 1994; Desjardins, 1995; Kozai and Lila Smith, 1995). *In vitro* culture vessels are designed to prevent microbial contamination, thus they are capped. In such relatively air-tight conditions, the air humidity is higher and irradiance lower than in conventional culture. There is decreased air turbulence, which increases leaf boundary layers and limits the inflow of CO_2 and outflow of gaseous plant products from the vessels. Cultivation media are often supplemented with saccharose as carbon and energy source, which results in reduced photosynthesis rate, and with growth regulators. In these conditions, plants tend to develop minute, with unfunctional stomata, weak root system and poorly developed cuticle and epicuticular waxes (Mathur et al., 2008). Thus, an acclimatization process is needed to gradually change these features to be compatible with life in greenhouse or field conditions (Hazarika, 2003). Physical and chemical environmental conditions must be progressively changed to induce increased photosynthetic capacity, controlled water losses, improved root absortion (rooting) and foliar reinforcement, and capacity to coexist with soil microflora (Mathur et al., 2008). This is achieved by subjecting plantlets to *ex vitro* conditions with increasing light intensity (to stimulate photosynthesis) and decreasing humidity (to slowly control stomatal and cuticular transpiration rates by stimulating development of cuticle and functional stomatal apparatus) (Pospisilova et al., 1999).

3.3. Selection of GM events

Transgenic plants regenerated from transformed cells (i.e. T0 plants) are hemizygous - meaning that the transgene forms a novel locus within the plant genome, which is absent in the homologous chromosome. The transgene is considered to segregate in the subsequent generations following the Mendelian laws. To achieve stability of the trait, homozygous plants need to be selected for downstream applications. In plant species such as rice, *Arabidopsis thaliana* and tobacco, T0 plants are self-pollinated to produce the T1 seeds, which in turn develop into T1 plants that carry the transgene in either hemizygous, homozygous positive or homozygous negative way (expected 2:1:1 Mendelian ratio if there is a single-copy insertion of the transgene). Similarly, GM seeds produced by transformation of T0 ovaries using floral dip are T1 seeds. T1 seeds need to be screened (e.g. using the correspondent selective agent) so that those carrying the transgene are selected. However, only one-third GM T1 seeds are homozygous for the transgene; and they are identified based on the T2 generation (i.e. all plants are GM).

Single-insert transgenic events tend to display stable transgene expression along generations. Multiple transgene copies are not normally associated to high expression levels but often lead to

gene silencing. In addition, they are prone to rearrangements (Linn et al., 1990; Hobbs et al., 1993). Thus, transgenic plants with single-copy insertions are preferred (Kohli et al., 2003; Jones, 2005). In contrast with methods such as particle gun, *Agrobacterium* based transformation usually leads to single-copy or low-copy insertions. Transgene copy numbers can either be experimentaly assessed by Southern blot (Bhat and Srinivasan, 2002) or real-time PCR (qPCR).

The integration site is also known to influence transgene expression (Gelvin and Kim, 2007). Traditional transformation methods lead to random insertion of the transgene into the plant genome (although they tend to evade certain types of regions such as centromere or telomere regions). Transgenes integrated in highly transcribed regions tend to achieve higher expression levels than those integrated within nontranscribed regions. Technical approaches to direct DNA integration include site-specific recombination (a promising technology that can be used either to direct transgenes towards specific locations or to efficiently remove it [Wang et al., 2011]), or use of zinc finger nucleases (chimeric proteins composed of a synthetic zinc finger–based DNA binding domain and a DNA cleavage domain). These processes can lead to homologous replacement of a given target gene, or introduction of site-specific mutations (Osakabe et al., 2010). Although these methods are very promising, they are not yet optimized for routine studies.

Transgene integration site can also be related to phenotypic modifications to the host plant. In some cases they will allow survival of the transgenic plants, but at the same time they will be directly linked to agronomic parameters such as plant height, number of tillers, number of panicles per plant, number of grains per panicle and grain weight. In general, events with the best yields are desirable.

4. Unintended effects of transgenes in the safety assessment of GM crops

The safety of GM crops is crucial for their adoption and has been the object of intense research work. As a result, guiding principles and regulatory frameworks have been established for safety assessment. The Principle of Substantial Equivalence was initially the leading principle for risk assessment. It was developed and agreed internationally (Codex, FAO, OECD, WHO) and established that the safety of a GM crop should rely on comparison with a non-genetically modified isogenic counterpart, that is, harbouring the same genotype except for the transgene(s). More recently, Kok and Kuiper, 2003 proposed the principle be rephrased into the Comparative Safety Assessment strategy, which better reflects its comparative nature and the various different tests involved, including agronomic, morphological and composition characteristics, covering a wide range of crop-dependent parameters such as macronutrients, micronutrients, anti-nutrients, toxins, and secondary compounds. The internationally adopted Codex Alimentarius defines a series of principles and guidelines to assess food safety of foods derived from recombinant-DNA derived plants, animals and microorganisms, including consensus documents on key compositional parameters for the analysis of new plant varieties. The objective of the OECD Task Force for the Safety of Novel Foods and Feeds is to promote international harmonisation in the safety assessment and regulation of novel foods and feeds, including the products of modern biotechnology. The major part of its current activities is the development of consensus documents that provide information on critical parameters of food safety and nutrition for each food crop. Any difference which falls within the range of the normal variability for the crop is considered safe (Colquhoun et al., 2006; EFSA, 2011).

Unintended effects are defined as statistically significant difference in the phenotype, response, or composition of the GM plant compared with the parent from which it is derived, but taking the expected effect of the target gene into account. They can be classified as expected (when the effects are expected and explicable in terms of the present knowledge on plant metabolism and physiology) and unexpected (which are changes falling outside our present level of understanding) (Cellini et al., 2004). Unintended effects can be positive, negative or null, so, they do not necessarily pose safety hazards, but they have to be taken into account.

Although unintended effects resulting from conventional breeding are significant, in the last years this concept has gained importance in relation to the application of recombinant DNA technology. The integration of a transgene in the plant genome is a random process with preference for gene-rich regions (Koncz et al., 1992), so disruption, modification or silencing of active genes and

production of new proteins might occur (transgene position effect). In addition, pleiotropic effects could appear as a result of transgene products interacting with the regulation of other genes or the activity of other proteins (Miki et al., 2009).

The targeted approach to Comparative Safety Assessment strategy has an obvious limitation in the number of compounds that are analysed. Therefore, unintended effects and specially unexpected effects could remain undetected. On the contrary, the so-called ‘‘–omics’’ approaches or profiling techniques permit to analyze a larger number of molecules for possible differences at various cellular levels, mainly gene expression (transcriptomics), proteins (proteomics) and metabolites (metabolomics) (Kier and Petrick, 2008). Although application of profiling techniques as standard procedure in risk assessment of GM crop still needs further development and validation, their usefulness has been demonstrated by numerous recent publications reporting the use of these technologies to evaluate possible unexpected effects of various transgenes in several plant species (Ricroch et al., 2011; Nicolia et al., 2013).

Over the last few years, several studies have been conducted to analyze GMP and isogenic transcriptome profiles (i.e. determination of the expression levels of a large number of genes) in order to detect unintended effects of GMOs. Microarray hybridization is one of the most used techniques so far, although next-generation sequencing (NGS) is rapidly becoming the method of choice for transcriptional profiling experiments. In contrast to microarray technology, high throughput sequencing allows identification of novel transcripts, does not require a sequenced genome and circumvents background noise associated with fluorescence quantification (McGettigan, 2013; Mutz et al., 2013). Transcriptomic studies comparing GM maize varieties of the commercial event MON810 with their near-isogenic counterparts revealed values of differential expression below 1.7%, both using in vitro- and in field-grown maize plants (Coll et al., 2008; Coll et al., 2009; Barros et al., 2010). Similar conclusions were reached for other species and transgenes, reporting limited differences between specific GMOs and comparable non-GM plants (Ammann, 2011; Ricroch et al., 2011) such as *Arabidopsis* (El Ouakfaoui and Miki, 2005; Abdeen et al., 2010), wheat (Gregersen et al., 2005; Baudo et al., 2006), soybean (Cheng et al., 2008), barley (Kogel et al., 2010) and rice (Dubouzet et al., 2007; Batista et al., 2008; Montero et al., 2011). In particular, transgenic rice lines expressing low amounts of two AMPs derived from BP100 had only minor transcriptomic changes compared to the untransformed line, i.e. 0.08% and 0.14% the analysed sequences, respectively (Montero, 2012). These differences were quantitatively similar to those described in other GM lines compared to their conventional counterparts. Moreover, the percentages of sequences with altered expression in GM and non-GM plants were lower than those regulated between different non-GM lines obtained by conventional breeding (Batista et al., 2008;

Cheng et al., 2008; Coll et al., 2008). Thus, transgenesis has smaller impact on the transcriptome than conventional breeding.

Gene expression variability should also be investigated in different locations, climates, seasons and farming practices (Van Dijk et al., 2009). Profiling gene expression studies carried out with MON810 GM and non-GM maize grown in real agricultural conditions report very low numbers of sequences with differential expression, and the identity of regulated sequences is not the same in different pairs of GM and non-GM comparators. Therefore, environmental conditions (growing seasons, agricultural practices and locations) had stronger effects than the genetic modification (Coll et al., 2010a; Barros et al., 2010).

A rice model was recently used to evaluate the possible causes of the unintended differences between GM and conventional isogenic plants, with the final objective of assisting the development of strategies to minimise unintended changes in GMPs (Montero et al., 2011). Analysis of transcriptomic differences between the conventional rice line Senia and the GM fungal-resistant rice line Senia-afp, which constitutively expresses the antifungal protein AFP, allowed distinguishing the impact of (i) the insertion site and associated rearrangements (15% of the sequences with altered expression), (ii) the processes to produce transgenic plants (35% of the sequences with altered expression) and (iii) the transgene expression (50% of the sequences with altered expression) (Montero et al., 2011). This proved that the transgene was not the sole cause of unintended differences between GM and non-GM isogenic rice plants; and that event selection and improvement of the transformation methods (e.g. through floral dip transformation) would result in plants with lower unintended effects.

Additional studies of possible alterations in a broad spectrum of proteins and metabolites in GM and non-GM comparators provide complementary information (Davies et al., 2010). Proteomics technologies allow qualitative and quantitative analyses of proteins, including posttranslational modifications. A two-dimensional gel electrophoresis (2DE) based proteomic approach has been used to compare protein profiles of transgenic *Arabidopsis* (Ruebelt et al., 2006; Ren et al., 2009), maize (Albo et al., 2007; Zolla et al., 2008; Coll et al., 2010b; Barros et al., 2010), tomato (Corpillo et al., 2004) and potato (Lehesranta et al., 2005; Khalf et al., 2010) lines and their non-GM counterparts. These studies revealed low percentages of proteins with significantly altered levels in transgenic and non-GM lines, and the differences in spot quantities fell within the range of natural variability (except for the intended trait). Similarly, metabolomics studies of defined sets of metabolites reported small differences between transgenic and non-GM samples (Piccioni et al., 2009; Davies et al., 2010; Dijk et al., 2010).

5. Strategies for production of recombinant proteins in plants

5.1. Host plants and expression platforms

The choice of a suitable host for molecular farming is crucial, because each plant species has unique characteristics that influence recombinant protein expression. Tobacco can be easily transformed and has a long history as a successful crop system for molecular farming. However, a large number of plant species can be used nowadays including tomato, potato, rice, maize, wheat, *Arabidopsis*, soybean, carrot, banana, pea, lettuce and alfalfa. Production of each recombinant protein in plant systems has its own requirements. The life cycle of the host, biomass yields and scale-up costs are deciding factors. The efficiency of a host depends on many biological and geographical factors, and needs to be evaluated on a case-by-case basis. Self-pollinating species, such as rice, are advantageous as compared to cross-pollinating crops like maize due to the lower probability of unintended gene flow through pollen (Obembe et al., 2011). Downstream processes needed to store and purify recombinant proteins can strongly affect the overall production cost, thus they need to be taken into account.

Many technologies for stable integration of the transgene into the host plant have been developed and used over the years. Tobacco has emerged as the leading species for production of recombinant proteins (Tremblay et al., 2010). The major advantages of this species include the well-established technology for gene transfer and expression, high biomass yield, prolific seed production and the existence of large-scale processing infrastructure (Fischer et al., 2004a; Biemelt and Sonnewald, 2005). Nevertheless, tobacco (except for some varieties like 81V9 [Menassa et al., 2001]) contains high amounts of toxic compounds, nicotine and other alkaloids which have to be removed during the purification process. Alternative leafy crops like alfalfa and lettuce have been explored as suitable hosts for molecular farming (Fischer et al., 2004b; Rosales-Mendoza et al., 2010). Although plant leaves are advantageous in terms of biomass yield, they are limited in terms of storage. Leaves have a short shelf life at room temperature and hence, processing needs to be carried out immediately after harvest to ensure product stability and quality.

In contrast, production of recombinant proteins in seeds can overcome limitations related to protein stability and storage (Lau and Sun, 2009; Boothe et al., 2010). Stöger and co-workers (2000) demonstrated that antibodies expressed in corn seeds remained stable at room temperature for more than three years. In addition, cereal seeds have no or low levels of phenolic compounds (Stöger et al., 2005), which makes the downstream process more efficient and less expensive. However, overall yields of recombinant proteins tend to be much lower in cereal seeds than in

tobacco leaves. Several different crops have been investigated for seed-based production systems, including cereals (rice, wheat, barley, and maize [Ramessar et al., 2008]) and legumes (pea and soybean [Stoger et al., 2005]), being maize the species producing the highest yields of recombinant proteins. Rice has many advantages in common with maize including high grain yields, the ease of transformation and manipulation in the laboratory, and the capacity for rapid scale up. Rice is self-pollinating and has the further advantage that, in the early stages of production, it can be grown in greenhouses. Oil-seeds are emerging as a promising platform for recombinant protein production due to their inherently low associated proteolytic activity and simplified protein isolation via oil body separations (Parmenter et al., 1995).

Transient expression systems are increasingly used to produce recombinant proteins in plants due to the short time of production, high protein yield and regulatory considerations. Production of biomass to transform in transient systems typically involves non-transgenic plants grown in contained environments. *Agrobacterium*-mediated transient assays have been used to produce considerable amounts of recombinant protein within few days or weeks (Pogue et al., 2010; Regnard et al., 2010; Whaley et al., 2011). Interestingly, Medrano and colleagues (Medrano et al., 2009) studied a direct comparison of stable versus transient expression of identical transgenes and obtained 4 to 20-fold higher yields using transient transformation of *Nicotiana benthamiana*. Recombinant protein production in transient expression systems has been developed to large scale and is suitable for industrial application. For example, the Fraunhofer Center for Molecular Biotechnology (Newark, DE) (http://www.fraunhofer.org) developed a scalable, automated plant-based factory using plant viral vector technology to efficiently produce large amounts of pharmaceuticals within weeks.

Large scale production of recombinant proteins can be carried out using *in vitro* culture systems, characterized by confinement of plant biomass in bioreactors under sterile conditions. Although plant cell cultures do not share the perspectives of unlimited scalability, culture in a sterile environment allows precise control over growth conditions and batch-to-batch product consistency (reviewed in Xu et al., 2011). Plant suspension cells, hairy roots and moss fall into this category (reviewed in Xu et al., 2012). Stable cell suspension cultures are generated from undifferentiated clusters of plant cells (callus) which can be dispersed and propagated in a liquid medium. A wide collection of biologically active recombinant proteins have been successfully expressed in plant cells, particularly in BY-2 (Bright Yellow-2) and NT-1 (*Nicotiana tabacum*-1). These strains are fast-growing, robust and readily unergo *Agrobacterium*-mediated transformation and cell cycle synchronization (Tremblay et al., 2010; Bortesi et al., 2012; Kirchhoff et al., 2012).

Aquatic plants are considered potential alternatives for production of recombinant proteins. The aquatic higher plant duckweed seems to be ideal to produce recombinant proteins since it is considered the smallest, fastest-growing and simplest flowering plant (Stomp, 2005). More than 20 therapeutic proteins including Plasminogen (Spencer et al., 2010), Aprotinin (Rival et al., 2008), monoclonal antibodies (Cox et al., 2006), avian influenza H5N1 Hemagglutinin (Guo et al., 2009) and Interferon α2 (De Leede et al., 2008) have been produced in duckweeds with yields up to 7% of TSP. Microalgae could potentially be used as well for large-scale and cost-effective production of recombinant proteins. Current work in this area maily focuses on the well-characterized green unicellular alga *Chlamydomonas reinhardtii* (Potvin and Zhang, 2010; Lauersen et al., 2013).

Adaptation of transgene codon usage to the host preferred genetic code is crucial to optimize recombinant protein production (Kang et al., 2004; Daniell et al., 2009). The genetic code uses 61 nucleotide triplets (codons) to encode 20 amino acids and three codons to terminate translation. Often a single amino acid can be encoded by more than one codon and different species tend to have different codon preferences. It has been reported that the presence of rare codons in mRNA may lead to formation of secondary structures that slow down or pause ribosome movement through that region (Wolin and Walter, 1988; Gustafsson et al., 2004). Horvath et al., 2000 achieved significantly higher levels of β-glucanase (prokaryote gene) in transgenic barley grains vegetalizing (making the sequences more like plant DNA sequences based on codon bias) the sequence of the transgene. In another example, a synthetic *ctx*B gene (originally containing prokaryote-adapted sequence motifs) was optimized by replacing certain codons with codons frequently used in tobacco. The modified *ctx*B gene was expressed in transgenic barley at about 15-fold higher levels than the native gene (Kang et al., 2004). Several codon usage optimization software programs are available like DNAWorks (Hoover and Lubkowski, 2002), Gene Designer (Richardson et al., 2006), and OPTIMIZER (Puigbò et al., 2007).

5.2. Regulation of transcription patterns

Transcription is the primary level at which transgene expression is controlled. This section examines factors affecting transcription rates and their use to increase or regulate transgene mRNA production.

One of the most important variables to design a gene construct for transformation is the promoter. Promoters are DNA sequences located upstream coding regions, which contain multiple *cis*-acting regulatory elements or transcription binding sites (TFBs), specific short DNA sequence motifs of

approximately 5–25 bp (Rani, 2007) involved in transcription regulation. The proximal (core) sequence generally comprises the TATA box (Molina and Grotewold, 2005), where the transcription initiation factor TFIID TBP (TATA-box-Binding Protein) subunit binds, and other *cis*-elements that are binding sites for the basic transcriptional machinery, including RNA polymerase II (Burley and Roeder, 1996; Lee and Young, 2000). Further upstream, the proximal promoter contains primary regulatory elements that are recognized by transcription factors. Although much of the regulatory portion of promoters is in the proximal 500 - 1000 base pairs, more distal sequences often contain regulatory elements. Today, a wide variety of promoters are available to drive the expression of transgenes in plants, either for monocotiledoneous or dicotcotiledoneous crops (Egelkrout et al., 2012). Based on their activity they can be:

i) **Constitutive promoters** are one of the most popular strategies to express proteins in the whole plant. They constantly make gene expression active, irrespective of the environment or developmental factors. They generally confer high levels of accumulation of the recombinant protein. In dicotiledoneous species, the most widely used constitutive promoters are the *Cauliflower mosaic virus* 35S promoter (CaMV35S) (Odell et al., 1985) and various derivatives (Kay et al., 1987) along with other alternative viral-based promoters (Samac et al., 2004). The CaMV35S is less effective in monocotiledoneous plants, probably because of the differences in quality/quantity of regulatory factors. Constitutive promoters suitable to transgenic monocotiledoneous species are e.g. maize and rice Ubiquitin promoters (Christensen and Quail, 1996; Wang and Oard, 2003; Breitler et al., 2004; Lu et al., 2008b), and the rice actin promoter (McElroy et al., 1990).

ii) **Tissue-specific promoters** regulate gene expression in specific tissues or developmental stages, and can be used in transgenic plants in order to exert a minimal effect on overall plant growth and development. Several promoters have been used to target transgene expression to specific organs or tissues. Seed specific promoters are the most widely studied in monocotiledoneous plants due to the ease of accumulation of storage proteins in a small volume, with compact biomass and in a stable environment (Stoger et al., 2005). The main storage tissue for monocot crop seeds is endosperm, which usually accounts for over 80% of the total seed weight and it is the main site for protein accumulation. *Gt-1* rice Glutelin, which confers specific expression in the endosperm in rice (Katsube et al., 1999; Yang et al., 2003; Qu et al., 2008) and other crops (Russell and Fromm, 1997) is frequently used. Other examples are the rice Globulin (Hwang et al., 2002), rice Prolamin (Furtado et al., 2008) and barley Hordein (Furtado et al., 2009) promoters. Other promoters, such as that of maize Globulin-1, target gene expression to the embryo (Belanger and Kriz, 1989; Hood et al.,

2003). Although the chosen promoter is often from the same host species, they often can work in different crops. As an example, the maize Bx7 promoter is active and endosperm specific in barley (Schünmann et al., 2003) and rice (Hwang et al., 2001). Finally, monocotiledoneous promoters to specifically drive transgene expression in tissues such as leaves (Thilmony et al., 2009), floral tissues (Cocciolone et al., 2005), epidermis (Altpeter et al., 2005) and roots (Li et al., 2013) have been developed as well for specific purposes. Tissue-specific promoters have also been widely used in dicots (Egelkrout et al., 2012).

iii) **Inducible promoters** specifically activate transgene transcription under specific conditions. Ideally, the inducible promoter should display nule or very low basal level of gene expression in the absence of the inducing agent, and expression should be reversible and dose-dependent. Moreover, the inducer should be relatively cheap, easy to apply, and non-toxic if the system is to be used for field or commercial purposes (Gatz and Lenk, 1998). Various types of stresses, both biotic and abiotic, induce the expression of a large number of genes. Biotic inducible promoters have been described and used as potential tools for engineering disease-resistance in plants. Some examples are transgenic rice plants conferring resistance to the blast fungus *M. grisea* through the expression of the antifungal *afp* gene under the control of ZmPR4 maize wounding inducible promoter (Moreno et al., 2005). The activity of the promoters of some wheat and rice defence genes has been studied in detail during plant development and in response to wounding (Kovalchuk et al., 2010). Abiotic factors inducing gene expression can be chemical compounds like alcohol (Garoosi et al., 2005), tetracycline (Bortesi et al., 2012) or steroids (Samalova et al., 2005); and as well environmental factors such as drought, salinity, temperature, light etc. The MeGA™ promoter was developed in tobacco by CropTech Corp company (Balcksburg, VA, USA) and enables activation of transgene transcription upon mechanical stress, i.e. without the need of any chemical agent. After plant harvest, recombinant protein production is induced and the newly synthesized protein can be recovered after 8-24 hours (Cramer et al., 1999). A number of plant promoters induced in response to abiotic conditions have been identified and characterized (Sahi et al., 2006; Huang and Xu, 2008; Mittal et al., 2009; Rai et al., 2009; Sarkar et al., 2009; Zou et al., 2009; Yi et al., 2010; Morran et al., 2011; Kovalchuk et al., 2013; Sarkar et al., 2013).

iv) **Synthetic or artificial promoters**. It has been found that the promoter strength depends on the *cis*-regulatory element types, copy number and spacing between them (Gurr and Rushton, 2005). Synthetic promoters can be engineered using *cis*-acting elements from various sources as building blocks. They can be designed using three different strategies.

Firstly, defined motifs can be combined with a strong constitutive promoter. For example, Rushton and co-workers (Rushton et al., 2002) designed pathogen inducible plant promoters by placing multiple *cis*-regulatory elements that can mediate gene expression under pathogen attack upstream of the strong constitutive CaMV 35S promoter. This synthetic promoter could be activated by salicylic acid and various stress types (Mazarei et al., 2008). Alternatively, *cis*-regulatory elements from different promoters can be combined (Sawant et al., 2001); and finally, two strong constitutive promoters can be fused to develop hybrids with either stronger activity or to develop bidirectional promoters (Chaturvedi et al., 2006).

Besides choosing the most suitable promoter, expression constructs should be designed to guarantee transcript stability and translational efficiency. This involves the introduction of 5' and 3' untranslated regions (UTRs), shown to significantly enhance the expression and translation levels of transgenes (Lu et al., 2008a; Sharma et al., 2008).

The 5'UTR is the mRNA region immediately upstream the initiation codon. It contains protein binding elements that modulate translation levels (Gebauer and Hentze, 2004) and has an important role in translation initiation and efficiency (Kozak, 1986; Koziel et al., 1996). The use of 5' UTR sequences from genes of plant virus significantly increases transgene translation efficiencies. It has been determined that the 5'UTR leader sequence of the alfalfa mosaic virus (AMV) strongly enhances transgene expression (Datla et al., 1993). Similar results were obtained in tobacco with the 5'-untranslated sequence of the tobacco mosaic virus (TMV) (Mitsuhara et al., 1996). Eukaryotic 5' UTR sequences, such as the 5'UTR introns of *Arabidopsis thaliana* polyubiquitin genes, can also improve chimeric gene expression (Norris et al., 1993).

The 3'UTR is the mRNA region immediately following the translation termination codon. The 3'UTR or terminator can also significantly influence mRNA stability (Chan and Yu, 1998). Heterologous 3'UTR regions from plants or plant viruses have been used to stabilize recombinant transcripts. Examples are the *Agrobacterium tumefaciens nopaline synthase NOS* and the *octopine synthase OCS* terminator sequences (De Greve et al., 1982; Shao et al., 2010); and the *Cauliflower mosaic virus* 35S terminator (Hirt et al., 1990). Besides virus and bacteria terminators 3'UTR sequences from plant genes have also been used for recombinant protein expression. Examples are the *Arabidopsis thaliana rubisco* small subunit (rbcS), which allowed higher yields of recombinant proteins than the *OCS* terminator in tobacco transgenic plants (Ingelbrecht et al., 1989); the soybean *vspB* and the potato *pin2* terminators, which were more effective than *NOS* in transgenic potatoes expressing the hepatitis B surface antigen (Richter et al., 2000).

5.2.1. Tools for *in silico* identification and characterization of regulatory sequences

As stated above, several promoters have been well characterized and can be used to regulate transgene expression in plants with different purposes. Presently, there is a vast amount of transcriptomic data available online, obtained by microarray hybridization and massive sequencing experiments which simultaneously measure the expression levels of large numbers of genes. This data can be used to identify sets of genes that are coordinately regulated in response to different treatments such as drought, salinity and suboptimal temperature stress, high concentration of nitrates or various hormones (Wang et al., 2003; Nemhauser et al., 2006; Peng et al., 2009; Lorenz et al., 2011; Liu et al., 2013; Rerksiri et al., 2013). Moreover, the transcriptomes of various tissues and organs have been characterized at different developmental stages in a number of plant species (Wang et al., 2010). In particular for rice, several expression databases are available that provide a wide range of gene expression data during development. They include the National Science Foundation (NSF) Rice Array Database (Jung et al., 2008), Yale Rice Atlas Database (Jiao et al., 2009), Collection of Rice Expression Profiles (CREP) Database (Wang et al., 2010) and RiceXPro database (Sato et al., 2011). Hence, the expression patterns of a gene of interest can be characterized *in silico* using these tools.

Moreover, with the rapid advances in sequencing and bioinformatics technology, and the availability of complete genome sequences for several plant species, a number of promoter databases have been developed in the last years to assist the study of transcriptional regulation. Most of the established plant promoter databases have been developed for a particular model species such as *Arabidopsis* (AGRIS [Yilmaz et al., 2011], ATCOECIS [Vandepoele et al., 2009], Athena [O'Connor et al., 2005] and AthaMap [Steffens et al., 2004]) or rice (Osiris database [Morris et al., 2008]). In contrast, promoter databases such as ppdb (Plant Promoter DB) (Yamamoto and Obokata, 2008; Hieno et al., 2013), PlantProm DB (Shahmuradov et al., 2003), PLACE (Higo et al., 1999) and PlantCARE (Lescot et al., 2002) gather information on various plant species. The DoOP database (Barta et al., 2005) holds promoter sequence data for chordates and different plant species, and is supplemented with a search tool capable to find conserved putative regulatory motifs (Sebestyén et al., 2009).

Although *in silico* tools display increasing potency, experimental validation of the functionality of any promoter and its regulatory elements is usually carried out using plant transformation and transgene expression analysis (Hernandez-Garcia and Finer, 2014). Placement of the promoter elements upstream of reporter genes prior to transformation allows validating their function. To

that end, transformation can be performed in a quick and simple transient system, or alternatively stable transgenic plants can be generated. Agro-infiltration of leaves with *Agrobacterium* cultures or particle bombardment are commonly used for rapid evaluation of transient gene expression (Chiera et al., 2007; Santos-Rosa et al., 2008; Hernandez-Garcia et al., 2010a; Hernandez-Garcia et al., 2010b). Stable transformation is a more complex and lengthy procedure, but it provides the most robust information on promoter function. The plant species used to characterize a promoter of interest is important. For example, some constitutive promoters such as those of actin and polyubiquitin have shown contrasting expression levels in different plant species including *Gladiolus*, freesia, Easter lily, tobacco, rice, and rose (Kamo et al., 1995; Joung and Kamo, 2006). In consequence, promoters and their contributing *cis*-elements intended for biotechnological applications should be functionally characterized, as far as possible, in the plant species of interest.

5.3. Subcellular location of the recombinant peptides and proteins

Achieving high levels of recombinant protein accumulation depends on the ratio between its synthesis and degradation. High mRNA levels do not necessary correlate with high product levels (Doran, 2006; Benchabane et al., 2008). The final protein subcellular location is the main aspect which determines the recombinant protein accumulation level.

In the absence of a targeting signal, the cytosol is the default location for protein accumulation. However, this location might not be the best for protein integrity and stability. In the cytoplasm there are high levels of proteases and Ubiquitin, which tags proteins for degradation (Hochstrasser, 1996), negative redox potential and insufficiency of proteins related to the post-translation modifications that may affect the protein folding and functionality (Benchabane et al., 2008). Studies such as those by Schillberg and co-workers (Schillberg et al., 1999) demonstrated that recombinant antibodies are commonly more stable and accumulate up to higher levels when they are targeted to the secretory pathway than in the cytosol.

Endoplasmatic reticulum (ER) is considered an effective subcellular location for recombinant protein accumulation if posttranslational modifications taking place in the Golgi, apoplast or vacuole are not needed (Doran, 2006). ER resident proteins have a C-terminal sequence, usually KDEL or HDEL, which binds to receptors in the Golgi complex, so they are retrieved back into the ER (Pelham, 1990; Gomord et al., 1997). The ER has very low hydrolytic activity, it is very plastic and it provides a neutral environment suitable for most proteins, thus it facilitates accumulation of high amounts of recombinant proteins. A number of reports have demonstrated the positive effect of ER retention

on the yields of recombinant proteins, which usually is 2 to 10-fold higher than the cytosol (Arakawa et al., 1998; Conrad and Fiedler, 1998; Stöger et al., 2000; Vaquero et al., 2002; Avesani et al., 2003; Ko et al., 2003; Moravec et al., 2007; Bortesi et al., 2009).

Alternatively the Zera motif, a prolamine-rich N-terminal domain derived from the maize (*Zea mays*) storage protein γ –zein, has been described to function similar to ER retention signals. In the ER of maize endosperm cells, γ-zein forms large complexes that accumulate in protein bodies (PB), together with other classes of zein polypeptides (Lending and Larkins, 1989). The ability of Zera to form PBs is not limited to native maize seeds, but can be extended to nonseed tissues of plants as well as non-plant eukaryotic hosts including cultured fungal, mammalian and insect cells (Torrent et al., 2009). Hence, fusion of Zera to proteins of interest has proven to induce formation of PBs that facilitate stable accumulation of very high levels of recombinant proteins. In an example, recombinant F1-V hybrid vaccine expressed in *N.benthamiana*, *Medicago sativa* (alfalfa) and *N.tabacum* NT1 cells had at least 3 times higher yields when it was fused to Zera than without this motif (Alvarez et al., 2010). Similar results were obtained with the human growth homorne expressed in *N.benthamiana* (Llompart et al., 2010).

Recombinant proteins can be targeted to other subcellular compartments along the secretory pathway, such as oil bodies. These organelles originate from the ER store plant seed oils. They are delimited by a phospholipid monolayer that is in direct contact with the lipid content (Zweytick et al., 2000) and embedded contain a huge quantity of the small structural protein Oleosin (Murphy, 1993). Recombinant proteins expressed in the seeds of transgenic plants as fusions with Oleosins are targeted to and strongly associated with the oil body (Parmenter et al., 1995; Li et al., 2012).

Seed vacuoles are also attractive compartments to accumulate recombinant proteins using the appropriate signal peptide (Koide et al., 1997; Frigerio et al., 1998; Brown et al., 2003; Jolliffe et al., 2004), although the precise mechanism of deposition of vacuolar storage is not fully understood. However, vacuoles of vegetative tissues tend to have high hydrolytic activity, which can degrade recombinant proteins. Seeds of most plants have protein storage vacuoles (PSVs) (Neuhaus and Rogers, 1998; Jiang and Sun, 2002; Stoger et al., 2005), with lower hydrolytic activity than vacuoles of vegetative tissues (Morita et al., 2002). Various vacuolar sorting determinants such as that of the barley lecitin, the common bean phaseolin, and soybean β-conglycinin α' subunit have been identified and allow targeting recombinant proteins to PSVs (Bednarek and Raikhel, 1991; Frigerio et al., 1998; Nishizawa et al., 2003; Robinson et al., 2005). Nevertheless, there is a high flow of seed storage proteins into PSVs and thus, recombinant proteins expressed in seeds tend to be driven to PSVs without any specific signal (Vitale and Pedrazzini, 2005).

High accumulation of recombinant proteins can be achieved into the cell wall and the intercellular space (apoplast). Some important recombinant secretory proteins, such as antibodies, can be secreted from plant cells and accumulated in the apoplast (Ma et al., 1995; Woodard et al., 2003). Finally, recombinant proteins can be targeted to chloroplasts or mitochondria to fulfill their function, as it occurs with the EPSPS that gives transgenic plants such as RR soybean tolerance to glyphosate herbicides. Alternatively, transgenes can be inserted into the chloroplast or mitochondrial genomes, which often lead to high yields of recombinant proteins (Lee et al., 2011b).

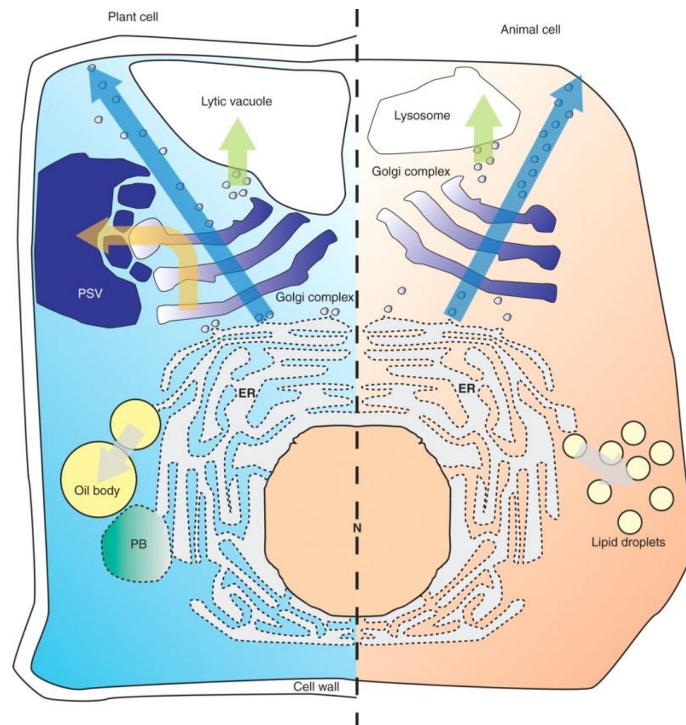

Figure 2. The secretory pathways of plant versus animal cells, and opportunities to exploit plants as bioreactors**.** Secretory proteins enter the ER (delimited here by the broken line, indicating membrane-bound ribosomes) in the two cell types and travel to the Golgi complex via vesicular trafficking. In the absence of ER-retention signals, soluble proteins are then secreted (blue arrows). Vesicular sorting to the inner hydrolytic compartments (lytic vacuoles in plants, lysosomes in animals; green arrows) requires signals that differ in plants and animals. In addition to lytic vacuoles, plant cells may also have protein storage vacuoles (PSV), which develop according to specific signals and protein sorting mechanisms (orange arrow) and may be useful in the biotechnological production of recombinant proteins. Vast amounts of proteins of cereals (e.g., the prolamin storage proteins) form protein bodies (PB) within the ER in aggregation processes that may also be exploited for biotechnology; the aggregation mechanisms are an area of active research and may involve prolonged interactions with chaperones, the formation of inter-chain disulfide bonds, and selective insolubility. Oil bodies and lipid droplets originate from the ER by the deposition of lipids in the space between the two monolayers of the ER membrane (gray arrows). (Extracted from Vitale and Pedrazzini, 2005).

5.4. Fusion proteins

Many different applications for fusion proteins have been reported in widespread areas of biotechnology, including decreasing proteolysis, increasing the yield, facilitating purification of

foreign proteins, and generating reporter molecules to monitor gene expression and protein localization.

5.4.1. Fusion proteins to increase recombinant protein accumulation and facilitate its purification

Although a wide range of plant host systems have been developed for production of recombinant proteins, two major challenges still limit extensive commercial use of plant-made recombinant proteins: inadequate accumulation levels and the lack of efficient purification methods. This is where fusion proteins, which can increase recombinant protein accumulation in heterologous expression systems and/or facilitate their subsequent purification, come into play.

The rationale for using fusion proteins can be as follows: by fusing a recombinant protein of interest to a plant protein that accumulates at high levels in plant cells, the stability of the recombinant fusion protein should be improved. Ubiquitin (Ub), a highly stable and abundant protein in eukaryotes, was the first protein employed as a fusion partner. Garbarino and coworders (Garbarino et al., 1995) and Hondred and colleagues (Hondred et al., 1999) demonstrated that the Ubiquitin fusion strategy resulted in 4 to 10-fold increased levels of recombinant protein. Other fusion partners with demonstrated beneficial effects on the stabilization and accumulation of recombinant proteins in plants include b-Glucuronidase (Gil et al., 2001; Dus Santos et al., 2002), cholera toxin B subunit (Arakawa et al., 2001; Kim et al., 2004; Molina et al., 2004), viral coat proteins (Cañizares et al., 2005) and human immunoglobulin (IgG) α-chains (Obregon et al., 2006).

Protein fusions can also protect recombinant proteins against proteolytic degradation through compartmentalization. Using a fusion partner that accumulates in a particular subcellular compartment that allows stable accumulation, the recombinant protein will be targeted to the same compartment. Some examples are discussed in section 5.3, such as the Zera motif. Other protein fusion partners have been shown to induce the formation of PBs and enhance the accumulation of recombinant proteins. As an example, fusion to Elastin-like polypeptides (ELPs), synthetic biopolymers composed of the repeated pentapeptide sequence 'VPGXG' (where the guest residue X can be any amino acid except proline) (Urry, 1988), resulted in 2- to 100-fold increased yields of recombinant human IL-10 and murine Interleukin-4 (Patel et al., 2007), spider silk proteins (Scheller et al., 2004; Patel et al., 2007), full-size anti-human immunodeficiency virus type 1 antibodies 2F5 (Floss et al., 2008) and 2G12 (Floss et al., 2009) and a scFv antibody (Joensuu et al., 2009) in tobacco leaves, and scFv antibodies in tobacco seeds (Scheller et al., 2006). Similarly, fusion

to Hydrophobins, small surface-active fungal proteins characterized by eight conserved Cys residues that form four intramolecular disulfide bridges and stabilize its structure (Hakanpää et al., 2004), significantly increased the expression of GFP in transiently transformed *N.benthamiana*, up to 51% of TSP (Joensuu et al., 2010).

On the other hand, recovery and purification of recombinant proteins can significantly increase its production cost. Fusion proteins can allow simple, low-cost and reliable purification of target proteins from plant materials, which can be scaled up to industrial levels of protein production (Conley et al., 2011). As an example, accumulation of recombinant proteins in PBs (e.g. via fusion proteins) allows using density-based separation methods (Torrent et al., 2009). Fusion to Oleosin partners and accumulation in oil bodies allows easy separation by flotation centrifugation (van Rooijen and Moloney, 1995). ELPs undergo a reversible inverse temperature transition. When the temperature is raised above the inverse transition temperature (T_t), the normally soluble ESP fused polypeptides form insoluble aggregates which can be separated by simple centrifugation. Subsequently, the aggregate can be resolubilized easily by a temperature shift below T_t (inverse transition cycling process) (Meyer and Chilkoti, 1999). Similarly, fusion to Hydrophobins permits nonchromatographic purification strategies: they are capable of altering the hydrophobicity of their respective fusion partners, thus enabling efficient purification using a surfactant-based aqueous two-phase system (ATPS) (Linder et al., 2004).

Addition of affinity tags or proteins with defined binding characteristics has been often used to assist protein purification (Streatfield, 2007). Many proteins of interest lack a suitable, specific and robust affinity ligand, which can be provided by a fusion partner. The affinity-tagged fusion protein can be easily purified using affinity chromatography techniques. The poly-histidine tag, usually comprising 6 consecutive histidine residues (6xHis), is the most widely used. Purification of His-tagged proteins is based on a chelated metal ion as an affinity ligand (Porath, 1992; Gaberc-Porekar and Menart, 2001; Charlton and Zachariou, 2008; Block et al., 2009; Cheung et al., 2012). The eight-amino acid StrepII epitope was shown to be an easy and fast means of purifying recombinant proteins from plants (Witte et al., 2004). The StrepII tag structurally mimics biotin and binds the StrepTactin streptavidin derivative. It can then be eluted by washing with biotin or desthiobiotin containing buffers (Schmidt et al., 1996; Schmidt and Skerra, 2007). Arg-tag, FLAG-tag, c-myc-tag, Glutathione S-transferase-tag, Calmodulin-binding peptide, Maltose-binding protein or Cellulose-binding domain (Terpe, 2003; Lichty et al., 2005; Rubio et al., 2005; Pina et al., 2013) have successfully been used as tags, not only to facilitate purification of recombinant proteins but also to detect and quantify fusion proteins using tag specific antibodies in western blot, flow cytometry and immunohistochemistry analyses.

It is not uncommon that fusion partners need to be cleaved after purification since they tend to interfere with the structural or functional properties of the recombinant protein of interest (Balbás, 2001). In the particular case of Ubiquitin, this is achieved through an additional proteolytic step driven by Ub-specific proteases. In other cases, this cleavage does not occur naturally and it may be complicated and result in reduced yields (Butt et al., 2005). However, short affinity tags are less likely to interfere with the structure and function of the target proteins and their removal can be avoided, decreasing thus the overall costs of the purification process (Hearn MT, 2001; Terpe, 2003; Hedhammar M, Gräslund T, 2005).

5.4.2. Fusion proteins for use as reporter

Fluorescent fusion partners have been used as interesting tools in cell biology through imaging applications. These fluorescent proteins can act as reporter proteins to monitor gene expression, protein localization and protein-protein interactions. Basically, fluorescence imaging enables visualization of live processes occurring in cells, tissues and organs in a wide range of organisms (van Roessel and Brand, 2002; Lippincott-Schwartz and Patterson, 2003). Depending on the particular purpose, these proteins can be expressed as fusions to heterologous partner proteins or on their own. Nowadays, a number of fluorescent proteins are commercially available (Shaner et al., 2005; Nowotschin et al., 2009), being the most popular β-Glucuronidase (GUS), Luciferase (LUC), Chloramphenicol acetyltransferase (CAT), Green fluorescent protein (GFP) and Discosoma Red fluorescent protein (DsRed). The last one is a tetrameric fluorescent protein (Figure 3) that was discovered and isolated by Martz and colleagues (1999) from reef corals (*Discosoma* sp.). It has 225 amino acid residues and a molecular mass of 25.4 kDa, an excitation peak wavelength around 555 nm (i.e. just above that of chlorophyll) and a maximum emission wavelength of 583 nm, emitting a bright red fluorescent light. Therefore, using the appropriate filter sets, it is possible to excite DsRed specifically thereby avoiding the chlorophyll autofluorescence (Jach et al., 2001). DsRed was first successfully used in plant cells by Más and co-workers (Más et al., 2000), who showed nuclear localized DsRed expression in tobacco BY2 protoplast cells. After that, the DsRed features have been studied and a number of desirable properties have been established: resistance to photobleaching, insensitivity over a wide pH range and extreme temperatures (it is more stable than EGFP, an enhanced GFP mutant, under extreme pH and temperatures, [Verkhusha et al., 2003]), stable conformation, and emission in the red region of the spectrum. However, there are some limitations, mainly its slow maturation, oligomerization and green emitting intermediate. The chromophore of the tetrameric DsRed takes 2-3 days to fully mature and be capable to emit red fluorescence (Matz

et al., 1999; Baird et al., 2000). High molecular weight DsRed aggregates have been reported, probably as a result of electrostatic interactions (as it was suggested based on computational studies). Recently, both random and site-directed mutagenesis approaches have permitted obtaining improved DsRed molecules (Gavin et al., 2002; Mirabella et al., 2004; Strongin et al., 2007).

Figure 3. Structure of DsRed tetramer. Monomers are labeled with uppercase A–D, and the carboxy termini are labeled with lowercase a–d (Yarbrough et al., 2001).

This Thesis focuses on the production of cationic α-helical AMPs derived from BP100 with potent activity against economically relevant phytopathogens, in plant biofactories. We demonstrated that high accumulation of BP100ders is toxic to host plant cells; and developed two alternative strategies to produce this type of peptides in transgenic plant biofactories. First, we proved that the haemolytic activity is an indicator of phytotoxicity and it was suitable to assist the design of BP100ders combining potent antimicrobial activities and minimal impact on the host: transgenic plants were obtained that expressed it with yields up to 0.5% total soluble protein. Secondly, highly phytotoxic BP100ders were produced in transgenic plants through strict control of transgene expression by using inducible promoters, reaching moderate yields. Recombinant peptides accumulated in new ER derived vesicles, which formation depended on the BP100 sequence.

CHAPTER II

OBJECTIVES

In an initial approach to produce AMPs in GM plants, our group transformed rice plants with transgenes encoding two and three BP100 tandem copies for accumulation in the ER and under the control of a constitutive promoter. An unusually low number of GM events were obtained that had very reduced transgene mRNA levels (Montero, 2012). This evidenced the complexity of producing AMPs such as BP100 in plant biofactories.

The **main objective** of this PhD Thesis was to develop strategies to produce cationic α-helical antimicrobial peptides, with potent activities against relevant phytopathogens, in rice plant biofactories. With that goal, the following specific objectives were pursued:

1. Evaluation of the possible phytotoxicity of cationic α-helical AMPs with high activity against economically important bacterial species using the synthetic BP100 and its derivatives as a proof-of-concept; and study of their toxicity against the host plant upon constitutive expression as recombinant peptides for accumulation in the ER.

2. Development of a strategy to select BP100 derived sequences displaying potent antimicrobial activity whereas at the same time, with minimal toxic effect against the host plant. Production of transgenic plants expressing the best candidate sequences.

3. In view of the particular physic-chemical characteristics of BP100, development of strategies based on fusion to fluorescent reporter or tag sequences to detect and quantify recombinant BP100ders produced in plants. Determination of subcellular localization of the recombinant peptides.

4. Development of a strategy to obtain transgenic plants producing recombinant proteins that are highly toxic to the host, by strictly limiting transgene expression to certain tissues and conditions, using heat-shock inducible promoters as an example.

CHAPTER III

Constitutive expression of transgenes encoding derivatives of the synthetic antimicrobial peptide BP100: impact on rice host plant fitness

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Constitutive expression of transgenes encoding derivatives of the synthetic antimicrobial peptide BP100: impact on rice host plant fitness

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Abstract

Background: The Biopeptide BP100 is a synthetic and strongly cationic α-helical undecapeptide with high, specific antibacterial activity against economically important plant-pathogenic bacteria, and very low toxicity. It was selected from a library of synthetic peptides, along with other peptides with activities against relevant bacterial and fungal species. Expression of the BP100 series of peptides in plants is of major interest to establish disease-resistant plants and facilitate molecular farming. Specific challenges were the small length, peptide degradation by plant proteases and toxicity to the host plant. Here we approached the expression of the BP100 peptide series in plants using BP100 as a proof-of-concept.

Results: Our design considered up to three tandemly arranged BP100 units and peptide accumulation in the endoplasmic reticulum (ER), analyzing five BP100 derivatives. The ER retention sequence did not reduce the antimicrobial activity of chemically synthesized BP100 derivatives, making this strategy possible. Transformation with sequences encoding BP100 derivatives (*bp100der*) was over ten-fold less efficient than that of the hygromycin phosphotransferase (*hptII*) transgene. The BP100 direct tandems did not show higher antimicrobial activity than BP100, and genetically modified (GM) plants constitutively expressing them were not viable. In contrast, inverted repeats of BP100, whether or not elongated with a portion of a natural antimicrobial peptide (AMP), had higher antimicrobial activity, and fertile GM rice lines constitutively expressing *bp100der* were produced. These GM lines had increased resistance to the pathogens *Dickeya chrysanthemi* and *Fusarium verticillioides*, and tolerance to oxidative stress, with agronomic performance comparable to untransformed lines.

Conclusions: Constitutive expression of transgenes encoding short cationic α-helical synthetic peptides can have a strong negative impact on rice fitness. However, GM plants expressing, for example, BP100 based on inverted repeats, have adequate agronomic performance and resistant phenotypes as a result of a complex equilibrium between *bp100der* toxicity to plant cells, antimicrobial activity and transgene-derived plant stress response. It is likely that these results can be extended to other peptides with similar characteristics.

Keywords: Antimicrobial peptide AMP, BP100, Transgenic rice, Oryza sativa, Hostplant fitness, Pathogen-resistant rice

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Background

Antimicrobial peptides (AMPs) are short sequence peptides, normally less than 50 amino acid residues, reported in living systems. They are components of the defense system against pathogens in plants and animals or are produced by microorganisms in antibiosis processes (see reviews in [1-3] bacteria; [4,5] fungi; [6,7] insects; [8-10] amphibian and mammals, and [11] plants). Around 1,000 AMPs have been reported [12]. They can structurally be linear peptides (often adopting α-helical structures); cysteine-rich open-ended peptides with disulfide bridges; cyclopeptides forming a peptide ring, or pseudopeptides. AMPs offer major perspectives as a novel class of therapeutic agents, especially against fungal infections and antibiotic-resistant bacterial pathogens in humans and animals [7,9]. This great potential extends to plant disease-protection products [13-15], as substitutes of antibiotics in animal feed, biopreservatives in food, cosmetics and biomaterials, and as antifouling agents [16,17]. AMPs have proved successful as biopesticides, with commercial development of several microorganisms secreting these compounds [14].

In recent years, novel peptides have been designed, based on natural AMPs, with the aim of optimizing the activity against selected target pathogens (including microorganisms against which no AMP or antibiotic are known) while decreasing toxicity to non-target organisms and increasing stability. Short truncated compounds (minimal domain), chimerical constructions and improved sequence analogs have been reported. Examples are mellitin derivatives blocking plant viruses [18], the anti-fungal and anti-bacterial lactoferricin B derivatives [13], antifungal cecropin A and cecropin A-mellitin derived peptides [19-21], and the de novo designed antifungal hexapeptide PAF26 [22,23] and bactericide cyclic decapeptide BPC194 series [19,20].

Genetically modified (GM) plants with different degrees of resistance to pathogens have been obtained by expression of native or synthetic analogues of AMPs, either constitutively or in response to pathogen attack (reviewed in [13,14]). These include AMPs naturally produced by insects [24-29] and amphibians [30]; fungal [15,31] and plant defensins [32-36]; and modified AMP analogues such as the magainine derived Myp30 [37] and MSI-99 [38-40], MsrA3, derived from temporin A [41] and MsrA2, derived from dermaseptin B1 [42], the chimeric peptides MsrA1 and CEMA derived from cecropin A and mellitin [43,44] and the synthetic D4E1 peptide [45,46]. The expression of these AMPs in plants including tobacco, rice, potato, tomato, grapevine and cotton, have been found to give moderate resistance to relevant plant pathogenic bacteria or fungi.

Combinatorial chemistry approaches have been used to assist the design of new AMPs with superior properties. The CECMEL11 peptide library, a 125-member linear undecapeptide library contains groups of sequences with high activity against a number of reporter bacterial and fungal phytopathogenic species, several also exhibiting low-sensitivity to protease degradation and hemolytic activity [47,48]. These peptides were cecropin A-mellitin hybrids and had the structure of an amphipathic α-helix with strong positive charge at neutral pH that can facilitate electrostatic attraction to phospholipid membranes of the target microorganisms, prior to insertion of the hydrophobic face into the membrane bilayer [49,50]. In particular, BP100 (KKLFKKILKYL-NH2) displays strong, selective bactericidal activity against three plant pathogenic bacteria at micromolar concentrations, with poor antifungal properties that inhibit infections by *Xanthomonas vesicatoria* in pepper, *Erwinia amylovora* in apple and *Pseudomonas syringae* in pear [47]. The efficacy of control is comparable to standard antibiotics and it is highly biocompatible, as assessed by acute oral toxicity tests in mice [51].

The envisaged phytosanitary applications of the BP100 series of peptides makes its expression in plant systems relevant, either as potent tools to confer plant phenotypes resistance to bacterial and/or fungal pathogen species, or as biofactories of plant protection products. Phytosanitary use of these peptides against bacterial diseases of plants is limited by the high cost of production. Large-scale chemical synthesis of peptides above around 6 amino acids is only economically viable for applications of very high added value; in view of its low toxicity against animal models, the use of GM plants as AMP molecular farms could putatively be an economic alternative. Production of a number of proteins using plants as biofactories has been reported in pharmaceutical applications [52-54], and a number of companies are currently using different approaches with various proteins [55].

However, expression of AMPs in plants requires specific strategies, due to their particular properties, and has proven challenging. The 17 and 19 amino-acid long D4E1 and MsrA3 are among the shortest peptides expressed in plants [41,45]. However, a number of AMPs with relevant properties are shorter. In particular, combinatorial chemistry approaches used to engineer improved synthetic peptides are usually based on smaller lengths [19,20,22,23,47,48]. For expression in plant systems, the length of these peptides should be increased above a minimum threshold while maintaining biological properties [56]. AMPs with high antimicrobial activity have been associated with high toxicity to transgenic plant cells, and peptides with moderate activity have often been expressed, including those especially modified to decrease the antimicrobial activity (e.g. MsrA1, [44]). Foreign AMPs expressed in plants can be prone to cellular degradation by endogenous peptidases, thereby

limiting the level of accumulation (see e.g. [57]). Targeting the endoplasmic reticulum (ER) has been suggested as a way to improve accumulation levels, due to either the low proteolytic activity in the lumen or the higher folding ability of ER resident chaperones [58,59]. Confining AMPs to the ER compartment would be expected to decrease their potential toxicity to the plant cell. Moreover, GM rice plants expressing ER targeted cecropin A had resistant phenotypes [29]. Interestingly, any change in the peptide sequence, such as enlargement, the introduction of hinge sequences, ER retention signal, or specific tagging, may affect the biological properties, and this would require a considerable level of screening.

To look at BP100 as a proof-of-concept of the possibility of expressing active AMPs of the BP100 series in plants, a number of BP100 derivatives were rationally designed to be produced in plants. The possible influence of these sequence modifications on the expected antibacterial activity was experimentally tested using chemically synthesized BP100 derivatives and specific bacterial growth inhibition assays. The possibility of producing BP100 derivatives with high antimicrobial activity in a plant system was investigated by stable transformation of rice, following a strategy based on constitutive expression and ER accumulation. The impact of this type of peptide on the fitness of the host plant was specifically evaluated. We envisage our results will be applicable to other short α-helical cationic peptides with high antimicrobial activity.

Results

Rationale of the approach - Design of BP100 derivatives suitable for expression in GM plants

BP100 is a synthetic linear polypeptide with only L-amino acids, which is compatible with standard protein synthesis in plant cells. However, BP100 is amidated at the C-terminal position [47]. An unmodified form of BP100 was chemically synthesized, retaining the same amino acid sequence. On analysis, it was found to have similar or slightly lower antibacterial activity and hemolytic activity as BP100 (Table 1), so was selected as a model undecapeptide from the CECMEL11 peptide library for AMP production in plants.

The length of the BP100 undecapeptide, a short molecule, needs to be increased above a minimum threshold to achieve expression in plant systems. Accumulation of this bactericidal peptide in plants could potentially result in BP100 degradation by plant proteolytic activities (e.g. in intercellular spaces) and/or produce plant cell damage even though cytotoxicity and protease susceptibility were minimized during the BP100 selection procedure. This effect is typically diminished in cell compartments such as the ER, where optimal yields of recombinant proteins have often been described. Five BP100 derivative peptides, including up to three copies of BP100 in the same or in opposite orientations, were specifically designed for expression in plants and accumulation in the ER. The derivatives were 15 to 48 amino acids long (see scheme in Figure 1).

It has been previously reported [47] that single amino acid changes in the BP100 sequence have a major effect on its biological characteristics. We tested the antibacterial activity of the newly designed BP100 derivatives by *in vitro* growth inhibition of the three model plant pathogen bacteria *Erwinia amylovora* (*Ea*), *Pseudomonas syringae* pv. *syringae* (*Pss*) and *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*). The different modifications incorporated in BP100 derivatives designed for expression in plants affected the properties of the resulting peptides (Table 1). BP100.1, BP100.2i and BP100.2mi had similar or higher antibacterial activity compared to BP100, and the increase in activity was higher against *Xav* than against *Ea* and *Pss*. BP100.2mi was the most active peptide and BP100.2 and BP100.3 were the least active BP100 derivatives in this assay.

Toxicity of BP100 derivatives

We initially assessed the possible toxicity to plant producer cells of BP100 derivatives by inoculation of chemically synthesized peptides in tobacco leaves in an *in planta* assay. Inoculation of 50 to 250 μM BP100.1, BP100.2, BP100.2i, BP100.3 and BP100.2mi induced lesions in tobacco leaves in a dose dependent manner (Figure 2A), with water as the negative control. The highly cytotoxic peptide mellitin was used as the positive control. BP100 derivatives produced similar or moderately larger lesions than BP100 (Table 1), while mellitin clearly produced more severe lesions at the same doses.

Further assays were carried out with rice. In a germination test, high concentrations of both BP100 and mellitin severely affected seedling development (Figure 2B). Remarkably, the effects of the two peptides were divergent. Mellitin predominantly inhibited root growth while allowing moderate shoot development. In contrast, BP100 barely effected root growth but almost completely inhibited shoot development. The phenotypic effects of BP100 derivatives were similar to those of BP100. Phytotoxic effects were quantified through shoot lengths of seedlings grown in the presence of decreasing concentrations of peptides, and minimal peptide doses causing significantly shorter seedlings were determined (Table 1). Toxic effects were dose dependent. BP100, unmodified BP100 and BP100.1 produced phenotypic effects at 64 μM and higher concentrations (one-way ANOVA P values at 64 μM were 0.001, 0.001 and 0.000, respectively). BP100.2 produced shorter seedlings at 32 μM and higher concentrations (one-way ANOVA P

Table 1 Antibacterial, phytotoxicity and hemolytic activity of BP100 derivatives

BP100 derived peptide	# Amino acids	Antimicrobial activity					Phytotoxicity (germination)	Phytotoxicity at 150 μM (inoculation)	Hemolytic activity at 150 μM
		Ea	*Pss*	*Xav*	*Dc*	*Fv*			
BP100	11	2.5 - 5.0	2.5 - 5.0	5.0 - 10	5-10	<2.5	64	0.90 ± 0.05^{a}	22.0 ± 2.8
unmodified BP100	11	5.0 - 10	5.0 -10	10 - 20	10 - 20	<2.5	64	0.80 ± 0.02^{a}	1
BP100.1	15	2.5 - 5	2.5 - 5	1.25 - 2.5	10 - 20	<2.5	64	1.17 ± 0.09^{ab}	<1
BP100.2	30	5.0 - 10	5.0 -10	5.0 - 10	>40	<2.5	32	1.15 ± 0.07^{ab}	3
BP100.2i	30	2.5 - 5	2.5 - 5	1.25 - 2.5	20 - 40	2.5 - 5	16	1.32 ± 0.06^{b}	4
BP100.3	45	>20	>20	10 - 20	>40	5-10	16	1.38 ± 0.04^{b}	4
BP100.2mi	48	2.5 - 5	2.5 - 5	<0.6	20 - 40	2.5 - 5	16	1.46 ± 0.12^{b}	5

Antibacterial activity was determined against *Erwinia amylovora* (*Ea*), *Pseudomonas syringae* pv. *syryngae* (*Pss*) and *Xanthomonas axonopodis* pv.*vesicatoria* (*Xv*) as reporter species, and against the rice pathogens *Dickeya chrysanthemi* (*Dc*) and *Fusarium verticillioides* (*Fv*).
Minimal inhibitory concentrations (MIC) are given in μM and were calculated with 10^8 bacterial CFU/ml or 10^4 conidia/ml. Phytotoxicity values in rice germination assays are given as minimal peptide concentrations (μM) causing significant alteration of shoot length. Phytotoxicity values in tobacco leaf inoculation assays are given as the mean of the lesion diameter (cm) and standard error upon inoculation of 150 μM peptide solutions. Letters indicate different groups in one-way ANOVA analysis and Tukey's b posttest with α = 0.05. Hemolytic activity is given as the ratio between each peptide and the reference peptide BP100 (calculated at 150 μM and given in % with confidence interval for α = 0.05 [47]).

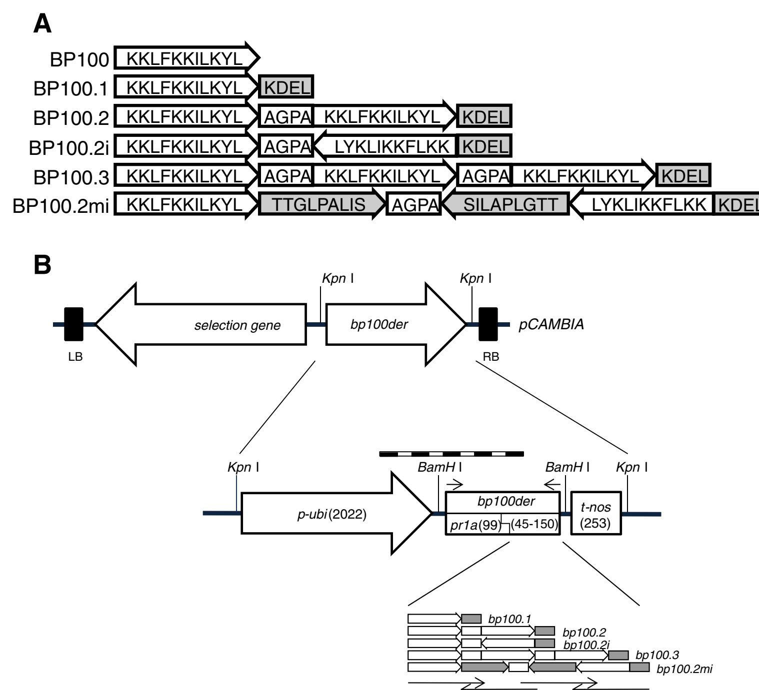

Figure 1 Schematic representation of the BP100 derivatives designed and the components of transgene cassettes in plasmids used for rice transformation. (A) White arrow, BP100; grey arrow, mellitin extension (nucleotides 19–18); white rectangle, linker sequence; grey rectangle, ER retention sequence. Arrows indicate the orientation of the sequences. **(B) Top**, schematic diagram showing the selection (*hptII*) and *bp100der* genes in a pCAMBIA1300 plant expression vector. LB and RB, *A. tumefaciens* left border and right border sequences. Expression of the *bp100der* genes is driven by the maize ubiquitin promoter (*p-ubi*), including the first exon and first intron (overall 1061 bp) and the nos terminator (*t-nos*). The hygromycin phosphotransferase *hptII* gene was used in combination with the cauliflower mosaic virus 35 S promoter and terminator sequences, *p-35 S* and *t-35 S* (overall, 2015 bp). *bp100der* length is between 2419 and 2569 bp. **Center**, regulatory and coding elements in the *bp100der* gene. The different sequence elements are not drawn to scale. Lengths (in base pairs) are indicated in brackets. Dashed box corresponds to the probe used in Southern analysis, and arrows above *bp100der* indicate RT-qPCR primers. **Bottom**, sequences encoding the BP100 derivatives: white, AGPA; grey, KDEL; light faded rectangle, BP100; dark faded rectangle, mellitin fragment. Color fading indicates the orientation of each amino acid sequence. Arrows below represent oligonucleotides (corresponding to *bp100.3*) used for recursive PCR. Restriction sites relevant for cloning are indicated.

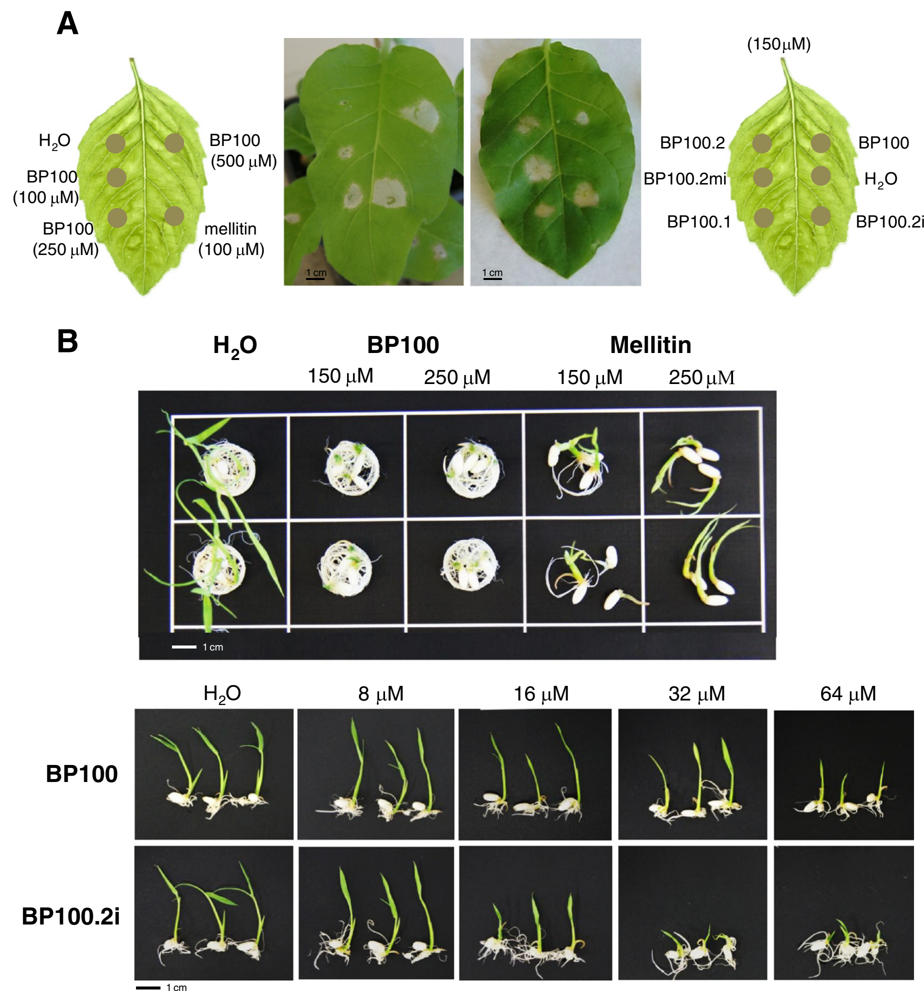

Figure 2 Phytotoxicity of chemically synthesized BP100 derivatives. (A) Representative examples of tobacco leaves, three days after inoculation with H_2O, BP100, BP100 derivatives and mellitin. **(B)** Inhibition of germination assay. **Top**, representative examples of rice Senia seeds germinated for 7 days in H_2O or high concentrations of BP100 and mellitin. **Bottom**, representative examples of rice Senia seeds germinated for 7 days in H_2O or decreasing concentrations of BP100 and BP100.2i. Scale bars: 1 cm.

value at 32 μM, 0.000); and BP100.2i, BP100.3 and BP100.2mi were toxic at 16 μM and higher concentrations (for each peptide at 16 μM, one-way ANOVA $P = 0.000$) (Figure 2C).

These results demonstrated that BP100 and its derivatives were toxic to plant cells when applied at high doses. The toxicity to mammalian cells of BP100 derivatives was tentatively assessed as the ability to lyse erythrocytes in comparison to a potent hemolytic agent, mellitin. As shown in Table 1, BP100 derivatives generally had higher hemolytic activities than BP100 (except for BP100.1), and this activity seemed to increase with peptide length.

Effect of *bp100der* transgenes on the efficiency of transformation

We assessed the feasibility of synthetic AMP production in plants using rice as the model, and on the basis of constitutive transgene expression and AMP accumulation in the ER to protect foreign peptides from potential degradation by proteolytic activities and plant cells from phytotoxic activity of peptides. To get a broad picture of the potential and possible limitations of this approach, all five BP100 derivatives were used for transformation, even though they had different characteristics (see above). Chimeric genes encoding all five BP100 derivatives (i.e. *bp100.1*, *bp100.2*, *bp100.2i*, *bp100.3* and *bp100.2mi*, collectively named *bp100der*) were synthesized and used in a series of transformation experiments designed to quantify the efficiency of the process in terms of amounts of transgenic calluses and plants that could be obtained for each BP100 derivative. A total of 1,200 rice calluses were transformed with each AMP plasmid and the empty vector, with the *hptII* selection gene and no *bp100der* sequence, and the numbers of

Table 2 Progress of the rice transformation with transgenes encoding BP100 derivatives

BP100 derived peptide	Hygromycin resistant calluses*	Regenerated plants*	Acclimated plants*	Plants producing seeds*	Overall efficiency of the process compared to control plasmid
control	110**	80%	100%	100%	
BP100.1	43 (39%)	21 (61%)	10 (48%)	9 (90%)	10%
BP100.2	23 (21%)	0 (0%)			<1%
BP100.2i	82 (75%)	20 (30%)	5 (25%)	2 (40%)	2%
BP100.3	20 (18%)	0 (0%)			<1%
BP100.2mi	83 (75%)	8 (12%)	8 (100%)	3 (38%)	3%

Absolute numbers and percentages relative to transformation with the control plasmid are given. Control transformation data is given in absolute numbers of hygromycin resistant calluses and percentages of transgenic plants, always relative to the previous step.
*A total of 1200 calluses were transformed with each plasmid in three independent experiments, each including transformation with the control vector. Only hygromycin resistant calluses and plants from different calluses (i.e. independent transformation events) were recorded.
**Only 15 of the 110 control hygromycin resistant calluses were further processed.

transgenic calluses were recorded. Regeneration and development of transgenic plantlets were also monitored (Table 2).

Transformation with all five *bp100der* plasmids resulted in growth of hygromycin-resistant transgenic callus. However, we found major differences in the efficiency of the transformation among plasmids with sequences encoding different BP100 derivatives. Transformation with control plasmids consistently gave the highest numbers of transgenic calluses in our experimental conditions. Slightly lower numbers of calluses showing the selection phenotype were obtained with plasmids carrying *bp100.2mi* or *bp100.2i*, while much reduced numbers of transgenic calluses carrying *bp100.1* and especially *bp100.2* and *bp100.3* were identified.

To confirm the functionality of *bp100der* transgenes in callus with the selection phenotype, we assessed transgene mRNA expression in three randomly chosen transgenic calluses for each construct by RT-qPCR (RNA extraction and cDNA synthesis were systematically performed in duplicate). All analyzed calluses had been subjected to various subculture steps to minimize non-transgenic cells. In a preliminary test we assessed the mRNA levels of three reference genes; elongation factor EF1α, β-actin and 18 S RNA ribosomal genes. In all our callus samples, β-actin had the best score upon application of the GeNorm algorithm (M value = 0.48), so was selected for normalization.

All analyzed calluses expressed both selection marker and (except for those with an empty vector) the corresponding *bp100der* transgenes (Additional file 1). As expected, transgene expression levels calculated by comparison to β-actin showed great variation among the different events, with relative standard deviations (RSD) of normalized transgene mRNA copy numbers of 63% and 117% for *hptII* and *bp100der*, respectively. Compared to β-actin, the amount of selection gene mRNA in GM callus was generally 1- to 10-fold higher, and that of *bp100der* mRNA 10- to 110-fold higher. The *hptII* selection gene was similarly expressed in transgenic callus transformed with plasmids carrying different *bp100der* sequences or the empty vector (5.8 ± 3.7 -fold those of actin; one-way ANOVA $P = 0.630$). Transgenes encoding distinct BP100 derivatives were expressed at statistically similar levels in calluses exhibiting the selection phenotype (30.3 ± 35.5 -fold those of actin; one-way ANOVA $P = 0.283$), although, the lowest *bp100der* expression values consistently corresponded to *bp100.2*.

Callus with the *hptII* selection gene and *bp100.2* grew slowly, suffered necrosis and never regenerated GM plants. Although they grew normally, none of the ~20 *bp100.3*-transformed hygromycin-resistant calluses achieved plant regeneration, so no GM plants expressing this AMP (S-bp100.3) could be obtained with this approach. In contrast, plant regeneration was possible for control, *bp100.1*, *bp100.2i* and *bp100.2mi* transformants (S-hgr, S-bp100.1, S-bp100.2i and S-bp100.2mi plants, respectively), although at decreasing rates. The presence of the *bp100der* transgene was assessed by qPCR in leaf samples of all regenerated plantlets. Two plants, transformed with *bp100.1-* and *bp100.2i*-containing plasmids, were qPCR negative and were discarded. An additional plant produced a *bp100der* qPCR product of different melting temperature than expected. Sequencing of the insert proved the *bp100.2mi* sequence in this event carried a copy of *bp100.2mi* with a deletion and was also discarded. Finally, 21, 20 and 8 T0 plantlets were obtained which carried the *bp100.1*, *bp100.2i* and *bp100.2mi* transgenes, respectively (Table 2). Less than half of these T0 plantlets completed acclimatization and were fertile (although all controls did). The capacity of transgenic cells carrying *bp100der* sequences to grow as callus, regenerate plants, survive greenhouse standard growth conditions and produce viable seeds was visibly inferior to controls with the empty vector. The efficiency of the whole transformation process was only 10% the control for S-bp100.1, and as low as 2-3% for S-bp100.2i and

S-bp100.2mi (no S-bp100.2 and S-bp100.3 GM plants were obtained). We concluded that *bp100der* had a negative effect on transgenic callus and plants; different *bp100der* sequences had different degrees of phytotoxicity in specific steps of the transformation process.

Molecular and cellular characterization of transgenic plants

A total of fourteen fertile transgenic plants were obtained carrying *bp100.2i* (2 independent events), *bp100.1* (9 independent events) or *bp100.2mi* (3 independent events). As all T0 transgenic plants originated from independent calluses, they corresponded to different transformation events. For selection of representative GM events, on the basis of insert copy number and *bp100der* expression levels, genomic DNA extracted from leaf samples of S-bp100.1 and S-bp100.2mi mature T0 plants was analyzed by qPCR targeting *bp100der*, *hptII* and actin, to determine approximate transgene copy numbers. Ratios of transgene to actin copy numbers were close to 0.5 for all events, suggesting they all had single copy insertions. Additional leaf samples of the same plants were subjected to RNA extraction and RT-qPCR analysis to compare *bp100der* mRNA levels. *bp100.1* was similarly expressed in the nine different S-bp100.1 events (24.9 ± 5.4 -fold actin mRNA), and *bp100.2mi* was expressed at similar levels in all three S-bp100.2mi events (2.1 ± 0.8 -fold). Three events carrying *bp100.1* (S-bp100.1-6, S-bp100.1-9 and S-bp100.1-10) and two with *bp100.2mi* (S-bp100.2mi-1 and S-bp100.2mi-9) were selected, in addition to the two events obtained for *bp100.2i* (S-bp100.2i-5 and S-bp100.2i-42).

The seven selected GM events were self-crossed to produce the homozygous T2 generation of GM plants: three independent homozygous lines harboring the *bp100.1* transgene and two with each, *bp100.2i* and *bp100.1* were obtained. Southern blot and qPCR analyses confirmed that all had single copies of the transgene (Additional file 2).

Transgene expression was assessed by RT-qPCR in leaves of homozygous plants grown under controlled conditions and sampled at the two-leaf stage. Three biological replicates of ten plants were analyzed per transgenic event. Every transgenic line expressed the corresponding *bp100der* mRNA in their leaves. Yields of transgene mRNA were calculated by comparison to actin as the reference gene (GeNorm M value < 0.5 in these samples) (Additional file 3). As expected, the expression levels of the transgenes varied in the different events: not only copy number but also the integration site is known to influence transgene expression levels [60].

We then compared transgene expression levels between plants transformed with different *bp100der* plasmids (Figure 3). S-bp100.1 and S-bp100.2i plants expressed higher amounts of *bp100der* mRNA in their leaves than S-bp100.2mi (one-way ANOVA *P* value, 0.000). *hptII* mRNA levels were similar in transgenic plants transformed with the different plasmids, including those with the empty vector (one-way ANOVA $P = 0.060$). The presence of *bp100der* or the specific *bp100der* incorporated in GM plants did not affect the expression levels of the selection gene.

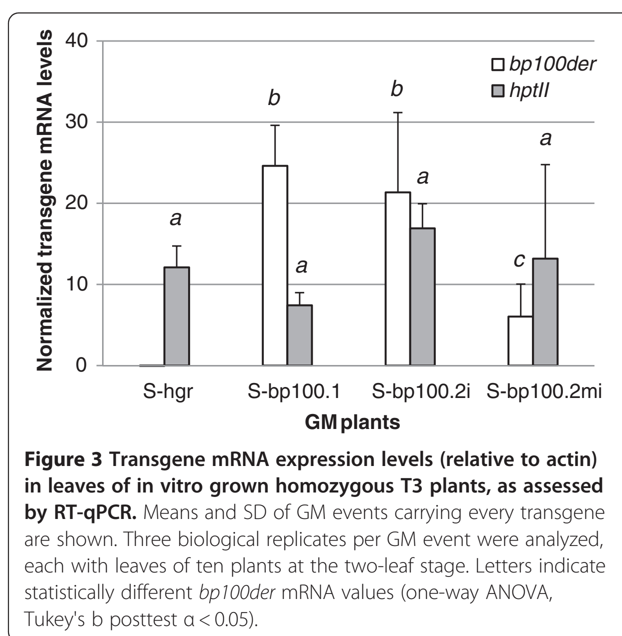

Figure 3 Transgene mRNA expression levels (relative to actin) in leaves of in vitro grown homozygous T3 plants, as assessed by RT-qPCR. Means and SD of GM events carrying every transgene are shown. Three biological replicates per GM event were analyzed, each with leaves of ten plants at the two-leaf stage. Letters indicate statistically different *bp100der* mRNA values (one-way ANOVA, Tukey's b posttest α < 0.05).

The *bp100der* transgenes encode highly cationic peptides and were designed with ER signal peptide and retention sequences. Accumulation of the *bp100der* product in the ER could affect the morphology of this organelle. S-bp100.2i.5, S-bp100.1.9, S-bp100.2mi.1 and Senia plants were compared at the ultrastructural level by TEM observation of the crown region of seedlings at the two-leaf developmental stage. Morphology of the ER was clearly altered in S-bp100der cells accompanying vascular cells (Figure 4), with wider and more variable intra-cisternal spaces of ER cisternae and an increase in dictysome vesicles. There was no clear morphological disorganization in organelles such as mitochondria (Figure 4C) and chloroplasts. An increasing number of vesicles were also formed, particularly in S-bp100.2mi cells, and an accumulation of numerous electron-dense granules was detected in parenchyma cells of S-bp100.2mi and S-bp100.1 (Figure 4D).

Resistance of GM plants to pathogen infection and tolerance to oxidative stress

An indirect approach was used to obtain evidence of the production of BP100 derivative AMPs in our transgenic rice lines, testing their resistance to infection with the bacterial pathogen *Dickeya chrysanthemi* and the fungus *Fusarium verticillioides*, and tolerance to oxidative stress. Seeds from all the homozygous lines obtained were incubated in the

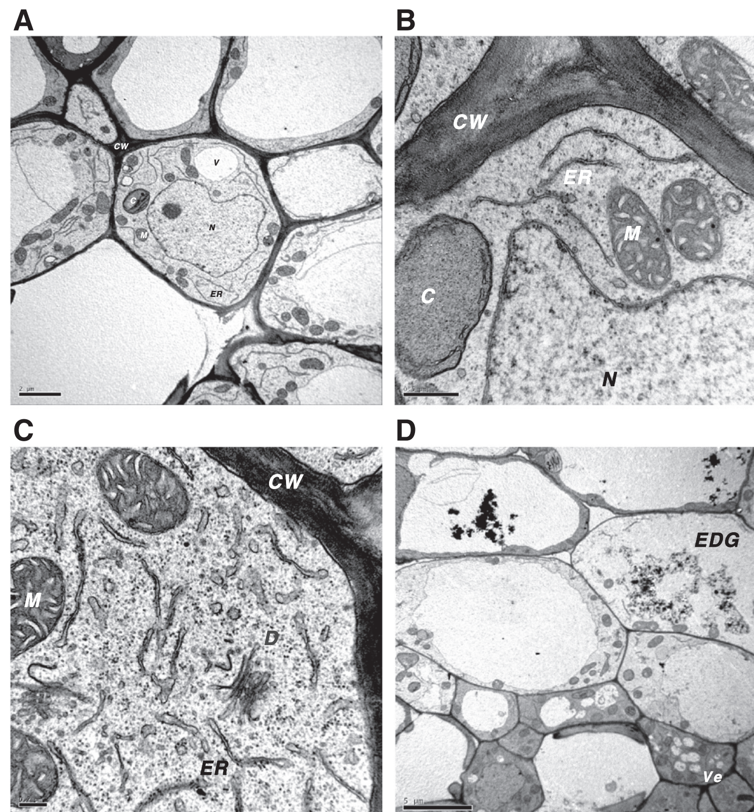

Figure 4 Transmission electron micrographs of rice cells expressing *bp100der* transgenes. (A) Control Senia vascular and surrounding, and parenchyma cells of the crown region showing normal morphology. **(B)** Detail of endoplasmic reticulum morphology of Senia cells. **(C)** Cells surrounding vascular cells, showing increased abundance of dictysome vesicles and distinct dilation of ER cisterna in S-bp100.2i cells. **(D)** Vesicles in S-bp100.2mi cells surrounding vascular cells and accumulation of electron dense granules in parenchyma cells. CW, cell wall; D, dictysome; EDG, electron-dense granules; ER, endoplasmic reticulum; M, mitochondria; N, nucleus; Ve, vesicle. Scale bars: 2 μm **(A)**, 0.5 μm **(B)**, 0.2 μm **(C)** and 5 μm **(D)**.

presence of *D. chrysanthemi* and *F. verticillioides*, and germination was monitored. Homozygous plants from all lines were treated with H_2O_2 in detached-leaf assays.

With Senia seeds, infection with 10^2 to 10^6 *D. chrysanthemi* CFU resulted in progressively smaller, brownish roots and shorter shoots, with germination almost completely inhibited at the highest dose. The pathogenic effect of *D. chrysanthemi*was estimated using a semi-quantitative scale (Figure 5A), and the values recorded seven days after infection ranged from 1.9 (negative control) to 0.4 (10^6 CFU).

We selected 10^5 CFU (with a value of 0.8) to assess susceptibility of S-bp100der seeds to this pathogenic bacterium. S-bp100.1 and S-hgr lines had similar sensitivity values as Senia (one-way ANOVA *P* values, 0.664 and 0.953, respectively), indicating that *hptII* or *bp100.1* did not significantly modify resistance to this bacterial pathogen. In contrast, S-bp100.2i and S-bp100.2mi lines were more resistant than untransformed plants (one-way ANOVA *P* values, 0.004 and 0.036, respectively) (Figure 5B and 5C), signifying that *bp100.2i* and *bp100.2mi* transgenes decreased rice susceptibility to *D.chrysanthemi*.

Seven days after *F. verticillioides* inoculation of Senia seeds, a cottony mycelium covered the surface of the remaining seed and seedlings showed severe growth effects. The primary embryonic root was shorter than that of uninfected controls, with a brownish tone, and there was little or no crown root production. Shoots were shorter in infected seedlings. The *F. verticillioides* pathogenic effect was estimated through a semi-quantitative index that reflected shoot height and root development (number and length of crown roots, and length of primary root).

There were clear differences in the effects of this fungus on plants harboring different transgenes. Those transformed with the control plasmid, *bp100.2i* and *bp100.1* had major morphology alterations with susceptibility values similar to Senia (one-way ANOVA *P* values, 0.562, 0.697 and 0.278, respectively). In contrast, all events harboring *bp100.2mi* had long shoots and well developed root systems (Figure 5D), which resulted in higher resistance

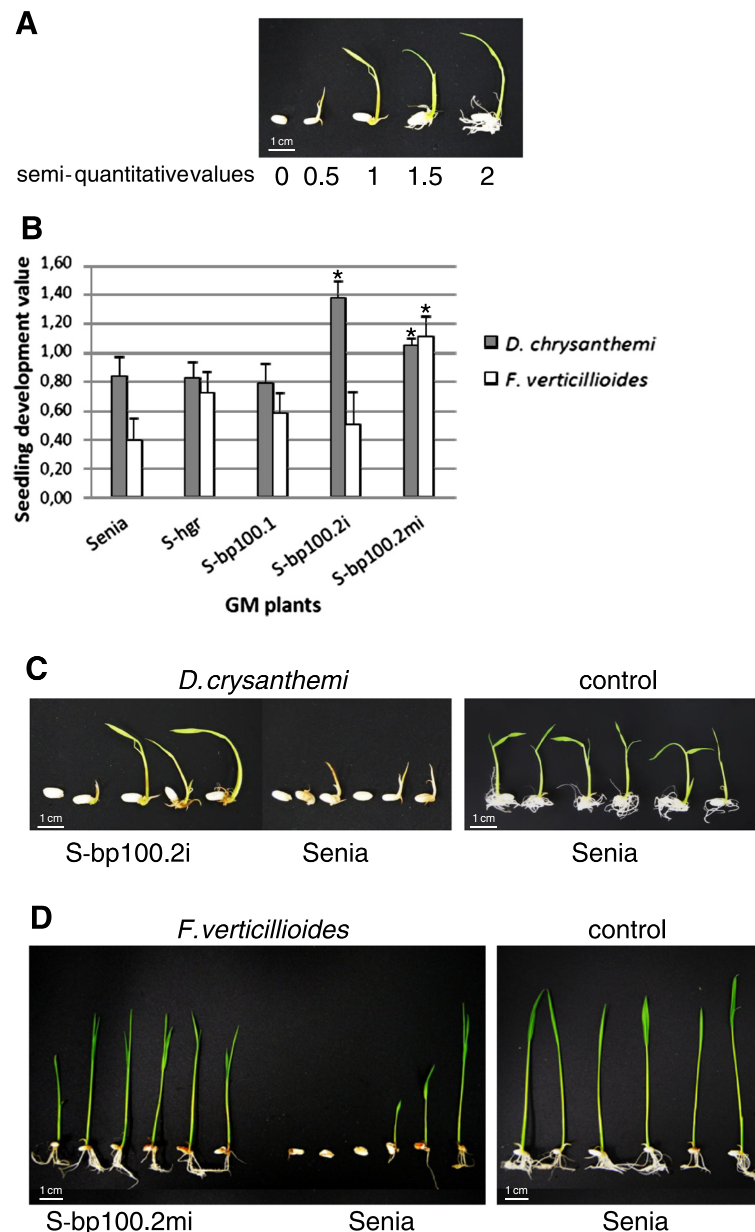

Figure 5 Susceptibility to *Dickeya chrysanthemi* (*D. chrysanthemi*) and *Fusarium verticillioides* (*F. verticillioides*) of control untransformed Senia and S-*bp100.1*, S-*bp100.2i*, S-*bp100.2mi* and S-hgr GM plants in a germination assay. (A) Representative examples of seedlings with different semi-quantitative development values to estimate *D.chrysanthemi* effects: 0, no germination; 0.5, cotyledon development; 1, standard shoot but no root development; 1.5, standard shoot development but short roots; and 2, standard growth. **(B)** Means and standard errors of seedling development values are represented as a function of the transgene in each line (every homozygous line obtained in this work was analyzed). Both for *D. chrysanthemi* and *F. verticillioides* susceptibility, a value of 0 indicated no germination while a value of 2 represented development similar to uninfected seeds. Asterisks indicate statistically significant differences compared to Senia (one-way ANOVA *P* values are given in the text). **(C)** Representative examples of S-bp100.2i (**left**) and Senia (**center**) seedlings grown in the presence of 10^5 CFU *D. chrysanthemi*. Germination of Senia control seeds (**right**). **(D)** Representative examples of S-bp100.2mi (**left**) and Senia (**center**) seedlings grown in the presence of 10^5CFU *F. verticillioides* and germination of Senia control seeds (**right**). Scale bars: 1 cm.

values (one-way ANOVA $P = 0.005$). This suggested that rice was protected against infection with *F. verticillioides* by *bp100.2mi* transgene but not *hptII*,*bp100.1* or *bp100.2i*.

Automated growth curve analysis demonstrated that the presence of BP100 derivatives in the culture broth resulted in inhibition of *D. chrysanthemi* and particularly

F. verticillioides growth in a concentration-dependent manner. The MIC values for the relevant BP100.1, BP100.3 and BP100.2mi peptides ranged from 10 to 40 and <2.5 to 5 μM, respectively (Table 1), which is indicative of their activity against this microorganism.

After treatment with H_2O_2, Senia untransformed leaves had the widest NBT stained leaf surface (7.3% ± 5.9), indicating accumulation of superoxide radicals (O_2^-). S-hgr rice lines carrying *hptII* but no *bp100der* sequences had slightly lower, statistically undistinguishable, NBT staining values (4.0% ± 3.9). NBT staining was somehow lower in S-bp100.1 lines (2.2% ± 2.0), but one-way ANOVA indicated they were similar to S-hgr ($P = 0.408$), suggesting that there was no clear improvement of stress tolerance with the *bp100.1* transgene compared to control leaves. The accumulation of O_2^- was considerably lower in S-bp100.2i and S-bp100.2mi rice lines than in untransformed and S-hgr control plants (0.2% ± 0.1 and 0.3% ± 0.7 stained area, respectively; one-way ANOVA $P = 0.001$; two groups in Tukey's b posttest $\alpha = 0.05$) (Figure 6), indicating *bp100.2i* and *bp100.2mi* transgenes resulted in increased tolerance to oxidative stress.

Performance of GM rice plants with *bp100der* transgenes

Plants from all S-bp100.2i and S-bp100.2mi homozygous lines obtained in this work were compared with control Senia in terms of agronomic parameters. For each line, three replicates of 10 plants each were analyzed. Growth and yield of control plants were as normal in our culture conditions. No significant differences were found between independent replicates of any particular GM line, and the different lines harboring the same *bp100der* transgene gave similar values for all studied parameters (one-way ANOVA P values above 0.05).

Slight differences were observed between control plants and those with different transgenes. S-bp100.2i plants were shorter than Senia (observations on 08/25/10), with mean height and standard error values of 90.6 ± 0.9 and 105.3 ± 1.1 cm, respectively (Additional file 4). In contrast, S-bp100.2mi plants were a similar height to the controls (107.8 ± 0.9 cm) (one-way ANOVA $P = 0.000$, two groups upon Tukey's b posttest with $\alpha = 0.05$). All lines had the same amount of tillers per plant (7.9 ± 0.2; one-way ANOVA $P = 0.854$). Leaf chlorophyll content was measured during plant growth and no significant differences

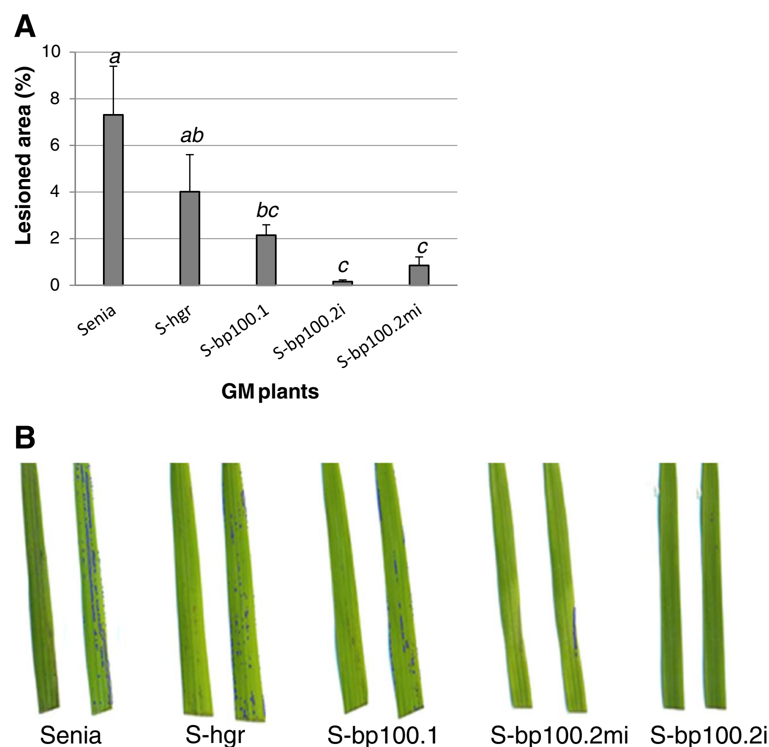

Figure 6 Tolerance to H_2O_2 treatment (oxidative stress) of control untransformed Senia and S- *bp100.1*, S-*bp100.2i*, S-*bp100.2mi* and S-hgr GM plants in a detached leaf assay. **(A)** Ten plants from every homozygous line obtained were analyzed. Means and standard errors of the percentages of affected areas are represented as a function of the transgene in each line. Letters indicate statistically significant differences (one-way ANOVA, Tukey's-b posttest with α value < 0.05). **(B)** Representative examples of leaves subjected to H_2O_2 treatment. The right of each pair, labeled in blue, shows NBT stained areas used to calculate percentages of lesion areas (APS assess tool).

were observed between S-bp100.2i and Senia lines. S-bp100.2mi exhibited slightly higher values, although they were all in a narrow range (42.8 to 44.6 spad units, observations on 08/25/10) (one-way ANOVA $P = 0.012$, two groups on Tukey's b posttest with $\alpha = 0.05$).

The Senia control line had better yield, corresponding to the weight of all panicles in a plant, than lines harboring *bp100der* sequences, and S-bp100.2mi had better yield than S-bp100.2i (Figure 7A). We refined our yield data by independently considering the number of panicles per plant, number of grains per panicle and grain weight. Senia grains were heavier than those of S-bp100.2mi and S-bp100.2i lines (one-way ANOVA $P = 0.05$), although overall differences were in a very narrow range (3.6 to 3.8 g/100 grains), as expected. As shown in Figure 7B Senia, S-bp100.2i and S-bp100.2mi had similar numbers of panicles per plant (mean and standard error, 6.2 ± 0.1 panicles/plant), but S-bp100.2mi and especially S-bp100.2i had less grains per panicle, which explained the lower yields.

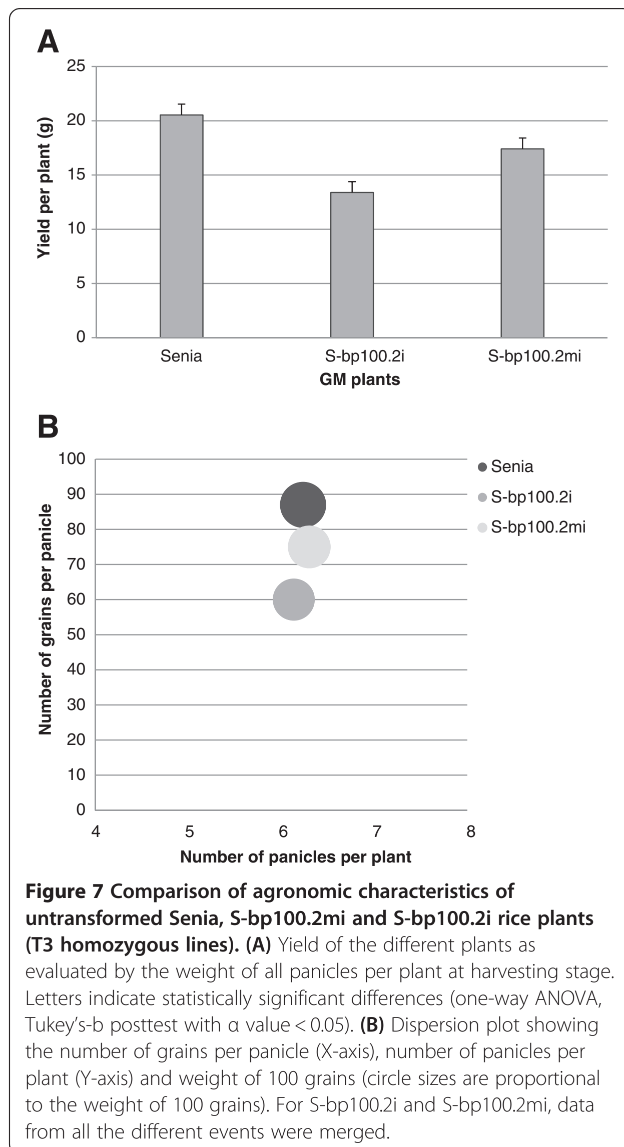

Figure 7 Comparison of agronomic characteristics of untransformed Senia, S-bp100.2mi and S-bp100.2i rice plants (T3 homozygous lines). (A) Yield of the different plants as evaluated by the weight of all panicles per plant at harvesting stage. Letters indicate statistically significant differences (one-way ANOVA, Tukey's-b posttest with α value < 0.05). **(B)** Dispersion plot showing the number of grains per panicle (X-axis), number of panicles per plant (Y-axis) and weight of 100 grains (circle sizes are proportional to the weight of 100 grains). For S-bp100.2i and S-bp100.2mi, data from all the different events were merged.

Complementary experiments were carried out under similar conditions to compare the performance of S-hgr lines (with the *hptII* selection gene) and untransformed Senia. A total of 5 independent S-hgr events were evaluated. Four were statistically undistinguishable from Senia in terms of all analyzed parameters. One single S-hgr line had similar chlorophyll contents to Senia but it was slightly smaller (99.9 ± 0.6 cm) and had more tillers (9.9 ± 0.4 tillers per plant). There were more panicles, with fewer grains per panicle but of the same weight as in Senia, which gave rise to the same yield as untransformed lines (20.7 ± 0.7 g/plant). These were considered event dependent features.

Discussion

Several synthetic linear undecapeptides of the CECMEL11 peptide library are of increasing interest for future development of fungicides and bactericides against plant pathogens [51]. The linear peptide BP100 is of particular interest due to its properties against major bacterial plant pathogens. Biotechnological production of this short, highly positively charged (pI = 11.02), α-helical amphipathic peptide, which could also damage producing plant cells, is challenging. The aim of this research was to investigate the feasibility of expressing transgenes encoding BP100-derived AMPs in transgenic plants using rice as the model host, particularly focusing on the putative impact on the fitness of the host plant. BP100 was used as a model for short cationic AMPs, which are recognized as a remarkable source of active substances with many different applications in diverse sectors besides plant protection [61]. We established the strategy of transgenic peptide accumulation in ER both to minimize the putative toxic effect and to protect it from plant proteases [29,57,62]. The length increase was addressed by designing larger peptides based on the BP100 sequence.

We found that any modification incorporated in BP100 derivatives designed for plant expression altered their properties in terms of antibacterial activity and range of target bacterial species. This is in agreement with previous data on how subtle changes in a peptide sequence of a given length influence antimicrobial activity [19,23,47], and with reports identifying length and sequence among the most relevant factors for biological activity of peptides [63]. It is therefore necessary to assess the biological activity and other relevant properties of any derivative of a given peptide prior to expression in plant systems.

The presence of the C-terminal KDEL element does not result in loss of antibacterial activity (see particularly the

comparison between unmodified BP100 and BP100.1), which makes the strategy of peptide accumulation in ER possible. Size increase based on BP100 tandem copies did not result in a proportional improvement of activity, but tended to reduce it. In contrast, peptides with two units in inverted orientation (BP100.2i and BP100.2mi, each unit of the latter elongated with a mellitin fragment) had higher activity than BP100 against the three reporter bacteria. This increase was especially relevant against *Xav*, demonstrating that sequence variations had an effect on target specificity, which could be related to membrane composition. In view of the antimicrobial activity of these BP100 derivatives, they were considered good candidates to evaluate the possibility of expressing synthetic, highly active antimicrobial peptides in plants.

Monitoring the *Agrobacterium* based transformation process showed that *bp100der* transgene products exerted a toxic effect on constitutively producer plant cells, although BP100 has very low toxicity in hemolysis tests [47] and in a mouse model [51]. The complete transformation process of *bp100der* genes was undoubtedly inefficient, below 10% of control transformations (mean value for all 5 *bp100der* sequences, 3.2% ± 4.2). Parallel transformation with the empty vector was as expected, so excluding technical errors. Remarkably, no GM plants were obtained carrying *bp100.2* or *bp100.3*, indicating transformation efficiencies of ≤1% that of the empty vector. AMP expression in plants has been accomplished for numerous peptides and host species ([13,14] and references therein [24-26,32-34]). Large numbers of GM rice events accumulating *Aspergillus giganteus* antifungal protein Afp [31] or cecropine A [29] have been reported with normal phenotypes. Recently, thanathin expression has been reported to have very few harmful effects to host plant cells [26]. However, to our knowledge this is the first report on detailed comparison of transformation efficiencies of different putatively toxic transgenes, as the objective is typically to obtain a few transgenic events and the impact of transgene expression at different stages of GM plant production is not addressed.

Not only did transformation with different *bp100der* sequences have different overall efficiency values but the effectiveness of specific steps during the transformation process varied. As an example, reasonable numbers of calluses harboring *bp100.2mi* and *bp100.2i* were obtained (around 80% of the control with only *hpII*) whereas only around 20 - 40% the expected was obtained with *bp100.1* and *bp100.3*. The *bp100der* transgene mRNA levels in callus could partially explain these results, with somewhat higher expression levels (e.g. *bp100.1* and *bp100.3*) in some way compromising host cell fitness. The number of calluses transformed with *bp100.2* was low and they suffered necrosis while expressing the transgene at the lowest *bp100der* levels. These phenomena can be interpreted on the basis of the different properties of the specific BP100 derivatives: lower levels of *bp100.2* product seem to be more toxic to the host cell than higher levels of other *bp100der* products.

The number of GM calluses regenerating plants was above 60% that of the control for *bp100.1* but extremely low for *bp100.2mi*. In contrast, all regenerated S-bp100.2mi plants but only one fourth of the S-bp100.2i plants survived the acclimatization stage, and virtually all S-bp100.1 plants in the greenhouse produced seeds but less than half S-bp100.2i and S-bp100.2mi developed and were fertile. As the *bp100der* transgene and mRNA were present in all plants obtained, regeneration of plants mainly from transgenic calluses having lost the *bp100der* transgenes could be discarded. However, for *bp100.2mi* only GM plants with low transgene expression levels were viable. Diverse *bp100der* transgene products seem to have different toxic effects on the producing cells, in particular affecting callus growth and capacity to regenerate plants, plant survival and/or development under standard greenhouse conditions. This is in agreement with the different characteristics of chemically synthesized BP100 derived peptides. Interestingly, length increment based on BP100 tandem repeats did not result in a proportional increase in activity but it greatly increased toxicity to the producer cell. This is in contrast to previous reports associating major antibacterial activity of cationic peptides to higher toxicity to eukaryotic cells [64].

Toxicity of BP100 derivatives to plant cells was confirmed in rice germination and tobacco leaf inoculation assays by comparison of the highly cytotoxic peptide, mellitin. BP100 phytotoxic effects were less intense than those of mellitin and they affected different biological pathways. BP100 reduced shoot development in seedlings while root growth was deficient with mellitin. The five BP100 derivatives had similar phytotoxic effects to BP100 (including BP100.2mi, with part of the mellitin sequence), in some cases with moderately higher intensity. Red blood cell-based assays have been used as an approach to estimate peptide toxicity to mammalian cells [65,66]. The hemolytic activity of the BP100 derived peptides was parallel to phytotoxic activity, with a tendency to increase with peptide length even though mellitin is just 26 amino acids long. Remarkably, toxicity to erythrocytes and plant cells could only be detected upon application of high peptide doses (i.e. 160 to 10-fold target pathogen MIC values). The mechanism of action of AMPs such as BP100 is based on electrostatic attraction and subsequent interaction with cell membranes [50,67]. This property has been exploited by using the BP100 sequence to internalize fused reporter proteins into eukaryotic cells [68]. Differences in membrane lipid composition between bacterial and eukaryotic cells (absence of acidic phospholipids and presence of sterols) drastically

reduce susceptibility of eukaryotic cells to many cationic peptides [69] and explains the specific antimicrobial properties of BP100 derivatives. Biologically synthesized *bp100der* products are expected to accumulate at lower concentrations than those exerting visible phytotoxicity. Phytotoxicity of BP100 derivatives could explain the reduced transformation efficiencies of *bp100der* transgenes. The *bp100der* products may cause stress in S-bp100der lines, but its expression certainly can be compatible with moderately decreased fitness and increased pathogen resistance phenotypes (see below).

The possibility of obtaining transgenic rice constitutively expressing *bp100der* transgenes cannot be directly inferred from the capacity to lyse erythrocytes, damage tobacco leaves upon inoculation or affect seedling development. Transgenic plants were obtained for expression of BP100 derivatives displaying high toxicity values in our assays (BP100.2mi and BP100.2i) but not e.g. BP100.2, with lower values. Exogenous application of peptides at very high concentrations causes these toxic effects, whereas the vulnerability of host plant cells to biologically synthesized *bp100der* products may differ, as these are produced within the plant cell and are likely to accumulate in a specific cell compartment.

Fertile GM lines with a correctly incorporated and constitutively expressed single copy of the transgene were obtained for some *bp100der* sequences. The specific chemical properties of BP100 (e.g. high isoelectric point, poor antigenicity, poorly detectable by mass spectrometry) make it extremely difficult to detect and purify from plant cellular material. We are currently working on this, but no operative protocols to directly detect BP100 derived peptides in plant tissues are yet available. In addition, among the prolific number of reports in the current literature on GM plants expressing AMPs only a few gave direct confirmation of AMP production [25,40,41,43]. Nevertheless *bp100der* mRNA expression in GM plants, low transformation efficiencies and other phenotypic evidences (ultrastructural, oxidative stress and pathogen resistant phenotypes) indirectly demonstrate the production of BP100 derivative molecules in these transgenic plants.

S-bp100der cells exhibit altered ER morphology, with distinct dilation of cisterna and abundant dictysome vesicles. Taking into account that *bp100der* sequences include signal peptide and ER retention motifs, these observations agree with synthesis of *bp100der* products and accumulation in this organelle. Additionally, numerous vesicles and electron dense granules were observed in S-bp100.1 and especially S-bp100.2mi cells. Disruption of the ER structure and the presence of increased vesicles or small vacuoles and in some cases, electron dense granules have been associated to exposure of plant cells to excess (toxic) levels of heavy metals [70,71]. This means that these observed morphological characteristics could be related to the cationic nature (as heavy metals) and/or toxic character of BP100 derivatives. Toxicity caused by the expression of *bp100.2mi* seems to be higher than that of *bp100.2i*.

Our indicator bacterial species *Ea*, *Xav* and *Pss* are not pathogens of rice so cannot be used to assess resistance of our GM rice plants. However, several antimicrobial proteins in GM plants have been reported to confer protection against a wide range of abiotic and biotic stress conditions. Transgenic expression of the *C. annuum* antimicrobial protein CaAMP1 in pepper has been shown to confer broad-spectrum resistance against pathogens [72]. In particular, overexpression of cecropin A in rice has been shown to be effective against the fungal blast *M. grisea* [29] and other conditions such as oxidative stress [73]. Expression of chimerical peptides including cecropin A has been found to give GM potatoes broad-spectrum antimicrobial activity [44], and other cationic α-helical peptides, such as the MsrA2 derivative of Dermaseptin B1 from *Phyllomedusa bicolor*, have been reported to confer resistance to a variety of fungal and bacterial phytopathogens in transgenic potato plants [42].

Activity of chemically synthesized BP100, BP100.1, BP100.2i and BP100.2mi peptides against the soft rot pathogen *D. chrysanthemi* (syn. *Erwinia chrysanthemi* [74]) was dose-dependent. Although they are mainly antibacterial, BP100 derivatives additionally exhibited antifungal activity against *F. verticillioides*, associated with the bakanae disease of rice [75]. This correlated well with the increased resistance of S-bp100.2mi lines to *F. verticillioides* and S-bp100.2mi and S-bp100.2i lines to *D. chrysanthemi*. Biotic and abiotic stresses are typically associated with the rapid production of reactive oxygen species (ROS), including H_2O_2 and O_2^-. ROS are known to play dual roles; they play a central role in the regulation of biological processes such as stress response, hormone signaling and development, but high ROS levels have been implicated in the damaging effects of various environmental stresses [76]. S-bp100.2mi and S-bp100.2i vegetative tissues subjected to oxidative stress showed decreased accumulation of O_2^- radicals, suggesting an increased ability to scavenge ROS. Overexpression of *bp100.2mi* and *bp100.2i* led to enhanced resistance to bacterial and fungal pathogens; and improved tolerance to oxidative stress.

The resistant phenotype of S-bp100.2mi and S-bp100.2i was not solely an outcome of the selection gene and/or the transformation itself as shown by (i) the different susceptibility phenotypes exhibited by S-bp100.2i and S-bp100.2mi lines; and (ii) the lack of resistance of untransformed Senia and the GM S-hgr and, unexpectedly, S-bp100.1 lines. Chemically synthesized BP100.1 was active against our

indicator bacterial species, *D. chrysanthemi* and *F. verticillioides*, but *bp100.1* was the less phytotoxic *bp100der* transgene. Taking into account its extremely small length (15 amino acids), we can speculate that this BP100 derivative was rapidly degraded in S-bp100.1 plants and/or produced in a modified form (e.g. not properly processed).

The expression of foreign genes in plants can trigger the activation of plant defense mechanisms normally activated only during pathogenesis [77]. Several resistant GM rice lines such as those constitutively expressing cecropin A [73], the antifungal protein AFP [78] or a pathogenesis-related (PR) protein [79] have been reported to overexpress endogenous defense genes in the absence of the pathogen. This means we cannot rule out transgene dependent overexpression of stress genes being the cause of the resistance phenotypes observed in S-bp100.2i and S-bp100.2mi lines. However, reverse transcription coupled to real-time PCR analyses of PR1b and PR5 coding genes [GenBank: U89895, X68197, widely used indicators of induction of plant defense responses [80]] showed they were expressed at similar levels in leaves of S-bp100.2i and S-bp100.2mi GM when compared to Senia *in vitro* grown plants. Additionally, plants overexpressing transgenes encoding ER driven proteins have been reported to constitutively express the unfolded protein response (UPR), activated by misfolded protein accumulation in the ER [73]. The UPR has recently been associated to plant resistance to abiotic stress and pathogen attacks ([81], and references therein), so the accumulation of transgene products (expected to be highly cationic peptides) in ER could result in increased-resistance phenotypes. In a preliminary experiment we showed that S-bp100.2mi and S-bp100.2i were somewhat resistant to the causative agent of rice blast *M. grisea*, while we could not detect clear *in vitro* activity of the corresponding peptides against *M. grisea* spores (data not shown). S-bp100.2i and S-bp100.2mi pathogen resistant and oxidative stress-tolerant phenotypes support the expression of active antimicrobial peptides in these plants, irrespective of whether these phenotypes are peptide-direct effects or indirectly derived from transgene dependent activation of other cellular pathways. The latter seems to have a role in S-bp100.2i decreased susceptibility to *F. verticillioides*, taking into account that BP100.2mi and BP100.2i have similar MIC values against this pathogen, whereas evidence from ultrastructural analysis and mRNA expression suggest the *bp100.2mi* product exerts higher toxicity on host cells than the *bp100.2i* product.

Although with low efficiency, we showed the feasibility of producing rice lines expressing at least some *bp100der* sequences (e.g. S-bp100.2i and S-bp100.2mi) and indirectly demonstrated the production of BP100 derivative molecules. Thorough assessment of agronomic characteristics showed the nutritional status of these GM plants was unaffected by *bp100der* transgenes, as demonstrated by leaf chlorophyll content: chlorophyll content has been directly related to nitrogen content, which is considered an indicator of plant nutritional status. Although S-bp100.2i plants were around 10% shorter than expected, the greatest reduction in the measured parameters was the number of grains per panicle in GM (especially S-bp100.2i) compared to control lines. It is well known that stress conditions have an effect on the numbers of grains per panicle, so it could be speculated that this was a consequence of the phytotoxic effect of BP100 derivative accumulation in plant cells. The slight reduction in grain weight is in agreement with it being a well conserved character under different cultural conditions. Despite this, the overall performance of rice lines expressing certain *bp100der* transgenes strongly resembled comparable untransformed lines. The best case was S-bp100.2mi, with similar vegetative features and only around 10% grain yield losses compared to Senia.

Conclusions

We found that transformation of rice with genes encoding α-helical cationic AMPs such as those derived from the synthetic 11 amino acids-long BP100 had, in many cases, a highly negative effect on the efficiency of transformation. Remarkably, GM lines constitutively expressing certain *bp100der* sequences were produced with resistance phenotypes and minimal impact on agronomic performance. BP100 tandem copies could not be constitutively expressed in GM plants, but sequences encoding BP100 inverted repeats either elongated or not with portions of a natural AMP (*bp100.2mi* and *bp100.2i*) greatly diminished transgene undesired effects. It is likely that GM plants constitutively expressing *bp100der* transgenes and displaying resistance to representative plant pathogens and fit phenotypes are the result of a complex equilibrium between *bp100der* product phytotoxicity, antimicrobial activity and transgene-dependent plant stress response. This approach could be considered feasible for expressing synthetic AMPs in plants either to establish disease resistant plants or to facilitate molecular farming.

Methods

Chemical synthesis of BP100 derived antimicrobial peptides

BP100 derivative peptides were designed with increasing lengths and inclusion of an ER retention signal (KDEL four-amino acid sequence). They included two or three copies of BP100, with a four amino-acid linker element, AGPA, which structurally links the α-helical active domain and the C-terminal domain in natural cecropin A

[82]. One of the designed peptides was further elongated by adding a portion of mellitin (amino acids 10 to 18), another another natural AMP. Two BP100 derivatives were designed with monomeric units in opposite orientations (see scheme in Figure 1).

BP100 derived peptides were manually synthesized by the solid-phase method using Fmoc-type chemistry as previously described [47]. Briefly, Fmoc-Rink-MBHA resin (0.64 mmol/g) was used as solid support, and side-chain protection was performed with *tert*-butyloxyrocarbonyl for Lys and Trp, and *tert*-butyl for Tyr. HBTU and DIEA (3 equiv. each) mediated couplings of Fmoc-amino acids (3 equiv.) in *N,N-dimethylformamide* (DMF), monitored by the ninhydrin test. After removal of the Fmoc group with piperidine-DMF (3:7) the peptidyl resin was washed with DMF. On completion of the sequence, peptides were cleaved from the resin with TFA-H_2O-TIS (95:2.5:2.5) and dissolved in H_2O and lyophilized after TFA evaporation and diethyl ether extraction. HPLC was used to assess their purity, which was above 90% in all cases. Peptide identity was finally confirmed by electrospray ionization mass spectrometry.

In vitro assessment of the properties of BP100 derivatives

Antimicrobial activity

Peptides were solubilized in sterile Milli-Q H_2O to a concentration of 1 mM and filter sterilized through a 0.22 μm pore filter. The plant pathogenic bacterial strains *Erwinia amylovora* PMV6076 (*Ea*, Institut National de la Recherche Agronomique, Angers, France), *Pseudomonas syringae*pv. *syringae* EPS94 (*Pss*, Institut de Tecnologia Agroalimentària, Universitat de Girona, Spain), *Xanthomonasaxonopodis* pv. *vesicatoria* 2133–2 and *Dickeya chrysanthemi* 1552-10-1 (*Xav*, *Dc*, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain) and the plant pathogenic fungal strain *Fusarium verticillioides* A-999 (*Fv*, ex-type *Fusarium* spp. collection deposited at the Department of Plant Pathology, Kansas State University Manhattan, Kansas, USA, provided by R. Jiménez-Diaz) were used for antimicrobial activity tests. Bacterial strains were stored at −80°C in Luria Bertani (LB) broth with glycerol (20%). After 24 h (*Ea*, *Pss* and *Dc*) or 48 h (*Xav*) growth in LB agar at 25°C, bacterial colonies were scraped off the surface, suspended in sterile H_2O and adjusted to 10^8 CFU/ml. The fungal strain was stored at 4°C in the particulate solid carrier perlite, on fresh potato dextrose agar (PDA). *Fv* cultures were prepared in potato dextrose broth (PDB) and incubated for one week at 25°C in the dark in a rotary shaker at 125 rpm. They were filtered through several layers of sterile cheesecloth to eliminate macroconidia and mycelial growth. The effluent was centrifuged at 8,000 rpm for 20 min at 4°C and the pellet was suspended in sterile water. Microconidia concentration was determined in a counting chamber.

Serial dilutions of synthetic peptides were prepared at 400, 200, 100, 50, 25, 12.5 and 6 μM. Twenty μl aliquots were mixed in a microtiter plate with 20 μl bacterial suspensions (10^8 CFU/ml) and 160 μl trypticase soy broth (TSB, BioMèrieux, France), or 80 μl fungal suspensions (10^4 conidia/ml) and 100 μl of double-concentrated PDB containing 0.006% (w/v) chloramphenicol. Three replicates were carried out per microbial strain, peptide and concentration. In the experiments, water was used as the positive control instead of the peptide, or as the negative control instead of the microbial suspension. Microplates were incubated at 25°C for 48 h (bacterial strains) or 22°C for 6 days (*Fv*), and optical densities at 600 nm were recorded hourly (every two hours for *Fv*) after 20 s shaking. Two experimental replicates were performed. The lowest peptide concentration showing negative microbial growth at the end of the experiment was taken as the minimal inhibitory concentration (MIC).

Phytotoxic activity

For each treatment, a total of 12 surface-sterilized Senia seeds were germinated in 500 μl water or peptide solution at the appropriate concentration (0, 8, 16, 32 and 64 μM) in a culture chamber (28 ± 1°C with a photoperiod of 16 h light / 8 h dark under fluorescent Sylvania Cool White lamps) for seven days. Seedling morphology was recorded and shoot length was measured. Mellitin and BP100 were also tested at 125 and 250 μM.

Tobacco (*Nicotiana benthamiana*) plants were grown from seed in a heated glasshouse and used between 20 and 30 days old. One hundredμl chemically synthesized peptides at 50, 100, 150 or 250 μM were inoculated into the mesophylls of fully expanded tobacco leaves (previously wounded with a needle), using a syringe without needle, and plants were kept at standard glasshouse conditions for three days. Up to six independent inoculations were carried out in a single leaf, and at least three independent inoculations were performed per peptide and concentration, randomly distributed in different leaves and plants. Toxicity was measured as the lesion diameter.

Hemolytic activity

Hemoglobin release from erythrocyte suspensions of fresh human blood (5% v/v) was used to evaluate hemolytic activity of synthetic peptides [47]. Briefly, aseptically collected blood (BD vacutainer K2E System with EDTA, Belliver Industrial State, Plymouth, UK) was centrifuged at 6,000 g for 5 min, washed with 10 mM TRIS, 150 mM NaCl, pH 7.2 and suspended in the same buffer. Fifty μl of peptide solutions at 300 μM were

mixed with 50 μl aliquots of erythrocyte suspensions and incubated for 1h at 37ºC with shaking. After centrifugation, the supernatants were transferred to microplate wells with 80 μl H_2O and the 540 nm absorbance was monitored with a Bioscreen plate reader. Hemolysis percentages were calculated relative to mellitin and TRIS buffer.

Construction of the chimeric *bp100der* genes and plant expression vectors

A wide variety of sequence-dependent factors influence gene expression levels and we took several critical ones into account. The deduced DNA sequences encoding BP100 derivatives were codon-optimized for more preferred codon usage in rice plants [83]. The distribution of codon usage frequency along the sequence was good, with codon adaptation index (CAI) values from 0.82 to 0.87 (CAI > 0.8 indicates a high level of expression). All five sequences had average GC contents close to 50% and no peaks outside the 30-70% range were found in 60 bp windows, which could reduce mRNA half-life. No internal ribosomal binding sites or other putatively destabilizing cis-acting elements were found. The DNA sequences encoding BP100 derivatives are shown in Additional file 5.

The signal sequence from the tobacco pathogenesis related protein PR1a [84] was fused to each AMP coding sequence to drive the BP100 derivative peptides to the secretory pathway. With the KDEL C-terminal sequence, it has been successfully used to drive foreign proteins to the ER in rice var. Senia [29]. Chimerical genes encoding BP100.1, BP100.2 and BP100.3 (*bp100.1*, *bp100.2* and *bp100.3* genes) were fully synthesized by recursive PCR [85] in a single reaction. Four oligonucleotides (i.e. two in sense and two in antisense orientations, Table 3 and Figure 1) were designed to cover the whole *bp100.3* sequence with partial overlapping. The outermost oligonucleotides incorporated *BamHI* restriction sites for subsequent cloning. They were synthesized on an Applied Biosystems 394 DNA synthesizer according to [31]. Recursive PCR was carried out in a final volume of 100 μl with 30 pmol external and 0.2 pmol internal primers, 300 μM dNTPs, 1x reaction buffer, 1.5 mM $MgCl_2$ and 1.75 units Expand High Fidelity DNA polymerase (Roche Diagnostics Corporation, Indianapolis USA). Reaction conditions were as follows: initial denaturing step (3 min 94°C); 10 cycles of 15 s at 94°C, 30 s at 45°C and 3.5 min 72°C; 20 cycles of 15 s at 94°C, 30 s at 58°C and 3.5 min 72°C; and a final elongation step of 10 min at 72°C. Due to the repetitive nature of *bp100.3*, this amplification consistently resulted in a mixture of three bands with lengths compatible with sequences containing one, two and three tandemly repeated *bp100* units. Recursive PCR products were cloned using the pGEM®T-Easy system (Promega, Wisconsin, USA) according to the manufacturer's instructions. Individual clones were subjected to PCR with primers *PR1a_for* and *KDELbam_rev* (Table 3) to individually amplify *bp100.1*, *bp100.2* and *bp100.3*. The PCR was in a final volume of 50 μl 1x reaction buffer with 1.5 mM $MgCl_2$, 200 nM primers, 200 μM dNTPs and 1 unit Expand High Fidelity DNA polymerase (Roche Diagnostics Corporation). Reaction conditions were 3 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C; and 10 min at 72°C. Synthetic *bp100.2i* and *bp100.2mi* chimerical genes, encoding BP100.2i and BP100.2mi, were purchased from GenScript (Piscataway NJ, USA) and included as well *BamHI* restriction sites, both at the 5' and 3' ends.

The newly synthesized *bp100.1*, *bp100.2*, *bp100.2i*, *bp100.3* and *bp100.2mi* genes were subcloned into the *BamHI* site of pAHC17 plasmid DNA [86], flanked by the promoter, first exon and first intron of the maize *ubiquitin* gene [87], previously shown to drive high expression levels of transgenes in rice [86,88], and the *Agrobacterium tumefaciens* nopaline synthase *nos* terminator sequences. After sequence verification by sequencing, the complete *bp100der* sequences (including promoter and terminator elements) were subcloned into the *KpnI* site of pCAMBIA1300, always in the opposite sense compared to the selection gene. The pCAMBIA derived binary vectors harboring *bp100der* cassettes were transferred into *Agrobacterium strain* EHA105 using the cold shock method [89]. DNA manipulations and transformation of the *Escherichia coli* strain XL1Blue with plasmid DNA were carried out using standard techniques [89].

Production of transgenic rice plants

Transformation was carried out using the Mediterranean elite *japonica* rice (*Oryza sativa L.*) cultivar Senia. Transgenic lines expressing one of the *bp100der* genes were produced by *Agrobacterium* mediated transformation of embryonic callus derived from mature embryos as described by [90]. The control plasmid was pCAMBIA 1300 (with the resistance to hygromycin selection gene). T0 plants were grown to maturity and a selection of events was further cultured in standard greenhouse conditions to obtain homozygous transgenic lines in the T2 generation.

Nucleic acid extraction

Genomic DNA was extracted from 1 g plant material using a CTAB based method [91]. Total RNA was extracted from 400 mg plant material, using a protocol based on the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and purified using the Qiagen RNeasy MiniElute Cleanup kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA and RNA concentration and quality

Table 3 Oligonucleotides used in this work

Oligonucleotide code	Sequence	Length
recursive PCR		
PR1a.1_for	5' ATA Ggg atc cGA GGC CAC *CAT GGG CTT CGT CCT CTT CTC CCA ACT CCC ATC CTT CCT CCT CGT C TC CAC CCT CCT CCT GT* 3'	80
BP100.1_ rev	5' GTA CTT GAG GAT CTT CTT GAA *GAG CTT C TT GGC GCG GCA GGA GTG GGA GAT CAC GAG GA A CAG GAG GAG GGT GGA* 3'	78
BP100.2_ for	5' *GAA GCT* CTT CAA GAA GAT CCT CAA GTA CCT CGC CGG CCC AGC CAA GAA GCT CTT CAA GAA GAT CCT CAA GTA CCT CGC C 3'	82
BP100.3KDEL_ rev	5' TAT Agg atc cAT TA**T CAG AGC TCG TC**C TTG AGG TAC TTG AGG ATC TTC TTG AAG AGC TTC TTG GCT GGG CCG GCG AGG TAC TTG AGG 3'	87
Cloning steps		
PR1a_for	5' ATA Ggg atc cGA GGC CAC CAT 3'	21
KDELbam_rev	5' TAT Agg atc cAT TAT CAG AGC TCG TC 3'	26
Southern blot probe		
SouthUBI_for	5' ACA TGT GAT GTG GGT TTA CTG ATG 3'	24
SouthBP_rev	5' GAG GTA CTT GAG GAT CTT CTT GAA G 3'	25
qPCR		
bp100der_for	5 ' TCC TCG TGA TCT CCC ACT CCT G 3'	22
bp100der_rev	5' CGG ATC CAT TAT CAG AGC TCG T 3'	22
hgr_for	5' CGA AAT TGC CGT CAA CCA AGC 3'	21
hgr_rev	5' CTG GAG CGA GGC GAT GTT C 3'	19

Underlined sequences indicate overlaps between two oligonucleotides used in recursive PCR, in italics is the *pr1a* signal sequence, the sequence encoding the KDEL retention signal is indicated in bold, and the *BamHI* and *KpnI* restriction sites introduced for cloning purposes are shown in lower case. Length of oligonucleotides is indicated in nt.

were systematically checked by UV absorption at 260 and 280 nm using a NanoDrop ND1000 spectrophotometer (Nanodrop technologies, Wilmington, DE, USA). All samples had appropriate values (mean and standard deviation [SD], 1.94 ± 0.15 and 2.08 ± 0.02 for DNA and RNA extracts, respectively).

Southern blot

Genomic DNA (25 μg) was digested with *Hind III* or *Eco RI*, electrophoresed on 0.8% agarose gels, transferred to nylon membranes (Hybond-N, GE Healthcare Life Sciences, UK) and hybridized with a thermostable alkaline phosphatase labeled probe. The *bp100der* sequences were detected by chemiluminescence using the CDP-Star™ reagent (GE Healthcare Life Sciences, UK). The *bp100der* probe was obtained from a 406 bp PCR product encompassing part of the *ubi* first intron, the *pr1a* signal sequence and the first *bp134* element (PCR amplified with primers *SouthUBI_for* and *SouthBP_rev*, Table 3 and Figure 1) using the AlkPhos Direct Labeling and Detection System (GE Healthcare Life Sciences, UK). Hybridization and washes were carried out at 65°C according to the manufacturer's instructions.

Reverse transcription and PCR analysis

Transgene expression was assayed by reverse transcription coupled to real-time polymerase chain reaction (RT-qPCR). Reverse transcription was performed on 2,000 ng total RNA, previously treated with Turbo DNase (Ambion, Austin, TX, USA) using 50U of MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamer primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. For each sample, cDNA was prepared at least in duplicate. The absence of remaining DNA targets was demonstrated by qPCR analyses (see below) of DNase-treated RNA samples.

qPCR assays targeting all five *bp100der* sequences and *hptII* were developed based on SYBR-Green technology. A single *bp100der* RT-qPCR assay was designed and optimized to detect and quantify *bp100.1*, *bp100.2*, *bp100.2i*, *bp100.3* and *bp100.2mi* based on two primers targeting conserved sites at the 5' and 3' ends of the coding sequences. PCR primers were designed using the Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA, USA). The qPCR assays were in a 20 μl volume containing 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 100 nM of primers (except for *hgr_for* and *hgr_rev*, 300nM and *bp100der_rev*, 50 nM) and 1 μl cDNA. Reaction conditions were: (1) initial denaturation (10 min at 95°C); (2) amplification and quantification (50 repeats of 15 s at 95°C and 1 min at 60°C) and (3) melting curve program

(60-95°C with a heating rate of 0.5°C/s). Melting curve analyses produced single peaks, with no primer-dimer peaks or artifacts, indicating the reactions were specific. All oligonucleotides (Table 3) were purchased from MWG Biotech AG (Germany). Reactions were run on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in triplicate. All reactions had linearity coefficient (R^2) and efficiency values [$E = 10^{[-1/slope]}$, [92]] above 0.99 and 0.95, respectively (for *bp100der* qPCR these values were assessed for each target).

The 18 S ribosomal RNA, β-actin and elongation factor (EF1α) housekeeping genes were analyzed as previously described [78]. Their suitability as internal standards was assessed in our samples through the geNORM v3.4 statistical algorithm, and β-actin had M values below 0.5 in all cases.

Pathogen infection assays and oxidative stress assay

A total of ten homozygous plants of each GM event (3 S-bp100.1, 2 S-bp100.2i and 2 S-bp100.2mi lines), plus two control lines (i.e. untransformed Senia and S-hgr, transformed with the empty plasmid) were grown in the greenhouse to the vegetative three-leaf stage. The second leaves were detached and 50 mM H_2O_2 applied for 8 h, with subsequent in situ detection of O_2^-radicals in plant tissues by overnight staining with nitro blue tetrazolium (NBT). Leaves were visually inspected and the percentages of stained area were calculated using the APS assess v2.0 software tool.

A total of 15 homozygous seeds of each GM event, plus Senia and S-hgr lines, were surface sterilized and germinated in sterile water in 24-well culture chambers. They were incubated in a culture chamber at 28 ± 1°C with a photoperiod of 16 h light / 8 h dark under fluorescent Sylvania Cool White lamps. After overnight pre-germination they were inoculated with increasing concentrations of the bacterial plant pathogen *D. chrysanthemi* (10^2, 10^3, 10^4, 10^5 and 10^6 for Senia; 10^5 for all GM events) and allowed to continue germination for seven days. The development of *D. chrysanthemi*-infected seedlings from wild-type and transgenic plants was determined using a semi-quantitative scale (Figure 5A). Control seeds incubated in water confirmed germination of over 98% seeds, with no statistical difference among different events (one-way ANOVA $p = 0.617$). Uninfected seedlings from all analyzed events consistently had maximum development values.

For *F. verticillioides* (anamorph stage of *Gibberella fujikuroi*, mating population A) assays, nine seeds of each GM event, plus Senia and S-hgr lines, were surface sterilized and incubated for 30 min in the presence of a suspension of *F. verticillioides* conidia (10^5 spores/ml) under agitation. They were allowed to germinate for seven days in sterile MS medium (Murashige and Skoog, 1962) supplemented with 0.7% agar in plastic tins under the same conditions as for *D.chrysanthemi*assays. Seedling development was estimated through a semi-quantitative index that considered shoot height (with values in the 0 to 1 range) and root development (number and length of crown roots and length of primary root, with values of 0 to 0.75 and 0 to 0.25, respectively). As uninfected control seeds consistently germinated and seedlings developed as expected they were given the highest possible values in this assay (i.e. 1, 0.75 and 0.25, respectively). Shoot height and primary root length were measured and values were assigned in the 0 to 1 and 0 to 0.25 ranges, respectively, in comparison to control seedlings. The number and length of crown roots were estimated by comparison with crown roots in control seedlings, and values were in the 0 to 0.5 and 0 to 0.25 ranges, respectively. The sum of the values for all seedlings was used to calculate the value of the seedling development index for each GM event. A global value of 0 indicated no germination and compact mycelium over the seed, while a value of 2 represented standard growth, the same as uninfected seeds.

Agronomic characterization of GM plants

Transgenic and control lines were compared in terms of agronomic parameters. For S-bp100.2i and S-bp100.2mi lines (i.e. those expressing *bp100.2i* and *bp100.2mi*), T3 homozygous plants from all the independent events obtained were assayed. The assay was carried out in the quarantine greenhouse from 29 April to 7 October 2010, i.e. during the conventional rice growing season in the region. For each line, a total of 45 seeds were sown and 81 to 91% germinated. At the vegetative three-leaf stage a total of 30 plants per line (three replicates of 10 plants each) were transferred to pots and grown in the greenhouse under standard conditions. Plant growth was monitored every two weeks in terms of plant height, number of tillers, chlorophyll contents (spat) and flowering (a total of 5 observations, the last on 08/25/2010). To estimate yield related traits, the number of panicles per plant, number of grains per panicle and the weight per 100 grains were measured on harvesting. Plant yield was calculated as the product of number of panicles, number of grains per panicle and grain weight, and corresponds to the weight of all panicles in a plant.

Bioinformatics

RT-qPCR data were normalized with the housekeeping gene and statistically analyzed using the SPSS software v.15.0 for Windows or the Genex software v.5.1.1.2 (MultiDAnalyses).

For pathogen resistance and oxidative stress tolerance assays, one-way ANOVA analyses were performed. The

analytical results (i.e. percentages of NBT stained area or seedling development values in the presence of *D. chrysanthemi* or *F. verticillioides*) were used as the dependent variables, and the transgene (*bp100.2i*, *bp100.1*, *bp100.2mi*, *nptII* or none) as factor. Statistical analyses were performed jointly comparing the different events carrying the same *bp100der* to Senia.

Additional files

Additional file 1. Expression levels of *hptII* and the corresponding *bp100der* transgenes in three randomly chosen transgenic calluses per construct, as assessed by RT-qPCR. Transgene mRNA copy numbers were normalized with actin values (GeNorm M values below 0.5). Means and SD of the three independent events are shown. No statistical differences were found.

Additional file 2. Transgene DNA copy numbers of S-bp100der plants. (A) Southern blot analysis of transgenic lines. Genomic DNA was digested with the restriction enzymes EcoRI or Hind III and subjected to electrophoresis through a 0.8% agarose gel. DNAs were transferred to nylon membranes and hybridized with a thermostable alkaline phosphatase labelled probe. The migration positions and sizes of markers are indicated in base pairs on the left (MW). (B) Determination of transgene copy number by qPCR. Means of six experimental replicates are shown. RSD values were consistently below 2.5%. Transgene DNA copy numbers were normalized with actin values.

Additional file 3. Homozygous T2 rice lines obtained in this work and transgene mRNA expression values (relative to actin) in leaves of in vitro grown homozygous T3 plants, as assessed by RT-qPCR. Mean and SD values corresponding to each particular GM event are shown. Three biological replicates per GM event were analyzed, each with leaves of 10 plants at the two-leaf stage.

Additional file 4. Examples of Senia and S-bp100.2i plants at maturity.

Additional file 5. DNA sequences encoding the BP100 derivatives designed in this work. The sequence encoding the Pr1a signal peptide is indicated in italics. The start and stop codons are underlined.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MP, AN and EM conceived and designed the study. MM carried out most experiments and participated in the analysis of the data. MP supervised the study and wrote the paper. AN participated in the production of transgenic plants,microscopy, discussion of all experiments and preparation of the manuscript. NC helped in qRT-PCR assays. EB performed the in vitro assessment of the properties of BP100 derivatives. JM performed the agronomic characterization of GM plants. LM carried out pathogen resistance assays. EM participated in the design of BP100 derivatives and helped to draft the manuscript. All authors read and approved the final manuscript.

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CHAPTER IV

The production of recombinant cationic α-helical antimicrobial peptides in plant cells induces the formation of protein bodies derived from the endoplasmic reticulum

Nuri Company, Anna Nadal, José-Luis La Paz, Sílvia Martínez, Stefan Rasche, Stefan Schillberg, Emilio Montesinos and Maria Pla

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The production of recombinant cationic α-helical antimicrobial peptides in plant cells induces the formation of protein bodies derived from the endoplasmic reticulum

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Summary

Synthetic linear antimicrobial peptides with cationic α-helical structures, such as BP100, are valuable as novel therapeutics and preservatives. However, they tend to be toxic when expressed at high levels as recombinant peptides in plants, and they can be difficult to detect and isolate from complex plant tissues because they are strongly cationic and display low extinction coefficient and extremely limited immunogenicity. We therefore expressed BP100 with a C-terminal tag which preserved its antimicrobial activity and demonstrated significant accumulation in plant cells. We used a fluorescent tag to trace BP100 following transiently expression in *Nicotiana benthamiana* leaves and showed that it accumulated in large vesicles derived from the endoplasmic reticulum (ER) along with typical ER luminal proteins. Interestingly, the formation of these vesicles was induced by BP100. Similar vesicles formed in stably transformed *Arabidopsis thaliana* seedlings, but the recombinant peptide was toxic to the host during latter developmental stages. This was avoided by selecting active BP100 derivatives based on their low haemolytic activity even though the selected peptides remained toxic to plant cells when applied exogenously at high doses. Using this strategy, we generated transgenic rice lines producing active BP100 derivatives with a yield of up to 0.5% total soluble protein.

Introduction

Antimicrobial peptides (AMPs) are key components of innate immunity in plants and animals and are also produced by microbes in antibiosis processes (Bulet and Stocklin, 2005; Cooter *et al.*, 2010; Degenkolb *et al.*, 2003; Ganz, 2003; Hancock, 2001; Jack and Jung, 2000; Lay and Anderson, 2005; Ng, 2004; Raaijmakers *et al.*, 2006; Tincu and Taylor, 2004; Toke, 2005; Zasloff, 2002). They are generally short peptides, which can have linear structures that often adopt an amphipathic α-helical conformation that binds to the phospholipid membranes of target microbes before the hydrophobic face is inserted into the membrane bilayer (Bechinger, 2004; Boman, 2003; Brogden, 2005; Ferré *et al.*, 2009; Huang, 2006; Marcos and Gandía, 2009; Tossi *et al.*, 2000). This unique mode of action helps to avoid the emergence of resistance in target pathogens (Brogden, 2005; Peschel and Sahl, 2006; Yeaman and Yount, 2003; Yount and Yeaman, 2005).

Antimicrobial peptides are valuable as novel therapeutic agents (Hancock, 2001; Marcos *et al.*, 2008; Montesinos, 2007; Moreno *et al.*, 2006; Rajasekaran *et al.*, 2012; Zasloff, 2002) because of their broad-spectrum activity against bacteria, fungi, viruses, parasites and tumour cells (Ajesh and Sreejith, 2009; Broekaert *et al.*, 1997; Brogden *et al.*, 2003; Bulet *et al.*, 2004; Jenssen *et al.*, 2006; Otvos, 2000; Torrent *et al.*, 2012; Zasloff, 2002). Natural AMPs have been optimized to increase their potency against selected pathogens while protecting nontarget organisms and enhancing stability (Badosa *et al.*, 2007; Cavallarín *et al.*, 1998; López-García *et al.*, 2002; Marcos *et al.*, 2008; Monroc *et al.*, 2006).

The synthetic CECMEL11 peptide library, a 125-member cationic α -helical undecapeptide library designed using a combinatorial approach, contains groups of sequences with potent and selective activity against a number of bacterial and fungal phytopathogenic reporter strains (Badosa *et al.*, 2007, 2009; Bardají, 2006). The synthetic peptide BP100 (KKLFKKILKYL-NH_2) was found to be effective at micromolar concentrations against *Xanthomonas axonopodis* pv. *vesicatoria* in pepper, *Erwinia amylovora* in apple and *Pseudomonas syringae* pv. *syringae* in pear (Badosa *et al.*, 2007). The efficacy was comparable to standard antibiotics, and BP100 is also highly biocompatible, as determined by acute oral toxicity tests in mice (Montesinos and Bardají, 2008).

Genetically modified (GM) plants with moderate resistance to pathogenic bacteria and fungi have been developed by expressing AMPs either constitutively or induced by pathogens (reviewed by Marcos *et al.*, 2008; Montesinos, 2007). Many different recombinant proteins have been expressed in plants (Fischer *et al.*, 2004; Hoja and Sonnewald, 2013; Twyman *et al.*, 2003), and the applications envisaged for CECMEL11 peptides suggest that plant-based expression would be preferable, to develop plants either that are disease resistant or that express AMPs for medical and industrial applications. Large-scale chemical synthesis of peptides above around six amino acids is only economically viable for applications of very high added value. Rice is a suitable

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platform for large-scale production, and the target product can be easily stored in the kernels for a long time allowing to decouple the production and the processing step.

BP100 was recently used as a proof-of-concept to show that the constitutive expression of short cationic α-helical synthetic peptides can have a strong negative impact on the fitness of transgenic rice plants (Nadal *et al.*, 2012). Transformation with five sequences encoding BP100 derivatives (BP100der) resulted in a transformation efficiency more than 100-fold less efficient than a control transgene. These BP100der peptides contained endoplasmic reticulum (ER) retention motifs to prevent peptide degradation in the cytosol and minimize toxicity to the host plant. This did not affect the antimicrobial activity of the products *in vitro* using bacterial growth inhibition tests. However, when applied at high doses (>10^2-fold of the minimal inhibitory concentration, MIC), the peptides exerted toxic effects such as erythrocyte lysis, leaf damage in *Nicotiana benthamiana* and the inhibition of rice seedling development. The extreme physicochemical properties and low immunogenicity of the recombinant peptides prevented their direct detection in GM rice, but low levels of transgene mRNA were detected and the plants were more resistant to oxidative stress and pathogens such as *Dickeya chrysanthemi* and *Fusarium verticillioides*. We speculated that BP100der toxicity was associated with constitutive transgene expression but was ameliorated by the conferred stress tolerance.

Here, we assessed the production of recombinant BP100 derivatives in transformed *N. benthamiana* and *Arabidopsis thaliana* plants directly, by expressing the peptides as fusions with the fluorescent marker protein DsRed. We also screened a series of BP100 derivatives elongated with sequences from natural AMPs and found that peptides with lower haemolytic activity achieved greater transformation efficiency. We were therefore able to confirm that peptides with potent antimicrobial and low haemolytic activity can accumulate in stably transformed rice plants at levels of up to 0.5% total soluble protein (TSP).

Results

BP100 derivatives can be produced by transient expression in *N. benthamiana* cells

We previously described transgenic rice plants constitutively expressing transgenes that encoded three BP100 derivatives: *bp100.1*, *bp100.2i* and *bp100.2mi* (Nadal *et al.*, 2012). The synthesis of BP100der in these plants could only be confirmed indirectly, that is, by demonstrating the presence of transgene mRNA, a resistant phenotype and ultrastructural changes in the plant cell. To confirm that active cationic α-helical antimicrobial peptides can be made to accumulate in transgenic plants, we developed a strategy based on the fusion of BP100 to the *Discosoma* spp. red fluorescent reporter protein DsRed (Matz *et al.*, 1999) and the epitope tag54 sequence for the detection of recombinant proteins using the specific antibody mAb54 (Rasche *et al.*, 2011).

Chimeric genes encoding BP100-DsRed-tag54-KDEL (hereafter described as BP100-DsRed-tag54) and a DsRed-tag54-KDEL control lacking AMP sequences (hereafter described as DsRed-tag54) were placed under the control of the constitutive *Cauliflower mosaic virus* 35S promoter for constitutive expression and product accumulation in the ER. The constructs were agroinfiltrated into *N. benthamiana* leaves together with HC-Pro silencing suppressor, and the accumulation of each recombinant protein was monitored by tracking the DsRed fluorescence by confocal microscopy. Three days postinfiltration (dpi), epidermal cells from *N. benthamiana* leaves transformed with the AMP and control constructs were both found to produce strong DsRed fluorescence signals in three agroinfiltrated fields representing the upper, medial and basal portions of different leaves. Cells expressing the BP100-DsRed-tag54 and DsRed-tag54 constructs had fluorescence intensities of 1004 ± 574 and 817 ± 189 fluorescence units per field, respectively, representing statistically similar expression levels as determined by one-way ANOVA ($P = 0.395$).

Western blot analysis using the tag54-specific monoclonal antibody revealed single bands of the anticipated sizes for DsRed-tag54 (29 kDa) and BP100-DsRed-tag54 (~33 kDa) as shown in Figure 1. This confirmed the presence of the BP100 peptide in the BP100-DsRed-tag54 fusion protein. The lack of a 29-kDa band in BP100-DsRed extracts showed that the BP100 peptide was not specifically cleaved from the fusion partner. There were no visible bands of the size expected for the tag54 alone, indicating the tag was not cleaved from the fusion protein either (data not shown). The observed differences in band intensity are likely to reflect the challenging extraction procedure for BP100 derivatives when dealing with complex matrices such as leaf samples. However, our data show clearly that BP100 derivatives can be synthesized in plants and directed to accumulate in plant cells.

BP100 induces the formation of ER-derived vesicles

The analysis of *N. benthamiana* leaves by confocal microscopy showed different patterns of fluorescence between the BP100

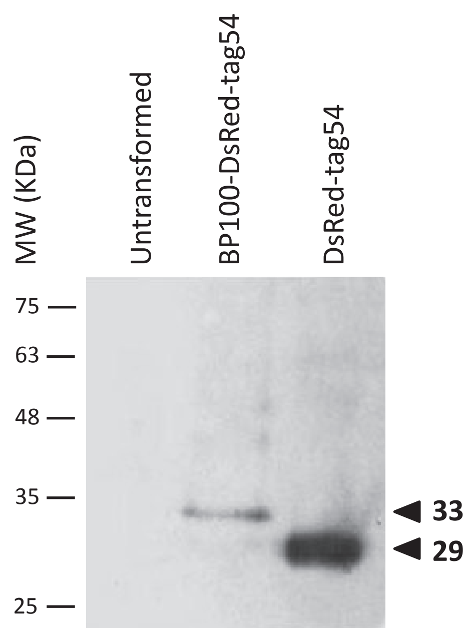

Figure 1 Transient expression of BP100-DsRed-tag54 and DsRed-tag54 in *Nicotiana benthamiana* leaves. Western blot of proteins extracted from *N. benthamiana* leaves agroinfiltrated with *bp100-dsred-tag54* or *dsred-tag54* as a control. Leaf tissue was collected at 3 dpi, and 1 μg of total protein was separated by SDS-PAGE before transfer to nitrocellulose filters. The recombinant proteins were detected using antibody mAb54k (diluted 1 : 1500) followed by the horseradish peroxidase-labelled anti-mouse IgG secondary antibody (diluted 1 : 10000) and ECL chemiluminescent detection. Arrowheads indicate BP100-DsRed-tag54 (32.9 kDa) and DsRed-tag54 control (28.5 kDa) proteins.

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and control constructs. As expected, DsRed fluorescence in the DsRed-tag54 control leaves displayed a characteristically reticulate pattern 2 days after infiltration, consistent with typical ER morphology in the plant epidermal cells. Although some cells transformed with BP100-DsRed-tag54 displayed a similar fluorescence pattern at 2 dpi, indicating that BP100 does not inhibit protein transit and retention in the ER, the majority contained numerous and widely distributed spherical structures ~1–2 μm in diameter showing intense fluorescence, obscuring the normal ER network. After 3–5 dpi, most cells expressing BP100-DsRed-tag54 contained larger (up to 15 μm) highly fluorescent and irregular structures accompanied by smaller punctate fluorescence.

To gain insight into the origin of these structures and the integrity of the ER in cells accumulating BP100-DsRed-tag54, we co-transformed *N. benthamiana* leaves with BP100-DsRed-tag54 and another construct encoding ER-localized cyan fluorescent protein (eCFP) (Joseph *et al.*, 2012). The merged fluorescence images in double-transformed cells at 3 dpi showed that BP100-DsRed-tag54 and eCFP co-localized in the induced vesicles described above while eCFP was also visible in the ER (Figure 2a–d). This confirmed that BP100-DsRed resides transiently in the ER lumen but rapidly accumulates in novel ER-derived vesicles that contain other luminal ER proteins such as eCFP. As expected, control cells co-transformed with the DsRed-tag54 and eCFP constructs showed co-localization of the proteins within the normal ER (Figure 2e–h). The observed changes in ER structure therefore appear to be driven by the BP100 component of the BP100-DsRed-tag54 fusion protein.

Stably transformed *Arabidopsis thaliana* seedlings accumulate BP100-DsRed-tag54 in vesicles but fail to develop into mature plants

Having confirmed the accumulation of BP100-DsRed-tag54 in large ER-derived vesicles following transient expression in plant cells, we set out to determine whether this could also be achieved in *A. thaliana* plants stably transformed with the same constructs using the floral dip method. Two weeks after *in vitro* germination, the radicles of hygromycin-resistant seedlings expressing the BP100 and control constructs were analysed by confocal microscopy. All analysed transgenic plants displayed red fluorescence, and those expressing BP100-DsRed-tag54 revealed fluorescent spots 1–10 μm in diameter, ranging from 1–3 μm in root tip cells to 2–10 μm in elongating epidermal and lateral root cells (Figure 3a,d). The morphology and distribution of the fluorescent vesicles were similar to the pattern observed in *N. benthamiana* epithelial cells.

Although we were able to recover fertile transgenic seeds producing each of the constructs, most of the seedlings expressing BP100-DsRed-tag54 did not survive acclimation, whereas those expressing the control construct developed normally. This emphasized that BP100 is phytotoxic at high concentrations even when it accumulates in vesicles. We further investigated BP100-DsRed-tag54 toxicity by incubating the transgenic radicles with SYTOX, a nucleic acid stain that can only cross damaged cell membranes. We found that DsRed and SYTOX were not co-localized at this developmental stage (Figure 3). Most of the cells expressing BP100-DsRed-tag54 and the control construct DsRed-tag54 were viable, and only a few epidermal cells above the lateral root cap revealed both SYTOX and DsRed fluorescence, and the distribution of SYTOX staining was similar in wild-type *A. thaliana* cells (data not shown), suggesting the cell damage reflected normal physiological processes rather than a BP100-specific effect (Truernit and Haseloff, 2008).

Haemolytic activity can be used to predict the feasibility of constitutive BP100 expression in stably transformed plants

To improve the accumulation of BP100 derivatives (BP100der) in transgenic plants, we screened a recently described synthetic

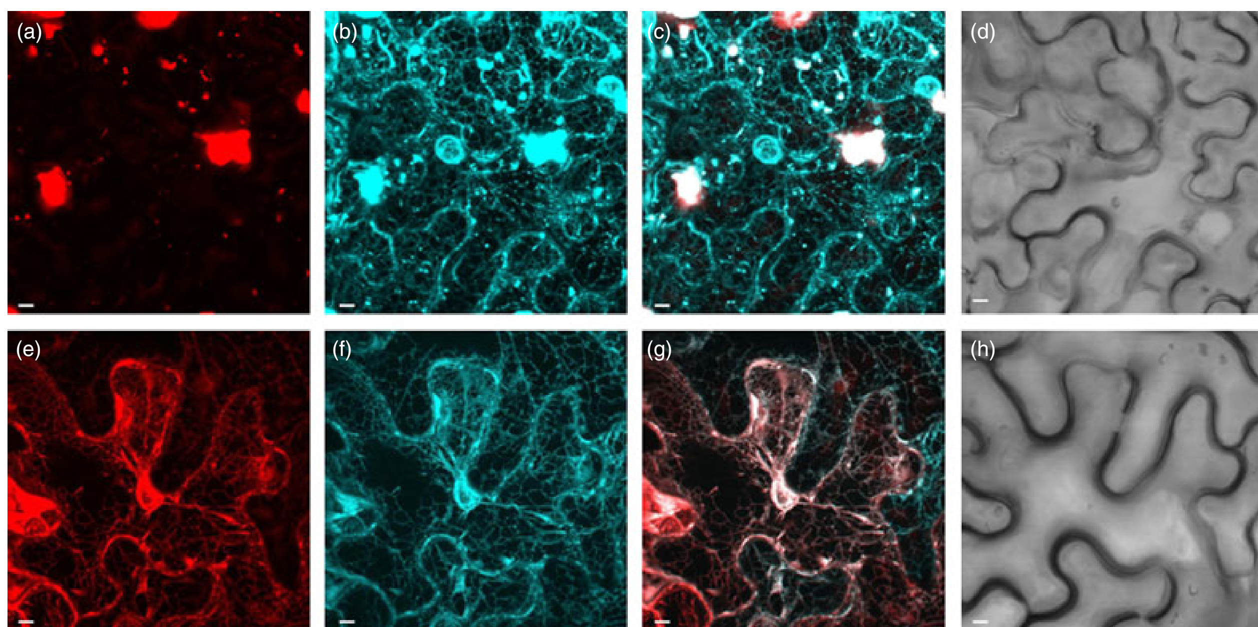

Figure 2 Confocal micrographs of *Nicotiana benthamiana* epidermal cells transiently expressing BP100der-DsRed-tag54 or the control DsRed-tag54. *Nicotiana benthamiana* leaves were co-transformed with constructs encoding ER-targeted eCFP and BP100-DsRed-tag54 (a–d) or DsRed-tag54 (e–h). (a,d), DsRed red fluorescence; (b,f) eCFP cyan fluorescence; (c,g), cyan and red merged images showing co-localization in white; (d,h), bright field. Scale bars, 5 μm.

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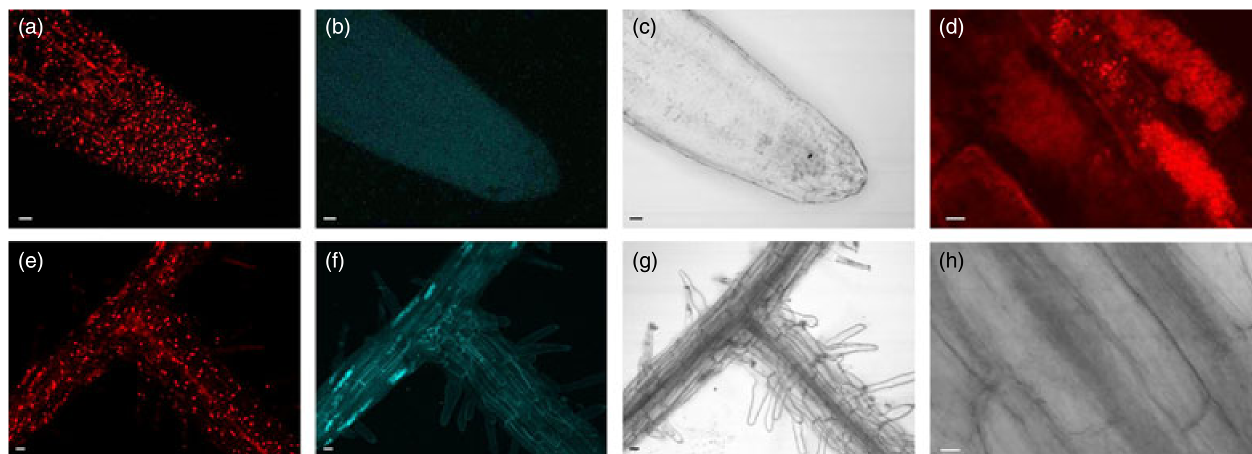

Figure 3 Confocal micrographs of transgenic *Arabidopsis thaliana* radicles expressing BP100-DsRed-tag54. *Arabidopsis thaliana* seeds obtained by floral dip transformation with recombinant bacteria carrying the BP100-DsRed-tag54 (a–c and e–g) or the DsRed-tag54 (d,h) constructs were germinated *in vitro* for a total of 16 days and observed by confocal microscopy following SYTOX staining. (a–c), radicle tip; (d–h), lateral root. (a,d,e), DsRed fluorescence; (b,f), SYTOX fluorescence; (c,g,h) bright field. Scale bars, 5 μm (d,h), 10 μm (a–c) and 20 μm (e–g).

library of BP100 derivatives to identify less phytotoxic peptides that maintained their potent antimicrobial activity (Badosa *et al.*, in preparation). The synthetic library of 40 BP100 derivatives included 20 carrying a C-terminal KDEL ER retention signal, and their haemolytic activity at 150 μM ranged from 0 to 100%. Because haemolytic activity has been widely used to determine the toxicity of peptides towards eukaryotic cells, we used this property as a tentative predictor of phytotoxicity and, by extension, of transformation efficiency in plants.

We therefore selected four BP100 derivatives with potent antimicrobial activity but the lowest haemolytic activity: BP100.m (15%), BP100.g (4%), BP100.g2 (4%) and BP100.c (1%), as shown in Table 1. All four peptides also showed stronger activity against the model plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) than the original BP100 based on *in vitro* growth inhibition assays, and their activities against *Erwinia amylovora* (*Ea*) and *Pseudomonas syringae* pv. *syringae* (*Pss*) were similar to BP100. The modifications involved elongation with sequences derived from natural AMPs: mellitin in BP100.m, magainin in BP100.g and BP100.g2, and cecropine A in BP100.c. We also produced the additional construct BP100.gtag, which was equivalent to BP100.g but contained three copies of the tag54 epitope (tag54×3) at the C-terminal end. This reduced the haemolytic activity of the peptide to 0% at 150 μM and increased its antimicrobial potency, even though the tag sequence alone had no antimicrobial activity (Table 1).

Constructs encoding the five BP100 derivatives (BP100.m, BP100.g, BP100.gtag, BP100.g2 and BP100.c, all including a C-terminal KDEL ER retention sequence) were introduced in-frame with the sequence encoding the *N. tabacum* pathogenesis-related protein PR1a signal peptide, under the control of the *ubi* constitutive promoter and *nos* terminator. All constructs were introduced in *Agrobacterium tumefaciens* and used to transform rice plants along with the *hpt*II marker for hygromycin selection. Each construct yielded hygromycin-resistant plants, most of which were shown to contain the BP100der transgene by testing leaf genomic DNA by PCR (data not shown). The number of transgenic events achieved with the BP100der constructs was approximately 35–50% of the number achieved using the selectable marker alone, specifically 48% for BP100.g and BP100.gtag, 41% for BP100.m, 36% for BP100.g2 and 34% for BP100.c, much better than reported for other BP100 derivatives in the same expression cassette introduced as well into rice using the same method (Nadal *et al.*, 2012).

Table 1 Sequence, and antibacterial and haemolytic activity of selected BP100 derivatives

AMP code	Sequence	MIC (μM) Xav	Pss	Ea	Haemolytic activity vs. BP100 (150 μM)
BP100	KKLFKKILKYL	5–10	2.5–5	2.5–5	22.0 ± 2.8
BP100.m	KKLFKKILKYL **AGPA** TTGLPALISW KDEL	5–7.5	2.5–5	5–7.5	0.7
BP100.g	KKLFKKILKYL **AGPA** KFLHSAK KDEL	5–7.5	7.5–10	2.5–5	0.2
BP100.gtag	KKLFKKILKYL **AGPA** KFLHSAK **AGPA** *KDWEHLKDWEHLKDWEHL* KDEL	2.5–5	2.5–5	2.5–5	0.0
BP100.g2	KKLFKKILKYL **AGPA** GIGKFLHSAK KDEL	2.5–5	2.5–5	2.5–5	0.2
BP100.C	KKLFKKILKYL **AGPA** VAVVGQATQIAK KDEL	1.25–2.5	2.5–5	2.5–5	0.1
Tag54	*KDWEHLKDWEHLKDWEHL* KDEL	>100	>100	>100	0.0

The KDEL ER retention sequence is underlined, the AGPA linker sequence is highlighted in bold, and the tag54 sequence in italics. Antibacterial activity was determined against *Erwinia amylovora* (*Ea*), *Pseudomonas syringae* pv. *syryngae* (*Pss*) and *Xanthomonas axonopodis* pv. *vesicatoria* (*Xv*) as reporter species. Minimal inhibitory concentrations (MIC) are shown in μM and were calculated with 10^8 bacterial CFU/mL. Haemolytic activity is shown as the ratio between each peptide and the reference peptide BP100, calculated at 150 μM and presented as a percentage value with confidence interval for α = 0.05 (Badosa *et al.*, 2007).

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The transgene copy number and expression profile were assessed in three independent events representing each of the five *bp100der* transgenes. The ratio of *bp100der* and *hpt*II DNA to the endogenous actin sequence was 0.5, as determined by quantitative PCR (qPCR) using leaf genomic DNA from T_0 plants, suggesting single-copy insertions. Transgene expression was verified by quantitative RT-PCR (RT-qPCR) in the same T_0 leaf samples, revealing that both *hpt*II and the *bp100der* transgene were expressed in all events, with mRNA levels ranging from 0.01 through to 350-fold the level of *actin* mRNA used for normalization (GeNorm M < 0.5 in these samples; Figure S1). The transformation efficiency and expression level of the *bp100.gtag* transgene were statistically similar to *bp100.g* lacking the *tag54* sequence.

BP100.gtag accumulates in stably transformed rice seedlings

Five independent transgenic events expressing *bp100.gtag* (S-bp100.gtag) were selected for further analysis. qPCR characterization confirmed that all five events contained single transgene copies (Table 2). They were self-crossed to obtain homozygous T_2 lines. Transgene expression was monitored by RT-qPCR using stage V2 leaves (two-leaf stage) from plants grown under controlled-environment conditions. The level of *bp100.gtag* mRNA varied from 2 through to 67-fold the level of *actin* mRNA (Table 2).

We used the tag54×3 sequence to measure the accumulation of the peptide in transgenic rice plants by Western blot. A ~10.1-kDa band was detected in total soluble protein (TSP) extracts from one-week-old seedlings in four of five S-bp100.gtag events (Figure 4). Chemically synthesized BP100.gtag migrates at ~5.7 kDa when mixed with nontransformed rice extracts, which is commensurate with its theoretical mass, and a minor ~10.1-kDa band is visible at higher peptide concentrations. Serial dilutions of chemically synthesized BP100.gtag mixed with 20 μg of nontransformed rice extracts (0.72–0.045% of rice TSP) were used as a reference in Western blots to determine peptide yields in the transgenic plants. Accordingly, biologically produced BP100.gtag was shown to accumulate at levels up to 0.5% TSP in some events (e.g. S-bp100.gtag-11). There was also a positive correlation between the levels of *bp100.gtag* mRNA and the peptide detected in the different S-bp100.gtag events (Pearson correlation coefficient = 0.9).

We further assessed the activity of the recombinant BP100.gtag by an *in vitro* bacterial growth inhibition test. *E. amylovora* growth was reduced in the presence of protein extracts obtained from S-bp100.gtag-11 homozygous T_2 seedlings in these assays, compared with protein extraction buffer and extracts of control seedlings harbouring the *hptII* selection gene. Inhibition levels were 63 ± 8% (one-way ANOVA P = 0.000) when compared with the extraction buffer and 51 ± 7% (one-way ANOVA P = 0.000) when compared with control seedlings. This demonstrated the antibacterial activity of the recombinant BP100 derivative.

Ultrastructural changes in rice cells induced by the accumulation of BP100.gtag

We investigated the ultrastructure of rice cells producing BP100.gtag by transmission electron microscopy (TEM) at stage V2. We found that the ER in S-BP100.gtag cells was morphologically distinct in the collar region (Figure 5a). Compared with nontransformed rice cells, there was some ER fragmentation and an increase in the abundance of dictyosome structures. As shown in Figure 5b, several cells also featured strongly dilated intracisternal spaces and ribosome-decorated ER-derived vesicles that were in some cases comparable to mitochondria in size. Dictyosome vesicles presented as a compact mass of vesicles in the cytosol of these cells.

The exogenous application of high doses (i.e. 50 μM) of chemically synthesized BP100.gtag also induced significant morphological changes in rice seedlings at the ultrastructural level. The ER, with swollen cisternae, was distributed in the cytosol near the cell wall. The nuclear envelope was more dilated than that of control cells, and there were numerous irregular vesicles containing electron-dense granules. No visible injury was observed in other cellular components (Figure 5c). However, in some cells, there was substantial disruption to the ER, including numerous irregular vesicles up to 1 μm across, containing electron-dense granules, as well as irregular mitochondria with altered cristae (Figure 5d). Membrane invagination and plasmolysis were observed in the worst-affected cells.

Discussion

Several synthetic linear undecapeptides from the CECMEL11 library are useful leads for the development of novel antimicrobial

Table 2 Transgene copy numbers and mRNA expression in S-bp100.gtag rice

	Copy numbers per qPCR			Normalized transgene DNA levels		Normalized transgene mRNA expression (RT-qPCR)	
GMO event code	bp100.gtag	hptII	actin	bp100.gtag	hptII	bp100.gtag	hptII
S-bp100.gtag-2	2.17E + 04	1.59E + 04	7.88E + 04	0.27	0.20	41.1 ± 2.48	83.85 ± 11.18
S-bp100.gtag-5	1.29E + 04	2.05E + 04	2.86E + 04	0.45	0.72	1.03 ± 0.22	101.98 ± 7.90
S-bp100.gtag-6	5.05E + 03	4.92E + 03	1.71E + 04	0.30	0.29	68.82 ± 2.28	153.84 ± 2.93
S-bp100.gtag-7	8.96E + 03	6.04E + 03	3.27E + 04	0.27	0.18	33.69 ± 3.96	81.52 ± 15.09
S-bp100.gtag-11	5.02E + 03	5.62E + 03	1.68E + 04	0.30	0.33	66.60 ± 10.97	189.97 ± 33.85

The transgene copy number was determined by qPCR using genomic DNA extracted from T_0 leaf tissue. Means of 10 qPCR experimental replicates are shown with relative standard deviation (RSD) values consistently below 2.5%. Transgene copy numbers were normalized against β-*actin.* Considering that the T_0 plants are hemizygous concerning the transgene, ratios close to 0.5 indicate single transgene copies. Transgene mRNA expression was assessed by RT-qPCR and normalized against β-*actin* (GeNorm M values below 0.5) using RNA extracted from leaves of homozygous plants. For each line, means and SDs are shown for three biological replicates of 10 plants per biological replicate.

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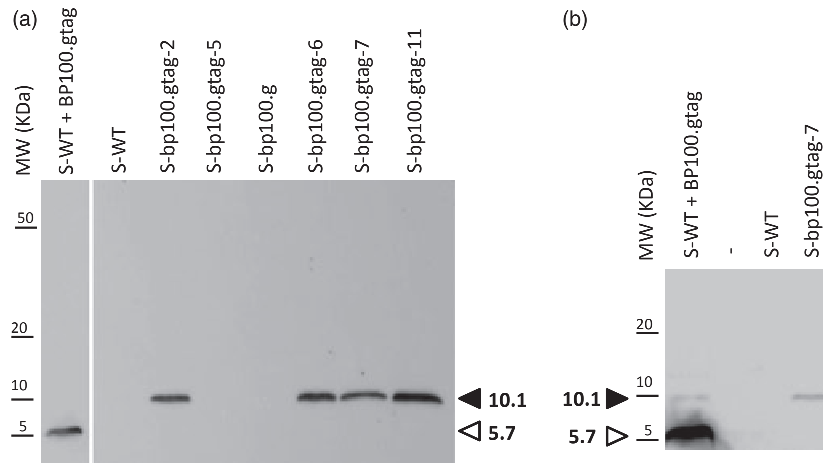

Figure 4 BP100.gtag accumulation in transgenic rice seedlings. Western blot analysis of proteins from five homozygous T_2 S-bp100.gtag rice events. Total soluble protein was extracted from rice seedling samples (five plants per event), and 20 μg (a) or 4 μg (b) of protein per lane was separated by SDS-PAGE before transfer to nitrocellulose filters. Recombinant proteins were detected using antibody mAb54k (diluted 1 : 1500) followed by the horseradish peroxidase-labelled anti-mouse IgG secondary antibody (diluted 1 : 10000) and ECL chemiluminescent detection with 30 ng (a) or 400 ng (b) chemically synthesized BP100.gtag mixed with wild-type rice protein extract as a control (S-WT). S-WT and S-bp100.g (i.e. transgenic rice with the same *bp100der* except for the tag54 sequence) were used as additional controls. Open arrowhead indicates chemically synthesized BP100.gtag (Mw = 5.7 kDa). Closed arrowhead indicates BP100.gtag produced in transgenic plants (Mw = 10.1 kDa).

agents, especially for the protection of plants against pathogens (Montesinos and Bardají, 2008; Montesinos *et al.*, 2012). These cationic, amphipathic α-helical peptides show potent activity against both microbial pathogens and tumour cells, but its toxicity at high concentrations towards eukaryotic cells makes them difficult to produce at high levels in plants (Nadal *et al.*, 2012). They are also difficult to detect and isolate due to their extreme physicochemical properties (highly cationic, pI = 11.5), low extinction coefficient (low contents or absence of aromatic amino acids) and lack of immunogenicity as determined by in silico prediction (OptimumAntigen™ Design Tool, GenScript, NJ) and the low antibody titres observed after immunization of rabbit with a BP100-KLH conjugate (data not shown).

BP100 is a member of the CECMEL11 library with potent activity against the major bacterial pathogens of plants (Badosa *et al.*, 2007). We expressed this peptide as a fusion with the fluorescent marker protein DsRed and the epitope tag54 to confirm that such peptides can be expressed transiently in *N. benthamiana* cells and can accumulate to similar levels as the nontoxic marker protein DsRed alone, when controlled by a strong constitutive promoter such as CaMV 35S (Piotrzkowski *et al.*, 2012).

We also showed that targeting BP100-DsRed-tag54 to the ER using a C-terminal KDEL tag resulted in the induction and rapid accumulation of ER-derived vesicles or protein bodies (PB) that merged to form structures up to 15 μm in diameter by 4 dpi. In contrast, the control protein DsRed behaved as a typical ER luminal protein, showing that the novel protein vesicles were induced by the BP100 component of BP100-DsRed-tag54. The BP100 peptide therefore appears to function in a similar manner to the highly tandemly repeated VPGXG elastin-like motif (ELP) and hydrophobins, which also induce the formation of novel ER-derived protein bodies when joined to ER-targeted fusion partners (Conley *et al.*, 2009; Joensuu *et al.*, 2010). The 93-residue Zera polypeptide derived from the maize seed storage protein γ-zein can also induce the formation of ER-derived protein bodies although in this case the capability appears to be intrinsic and does not require an additional KDEL sequence. This property has been used to facilitate purification of the recombinant protein (Torrent *et al.*, 2009). Fusions with peptides or polypeptides that induce vesicle formation may be suitable for hard-to-express and toxic protein candidates (Conley *et al.*, 2011).

When we expressed an ER-targeted cyan fluorescent protein along with the DsRed constructs, this not only accumulated in the typical ER along with DsRed in the control experiments, but also co-localized with BP100-DsRed-tag54 in the novel vesicles confirming that the vesicles are derived from the ER compartment and contain typical ER-resident proteins. The proteome of Zera-DsRed protein bodies induced by transient expression in tobacco leaves has recently been characterized (Joseph *et al.*, 2012), revealing the presence of nearly 200 additional proteins including typical ER-trafficking and ER-resident proteins that appear to be recruited to the new vesicles as if they represent extensions of the typical ER. The vesicles induced by BP100 appear to behave in a similar manner. BP100, ELP and Zera share an amphipathic structure that facilitates self-assembly (Llop-Tous *et al.*, 2010) and membrane interactions (Alves *et al.*, 2010). Therefore, although BP100 is much smaller and has a pI of 11.5, its propensity for self-assembly may promote its ability to induce the formation of novel vesicles.

BP100-derived peptides are phytotoxic at concentrations 100-fold higher than the MIC for target microbes, and this was confirmed by the expression of BP100-DsRed-tag54 in stably transformed *A. thaliana* plants. Two-week-old plants demonstrated the same vesicular properties as *N. benthamiana* leaves transiently expressing the same construct, but they were unable to develop fully suggesting that long-term exposure to the peptide has a deleterious impact. Confocal micrographs of radicles expressing BP100-DsRed-tag54 and stained with SYTOX showed the same pattern of cell death as control radicles expressing DsRed alone and wild-type plants (Truernit and Haseloff, 2008). It is therefore clear that BP100 can be expressed transiently in plants without harmful effects but that long-term exposure interferes with normal growth and development.

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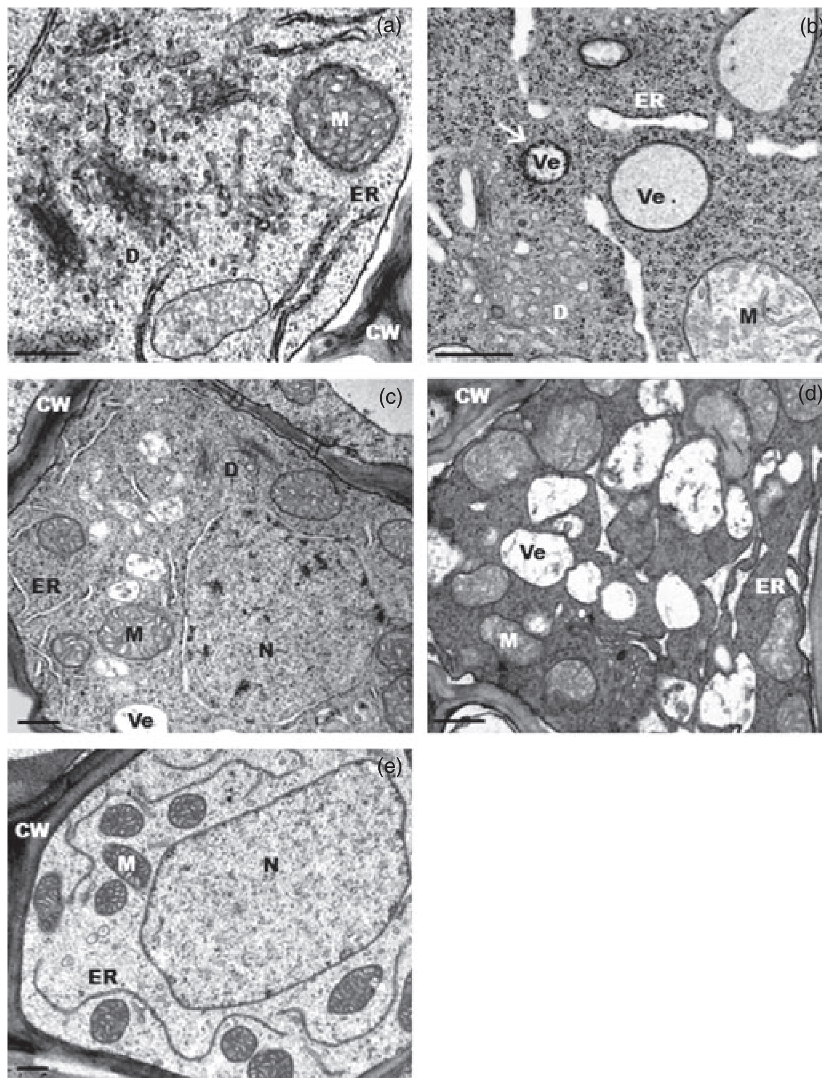

Figure 5 Transmission electron micrographs showing the effects of recombinant or exogenous BP100.gtag on the ultrastructure of rice cells. Surrounding vascular cells of the crown region of S-bp100.gtag.11 seedlings producing BP100.gtag (a,b) and untransformed rice seedlings either grown in the presence of 50 μM chemically synthesized BP100.gtag for 7 days (c,d) or in control conditions (e). A, detail showing increased abundance of dictyosomes; (b), detail showing dilation of ER cisternae, ER-derived vesicles and more abundant dictyosome vesicles; (c), placement of the ER near the cell wall, with some dilation of flattened cisternae, swelling of the nuclear envelope and appearance of vesicles containing electron-dense granules; (d), altered ER morphology, numerous large vesicles with electron-dense granules, altered mitochondria. CW, cell wall; (d), dictyosome; ER, endoplasmic reticulum; M, mitochondria; N, nucleus; Ne, nuclear envelope; Ve, vesicle; Arrow, ribosomes decorating vesicles. Scale bars, 0.5 μm.

The long-term toxicity of BP100 means that it is only suitable for short-term applications such as transient expression and local delivery, for example BP100 has been developed as an efficient cell-penetrating agent to deliver functional cargoes such as the actin-binding Lifeact peptide (MGVADLIKKFESISKEE) into tobacco cells (Eggenberger *et al.*, 2011). However, longer-term applications such as stable expression in plants for molecular farming or to provide pathogen resistance in crops would benefit from rational modification to achieve a targeted reduction in phytotoxicity without affecting antimicrobial potency. We have previously noted that modified BP100 peptides (BP100der) with potent antimicrobial activity against *E. amylovora*, *X. axonopodis* pv. *vesicatoria* and *P. syringae* pv. *syringae* can vary significantly in terms of their haemolytic activity (Badosa *et al.*, in preparation). Therefore, we hypothesized that this property could be used as a marker for phytotoxicity, allowing the selection of potent derivatives suitable for stable expression in plants based on our previous experiments in rice (Nadal *et al.*, 2012).

We found an inverse correlation between the haemolytic activity and transformation efficiency of nine BP100der constructs, with a Pearson coefficient of –0.951 and a bilateral significance of 0.000. One-way ANOVA distinguished two groups of sequences based on their haemolytic activity: those with haemolytic activities up to 15% at 150 μM achieved transformation efficiencies of 30–50% that of the control plasmid, whereas those with haemolytic activities in the 68–99% range at 150 μM achieved only minimal transformation efficiencies (Figure 6). BP100 derivatives selected on the basis of low haemolytic activities therefore appear to provide the greatest likelihood of successful expression in stably transformed plants.

Five BP100 derivatives with low haemolytic activities were expressed in stably transformed rice, including an epitope tag to

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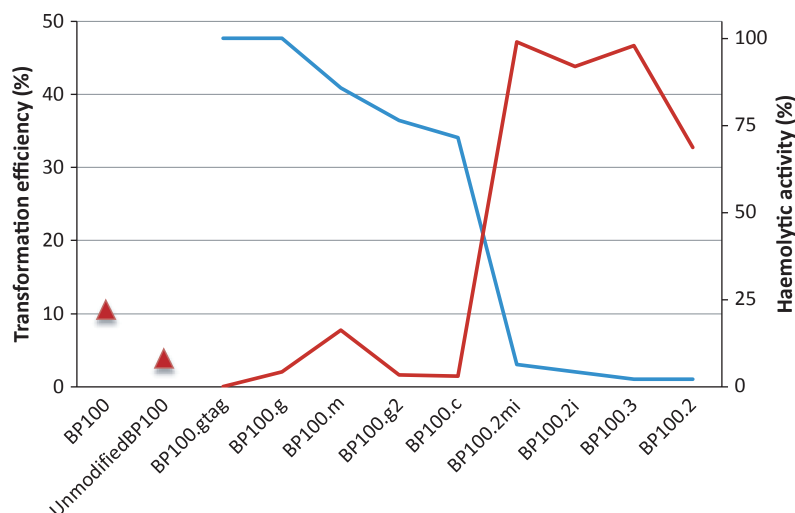

Figure 6 Haemolytic activity of BP100 derivatives and corresponding transformation efficiencies. Transformation efficiency is expressed as a percentage compared to the empty vector (*hpt*II, blue line). Haemolytic activity is expressed as a percentage at 150 μM peptide (red line). Triangles represent BP100 (amidated) and unmodified BP100, for which no transformation efficiency data are available.

facilitate detection. A direct comparison of BP100.g and BP100.gtag confirmed that the tag did not affect the antimicrobial profile or transformation efficiency of the peptide. Although the expression levels varied considerably among independent transformants with the same construct, we found a good correlation between transgene mRNA and recombinant BP100.gtag peptide levels in extracts from the transgenic plants and that the best performing lines produced up to 0.5% BP100der as a proportion of TSP and, importantly, had antibacterial activity. Similar yields of recombinant proteins have been reported using constitutive promoters in *Triticum aestivum* and *N. tabacum* (Khan *et al.*, 2012). We cannot completely exclude the possibility that our yield estimates were affected by the unusual properties of the BP100der peptides, which result in nonspecific interactions with many components of the plant cell, thus reducing the efficiency of isolation from complex matrices. BP100 yield decreases due to unspecific adherence to plant-derived molecules and surfaces of materials used during extraction and purification have been observed (L. Montesinos, personal communication). The recombinant BP100.gtag was larger than its chemically synthesized counterpart used in the control lanes (10.1 vs 5.7 kDa), a phenomenon that has been described for other AMPs such as Sarcotoxin IA and Cecropin A when targeted to the ER in tobacco and rice, respectively (Mitsuhara *et al.*, 2000; Coca *et al.*, 2006). This gain in electrophoretic mobility did not affect the activity of the peptide and may potentially reflect the proposed higher stability of these peptides as dimer.

BP100.gtag was produced in stably transformed rice plants without significantly impairing fitness, suggesting lower toxicity to the host plant than other BP100 derivatives such as BP100.2 that could not be expressed in rice plants using the same approach (Nadal *et al.*, 2012). However, the exogenous exposure of plant tissues to high concentrations of chemically synthesized BP100.gtag did have a significant deleterious impact, as demonstrated in *N. benthamiana* leaf microinfiltration and rice seedling development assays. Indeed, all nine chemically synthesized BP100der peptides with available transformation efficiency data produced similar results in plant toxicity tests, statistical analyses (ANOVA and Tukey B post-test) clustering most peptides together (Figure S2). There was no correlation between the *N. benthamiana* leaf lesion size or inhibition of seedling development and transformation efficiency (Pearson coefficient of −0.488 and 0.586, respectively). The assays discussed above therefore should not be relied upon to predict plant transformation efficiencies when using constitutive expression constructs and ER-targeted peptides. It may be possible to extrapolate the data obtained with BP100.gtag to other cationic α-helical antimicrobial peptides that show some toxicity towards plant cells when applied at high concentrations.

The exogenous application of BP100.gtag to the roots of rice plants for 7 days resulted in ultrastructural changes similar to those observed in other plants exposed to heavy metals, for example modified ER and dictyosome morphology, modified nuclear envelope structure, the appearance of numerous vesicles containing electron-dense granules and in some cases the degeneration of other organelles and plasmolysis, perhaps reflecting a common response to strongly cationic molecules (Fan *et al.*, 2011; Jiang and Liu, 2010; Liu *et al.*, 2009). Conversely, the ultrastructure of rice cells stably transformed with *bp100.gtag* was less severely affected, and the dictyosome and ER modifications appeared similar to those associated with a highly active endomembrane system or the morphological changes observed during very short exposure to metal ions. The accumulation of recombinant peptides in ER-derived vesicles probably protects the cell from the worst effects, thus minimizing the residual toxicity of these BP100-derived peptides. Similarly, the antimicrobial peptide Cecropine A could be expressed in rice when targeted for accumulation in the ER but not when targeted to the apoplast (Coca *et al.*, 2006).

In conclusion, cationic α-helical peptides such as BP100 can be produced as recombinant proteins in plant cells, using an approach based on constitutive expression and ER targeting. Using a fluorescent fusion partner, we demonstrated that BP100 derivatives accumulate in ER-derived vesicles when transiently expressed in *N. benthamiana* and when stably expressed in *A. thaliana* and that the BP100 component induces the formation of these vesicles. BP100 expression in transgenic plants does not cause cell death in young tissues, but long-term expression nevertheless impairs normal growth and development. We

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demonstrated that antimicrobial peptides such as BP100 could be produced in a transient-expression plant-based platform. We also showed that BP100 derivatives with low haemolytic activity but high antimicrobial potency retain a degree of phytotoxicity at high doses but can nevertheless be produced in stably transformed rice plants at levels of up to 0.5% TSP when targeted to the ER.

Experimental procedures

Chemical synthesis of BP100-derived peptides and *in vitro* characterization

The BP100-derived peptides were synthesized using solid-phase Fmoc-type chemistry as previously described (Badosa *et al.*, 2007) and assessed for purity by HPLC (the purity was >90% in all cases). Peptide identity was confirmed by electrospray ionization mass spectrometry. Peptides were solubilized in sterile Milli-Q H_2O to a concentration of 1 mM and filter-sterilized (0.22-μm pore filter).

Antimicrobial activity tests were carried out as previously described (Nadal *et al.*, 2012) using the plant pathogens *Erwinia amylovora* PMV6076 (INRA, Angers, France), *Pseudomonas syringae* pv. *syringae* EPS94 (UdG, Girona, Spain) and *Xanthomonas axonopodis* pv. *vesicatoria* 2133-2. Positive (water instead of peptide) and negative (water instead of microbial suspension) controls were included in each experiment. Three biological replicates were performed, each comprising two experimental replicates. The lowest peptide concentration inhibiting microbial growth at the end of the experiment was established as the minimal inhibitory concentration (MIC). Haemolytic activity was assessed by measuring the release of haemoglobin from erythrocyte suspensions prepared from fresh human blood as previously described (Badosa *et al.*, 2007). The percentage haemolysis was calculated relative to mellitin and Tris buffer. Each experiment was carried out three times.

Phytotoxic activity was determined using a rice seedling development test and a *N. benthamiana* leaf inoculation assay as previously described (Nadal *et al.*, 2012). Toxicity in the first assay was expressed as the inverse shoot length of 12 plantlets per treatment. Toxicity in the second assay was expressed as the mean of the lesion diameters of six replicates infiltrated with 100 μl of 50 μM peptide.

Construction of vectors

The *bp100:dsred:tag54* and *dsred:tag54* constructs included the *Petrosilinum hortense* chalcone synthase 5′ untranslated region and a sequence encoding the codon optimized signal peptide from the heavy chain of monoclonal antibody 24 (Vaquero *et al.*, 1999) upstream of BP100-DsRed or DsRed (Matz *et al.*, 1999), and the epitope tag 12-tag54 (Rasche *et al.*, 2011) and KDEL ER retention motif downstream, all placed under the control of the double-enhanced *Cauliflower mosaic virus* 35S promoter and terminator (Piotrzkowski *et al.*, 2012). The constructs were directionally inserted into the KpnI and SbfI sites of pCAMBIA1300 to obtain pCbp100-dsred-tag54 and pCdsred-tag54. After sequencing the whole insert, these plasmids were transferred into *A. tumefaciens* strain GV2260 by electroporation and infiltrated into *N. benthamiana* leaves or used to transform *A. thaliana* plants by floral dipping. Subcloning procedures and the transformation of *Escherichia coli* strain XL1Blue were carried out using standard techniques (Sambrook and Russell, 2001).

Sequences encoding the five BP100 derivatives fused in-frame to the *N. tabacum* pathogenesis-related protein PR1a signal peptide sequence were designed according to rice codon usage. Synthetic *bp100.c*, *bp100.g*, *bp100.g2*, *bp100.gtag* and *bp100.m* genes were prepared by GenScript (Piscataway NJ) and included terminal BamHI restriction sites to facilitate insertion into pAHC17 (Oh *et al.*, 2000). The constructs were flanked by the promoter, first exon and first intron of the maize *ubiquitin-1* gene (Huang, 2000) and the *A. tumefaciens* nopaline synthase (*nos*) terminator. After verification by sequencing, the complete cassettes were introduced into the KpnI site of pCAMBIA1300 in the opposite orientation to the *hpt*II gene to generate five pCBP100der vectors for the stable transformation of rice. The resulting binary vectors were transferred into *A. tumefaciens* strain EHA105 by cold shock (Sambrook and Russell, 2001).

Agroinfiltration of *N. benthamiana* leaves

Wild-type *N. benthamiana* plants were cultivated for 4–5 weeks in a greenhouse at 18–28 °C with a long-day (16-h) photoperiod of light. And the bacteria cultures containing the expression vectors discussed above as well as the one encoding eCFP (pCSPECFPKDEL, Joseph *et al.*, 2012; kindly provided by D. Ludevid, CRAG) were mixed with cultures carrying the HC-Pro suppressor of silencing (Goytia *et al.*, 2006). Agroinfiltration of the abaxial side of the upper leaves was carried out using a syringe without needle.

Stable transformation of *A. thaliana*

Arabidopsis thaliana plants were transformed with *A. tumefaciens* cultures carrying the expression vectors discussed above by floral dipping (Clough and Bent, 1998). Seeds were surface-sterilized and allowed to germinate on MS medium with 20 mg/L hygromycin B in culture chambers for 3 days in the dark at 4 °C (stratification) and 13 days under long-day conditions (16-h photoperiod) at 22 °C. Plantlets were analysed by confocal microscopy or allowed to grow to maturity under standard conditions.

Stable transformation of rice

Embryonic rice callus (*Oryza sativa* L., ssp *japonica*, cv Senia) derived from mature embryos as described (Pons *et al.*, 2000) was transformed with the constructs discussed above and pCAMBIA 1300 (containing *hpt*II) as a control. We transformed 500 callus pieces with the constructs containing the *bp100.gtag, bp100.m, bp100.g* and *bp100.c* genes and 1000 callus pieces with the constructs containing the *bp100.c2* and *bp100.g2* genes. Hygromycin-resistant, fertile T_0 plants were grown to maturity, and leaves were tested for transgene insertion by qPCR. The efficiency of transformation was calculated by comparing the number of transformants obtained with each BP100der construct compared with the pCAMBIA 1300 control. S-bp100.gtag plants were cultured under standard greenhouse conditions to obtain homozygous transgenic lines in the T_2 generation.

Protein extraction and Western blot analysis

Total soluble protein was extracted from three *N. benthamiana* leaf sections (~1 cm^2 agroinfiltrated tissue) or shoots from six rice seedlings germinated in a culture chamber at 25 ± 1 °C with a photoperiod of 16 h light/8 h dark under fluorescent Sylvania Cool White lamps. Tissue was extracted in lysis buffer (10 mM Tris-HCl pH 6.2, 50 mM KCl, 6 mM $MgCl_2$, 0.4 M NaCl, 1% (v/v) Triton X-100 and 10 mM EDTA) and centrifuged at 16 000 ***g*** for 15 min at 4 °C, and the protein concentration in the supernatant was determined using the Bradford method. The extracted

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protein was separated by 12% or 15% (w/v) SDS polyacrylamide gel electrophoresis (20 or 4 μg per lane) and transferred to nitrocellulose filters, and the tag54 epitope was detected with monoclonal antibody mAb54k (Rasche *et al.*, 2011) diluted 1 : 1500, overnight at 4 °C. Binding was detected using a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (GE Healthcare Life sciences) diluted 1 : 10000 for 1 h at room temperature. The signal was detected by ECL chemiluminescence (Supersignal® West Femto, Thermo Scientific, Waltham, MA) and quantified using Multi Gauge v3.0 software based on 25.0, 12.5, 6.25, 3.125 and 1.5625 pmol standards corresponding to 143.4–9.0 ng of chemically synthesized BP100.gtag mixed with protein extracts from wild-type rice, or 0.72–0.045% rice TSP, run on the same gel.

Antimicrobial activity of recombinant peptides

Protein extracts were obtained from 30 rice seedlings germinated for 4 days in a culture chamber at 25 ± 1 °C with a photoperiod of 16 h light/8 h dark. Tissue was ground in liquid nitrogen and treated with 10 mM phosphate buffer pH 7.5 and 0.6 M sucrose buffer, previously sterilized through a 0.2-μm pore filter. The extract was centrifuged at 200 ***g*** for 10 min at 4 °C, and the supernatant was re-centrifuged at 2000 ***g*** for 10 min at 4 °C to precipitate a fraction enriched in vesicles and protein bodies, which was re-suspended in 10 mM phosphate buffer pH 7.5 with protease inhibitor cocktail (Sigma-Aldrich, Munich, Germany). Protein concentrations were determined using the Bradford method and were consistently in the 12–15 mg/mL range. Preliminary experiments showed that the protein extraction buffer had no effect on *E. amylovora* growth, but protein extracts from control rice samples inhibited bacterial growth. Dilution of protein extracts to 1/3 in the same buffer permitted growth of the indicator bacteria after a short lag phase. To evaluate the expected inhibition of the recombinant BP100.gtag, controls were carried out with extracts from rice seedlings harbouring only the *hptII* selection gene. Three biological replicates were performed, each comprising three experimental replicates. The area under the growth curve (AUC) was used to calculate the inhibition effects of the extracts from transgenic seedlings expressing BP100.gtag compared with control seedlings or the buffer control: (Ac–At)*100/Ac, where Ac and At are the AUC of control and test seedlings, respectively.

Imaging

Confocal microscopy

Nicotiana benthamiana leaves and *A. thaliana* leaves/radicles were analysed by confocal microscopy using an FV1000 Olympus microscope. Red fluorescence images were collected at 559 nm excitation and 570–670 nm emission, whereas cyan fluorescence images were collected at 405 nm excitation and 460–500 nm emission. ImageJ software (http://rsb.info.nih.gov/ij/) was used to calculate the number and size of fluorescent spots or aggregates. SYTOX staining was carried out by incubating plantlets with 2.5 μM SYTOX (Invitrogen Life Technologies, Carlsbad, CA) for 24 h prior to analysis at 559 nm excitation and 570–670 nm emission. A total of three agroinfiltrated fields in the upper, medial and basal portions of different leaves were analysed using Olympus Fluoview v3.1 software with standard parameters. IBM SPSS Statistics19 software was used to compare means by one-way ANOVA and Tukey B post-test.

Transmission electron microscopy

Rice seeds were allowed to germinate for 7 days in distilled water under controlled conditions, with or without 50 μM chemically synthesized BP100.gtag. Sections of the collar region were excised and fixed in 1% (v/v) glutealdehyde and 2.5% (v/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at room temperature. TEM sections were prepared by the Service for Microscopy at the Autonomous University of Barcelona, and images were collected on a GEM-1400 TEM. Wild-type rice and plants transformed with the pCAMBIA 1300 vector (containing *hpt*II) were used as controls.

Nucleic acid extraction, qPCR and RT-qPCR

To assess transgene copy number, genomic DNA was extracted from 1 g of mature hemizygous T_0 leaf tissue using a CTAB method (Coll *et al.*, 2008) and analysed by qPCR targeting conserved sequences in each construct, with 10 experimental replicates (Nadal *et al.*, 2012) and the rice β-*actin* gene as a standard for normalization (Montero *et al.*, 2011). All oligonucleotides were purchased from MWG Biotech AG (Germany).

Transgene expression was measured in leaf samples from three independent T_0 transgenic rice plants representing each construct and in five homozygous lines expressing *bp100.gtag*. Leaves of the homozygous T_2 plants were sampled at the two-leaf vegetative stage, and three biological replicates of 10 plants (i.e. a total of 30 plants) were analysed per line. Total RNA was extracted from 400-mg samples using Trizol reagent (Invitrogen Life Technologies) with DNase I treatment (Ambion, Grand Island, NY), according to the manufacturer's instructions. The concentration and quality of the RNA were confirmed by UV absorption at 260 and 280 nm using a NanoDrop ND1000 spectrophotometer (Nanodrop technologies, Wilmington, DE). RT-qPCR was carried out as previously described (Nadal *et al.*, 2012). For each sample, cDNA was prepared with random primers in duplicate, and the reactions were performed in triplicate. The absence of DNA targets was demonstrated using DNase-treated samples. All reactions had linearity coefficients exceeding 0.99 and efficiency values above 0.95. The β-actin gene was used for normalization, its suitability having been confirmed using the geNORM v3.4 statistical algorithm (Vandesompele *et al.*, 2002; M values below 0.5 in our samples).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Transgene mRNA expression levels (relative to actin) in the leaves of transgenic rice plants grown *in vitro*, as assessed by quantitative real-time RT-PCR.

Figure S2 Phytotoxicity of chemically synthesized BP100 derivatives as determined using *N. benthamiana* leaf inoculation (dark grey) and rice germination (light grey) assays (Nadal *et al.*, 2012).

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CHAPTER V

Production of phytotoxic cationic α-helical antimicrobial peptides in plant cells using inducible promoters

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Production of phytotoxic cationic α-helical antimicrobial peptides in plant cells using inducible promoters

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Abstract

Synthetic linear antimicrobial peptides with cationic α-helical structures, such as BP100, have potent and specific activities against economically important plant pathogenic bacteria. They are also recognized as valuable therapeutics and preservatives. However, highly active BP100 derivatives are often phytotoxic when expressed at high levels as recombinant peptides in plants. Here we demonstrate that production of recombinant phytotoxic peptides in transgenic plants is possible by strictly limiting transgene expression to specific tissues and conditions. Specifically, minimization of this expression during transformation and regeneration of transgenic plants is essential to obtain viable plant biofactories. On the basis of whole-genome transcriptomic data available online, we identified the Os.Hsp82 promoter that fulfilled this requirement and was highly induced in response to heat shock. Using this strategy, we generated transgenic rice lines producing moderate yields of severely phytotoxic BP100 derivatives on exposure to high temperature. In addition, a threshold for gene expression in selected tissues and stages was experimentally established, below which the corresponding promoters should be suitable for driving the expression of recombinant phytotoxic proteins in genetically modified plants. In view of the growing transcriptomics data available, this approach is of interest to assist promoter selection for specific purposes.

Key Words: AMP (antimicrobial peptide); cationic peptide; BP100; recombinant peptide; transgenic plant; phytotoxicity; heat shock; inducible promoter.

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Introduction

Antimicrobial peptides (AMPs) are key components of innate immunity in plants and animals, and are also produced by microbes in antibiosis processes. A significant proportion are strongly cationic and have linear structures that adopt an amphipathic α-helical conformation that binds to the phospholipid membranes of target microbes before the hydrophobic face is inserted into the membrane bilayer. This unique mode of action explains the lack of resistance in target pathogens. This makes AMPs valuable novel therapeutic agents against bacteria, fungi, viruses, parasites and tumor cells. Novel improved or synthetic AMPs have been designed with increased potency against selected pathogens [1–5]. As an example, the synthetic undecapeptide BP100 (KKLFKKILKYL-NH_2) is effective against *Xanthomonas vesicatoria* in pepper, *Erwinia amylovora* in apple and *Pseudomonas syringae* in pear [3] with the same efficacy as standard antibiotics, while also being biocompatible, as determined by acute oral toxicity tests in mice [6]. Plant expression of recombinant cationic α-helical peptides such as BP100 is preferable for industrial and phytosanitary applications. We have recently showed that BP100der peptides can be expressed, at high levels, as recombinant peptides in plants [7,8]. The endoplasmic reticulum (ER) retention motifs of these recombinant BP100der peptides, to minimize toxicity to the host plant, did not affect the antimicrobial activity of the products in vitro, using bacterial growth inhibition tests. Following transient expression in *Nicotiana benthamiana* and stable expression in *Arabidopsis thaliana* seedlings, the peptides accumulated in large ER-derived vesicles, along with typical ER luminal proteins [8]. However, the recombinant peptides were often toxic to the host during later developmental stages. Similarly, most transgenic *Oryza sativa* plants constitutively expressing recombinant ER-targeted BP100der peptides failed to achieve maturity. Only a few peptides coupling potent antimicrobial and low hemolytic activity accumulated in transgenic rice lines, with a yield of up to 0.5% total soluble protein (TSP) [8]. Many cationic α-helical peptides cannot be expressed in transgenic plants following this strategy, although they have potent activities against other types of pathogenic cells making them valuable as novel therapeutics and preservatives.

High temperature stress is one of the most common abiotic stresses among many world crops. Plants have evolved various physiological and molecular mechanisms to resist heat stress. Based on the expression data from different plant species, it is estimated that high temperatures affect approximately 2% of the plant genome (review in [9]). Exhaustive identification of heat stress-responsive genes has been carried out by means of transcriptomics [10–15]. Two groups of genes have been found, signaling components (e.g. protein kinases and transcription factors) and functional genes such as heat shock proteins (Hsps) [16], although many heat shock genes still have

unknown functions. Hsp are functionally linked to molecular chaperones that are essential for maintenance and restoration of protein homeostasis. Protein denaturation occurring during stress triggers high transcription of *hsp* genes by the binding of active heat shock factors (Hsf) to heat shock elements. Alterations in expression of a high number of genes in response to stress occur in complex systems. Cross-talk seems to exist between the regulatory pathways in response to different abiotic stresses such as temperature, and osmotic or oxidative, and biotic stresses [17]. On fusing several heat-shock promoters to reporter genes, they have been found to be regulated during various stress situations, have organ and developmental stage-specific basal expression levels and are induced by stress [18].

Heat-shock induced genes can be highly up-regulated at the transcriptional level by exposure to temperatures above those of normal plant growth. Therefore, induction can be easily carried out, is inexpensive and does not require the use of hormones or chemicals. Here we explored the use of heat-shock inducible promoters with minimal basal activity during normal plant development, to allow expression of recombinant phytotoxic peptides in transgenic rice. The exponential growth of profiling studies reporting gene expression data for rice, and the development of databases and bioinformatics tools, provide a possible way to identify genes related to heat stress responses, and with specific expression patterns.

Experimental procedures

In silico identification of candidate heat shock promoters

Miamexpress-EMBL-EBI (http://www.ebi.ac.uk/miamexpress/) and GEO-NCBI (http://nlmcatalog.nlm.nih.gov/geo/) were used to identify published microarray experiments questioning the late response of rice seedlings to heat shock stress. In the experiment with accession number GSE14275, the authors [10] state that rice *Affymetrix* microarrays were hybridized with RNA extracted from 14-day-old rice seedlings grown in a growth chamber with a daily photoperiodic cycle of 14 h light and 10 h dark, at between 28-30ºC, either or not subjected to 42 ºC for 3 h. The data from this experiment was extracted using the RMA software [19], which includes background adjustment, quantile normalization and summarization, and used to identify probes for subsequent analysis.

Rice gene expression data in different organ and developmental stages, and in response to various stress conditions, was obtained from the CREP database (Collection of Rice Expression Profiles; http://crep.ncpgr.cn/crep-cgi/home.pl) and the microarray hybridization results available at the Miamexpress-EMBL-EBI and GEO-NCBI websites.

The *NetAffx-analysis center* (www.affymetrix.com) allowed identification of the gene associated to every probe, and the sequence was retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank). Likely promoter sequences were identified using the PlantProm DB, an annotated and non-redundant database of proximal promoter sequences [20,21].

Primer design and amplification of rice promoter sequences

The Primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primer pairs specifically targeting the identified promoter sequences, blocking the reverse primer at the -1 position (immediately upstream to the ATG translation start codon). Two primer pairs (Table 1) were designed to amplify 553 and 1016 bp fragments of the pHsp18 and pHsp82 promoters. They each included an enzyme restriction site (*Kpn*I and *Spe*I at the distal and proximal promoter end, respectively) to facilitate the subsequent cloning steps.

Oryza sativa L., ssp *japonica*, cv Senia genomic DNA was extracted from 1 g of leaf samples obtained from young plants grown under controlled conditions, using the commercial NucleoSpin®Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

PCR was in a final volume of 50 µl 1x reaction buffer with the appropriate concentrations of $MgCl_2$ and primers (Fisher Scientific SL, Madrid) (Table 1), 200 µM dNTPs and 1 unit Expand High Fidelity DNA polymerase (Roche Diagnostics Corporation). The reaction conditions were as follows: 3 min at 94°C; 10 cycles of 15 s at 94°C, 30 s at the appropriate annealing temperature (Table 1) and 1 min at 72°C; 20 cycles of 15 s at 94°C, 30 s at the same annealing temperature and 1 min, plus an additional 5 s for each successive cycle, elongation at 72°C; and a final extension of 10 min at 72°C.

Table 1. Primer sequences and PCR conditions used. Additional restriction sites are underlined.

Target sequence	Primer	Primer sequence	$MgCl_2$ (mM)	Primer conc. (nM)	Annealing temperature (ºC)
Os.hsp18 promoter	pHsp18_Kpn	TATAGGTACCAGGATATAAATTAACAGCGC	3	500	49 / 59
	pHsp18_Spe	GTAGACTAGTCTCTCGATCGCCTCTTC		500	
Os.hsp82 promoter	pHsp82_Kpn	TATAGGTACCATACGTTTATTAGCAATAATAGTTT	3	500	50 / 62
	pHsp82_Spe	GTAGACTAGTGTTGATCTCTGCCTGGAA		500	
bp100.2 coding sequence	PR1a_Spe	TATAACTAGTGAGGCCACCATGGG	1.5	300	49 / 58
	BP100KDEL_Bam	TATAAAGCTTGGATCCATTATCAGAGCTC		300	
bp100-dsred-tag54 and dsred-tag54 coding sequence	CHS_Spe	TATAACTAGTGAATTCACAACACAAATCAGA	1.5	300	49 / 58
	TagKDEL_Bam	TATAGGATCCTACTAAAGCTCATCCTTCTCA		300	
β-actin rice gene	ACTrice_for*	CCTCTTCCAGCCTTCCTTCATA		100	60
	ACTrice_rev*	GCAATGCCAGGGAACATAGTG		100	
ef-1α rice gene	EFrice_for*	TTTCACTCTTGGTGTGAAGCAGAT		300	60
	EFrice_rev*	GACTTCCTTCACGATTTCATCGTAA		300	
bp100.2 transgene	bp100der_for*	TCCTCGTGATCTCCCACTCCTG		100	60
	bp100der_rev*	CGGATCCATTATCAGAGCTCGT		50	
dsred transgene	SYDsRed_for	TGGCAAAGAAGCCTGTGCAGC		100	60
	SYDsRed_rev	TGGTGGCGTCCCTCGGTTCT		100	
Os.hsp18 rice gene	P17,5SYR_for	AACGCAGACGTCGACAAGAT		100	60
	P17,5SYR_rev	CGACGCAGATGCAGAGAGAT		100	
Os.hsp82 rice gene	P82SYR_for	TCGACGACCCAAACACCTTC		100	60
	P82SYR_rev	CATTTCGACATGGCAGCGAG		100	
T_1 leaf material (dsred)	DsRed_for	GTCATGAACTTTGAAGACG	1.5	500	55
	Nos.te_rev	TAATCATCGCAAGACCGGCA		500	
T_1 leaf material (bp100.2)	P82_for	ATCGGAGCAGTGAACTGA	1.5	500	57
	Nos.te_rev	TAATCATCGCAAGACCGGCA		500	

*[7]

Construction of plant transformation vectors

The pHsp18 and pHsp82 promoters were separately subcloned into the *Kpn*I and *Spe*I sites of a pBluescriptIIKS+ derived vector having a polylinker fragment with the *Kpn*I, *Spe*I and *BamH*I restriction sites, followed by the *A. tumefaciens* nopaline synthase *nos* terminator sequence. The sequences encoding BP100.2, BP100-DsRed-tag54 and DsRed-tag54 were subcloned into these plasmids using the *Spe*I and *BamH*I restriction sites.

For the BP100.2 plasmids, the sequence encoding the *N. tabacum* pathogenesis related protein PR1a signal peptide [22] fused to BP100.2 [7] was PCR amplified using pAHC17-bp100.2 [7] as template and the primers PR1a_Spe and BP100KDEL_Bam, with the *Spe*I and *BamH*I restriction

sequences, respectively. For DsRed-tag54 control plasmids, the sequence encoding the *Petrosilinum hortense* chalcone synthase 5′ untranslated region plus the codon optimized leader peptide derived from the heavy chain of the murine mAb24 monoclonal antibody [23] fused to DsRed [24], the epitope tag54 [25] and the KDEL ER retention motif, were PCR amplified using pCdsred-tag54 [8] as template and the primers CHS_Spe and TagKDEL_Bam that included the required restriction sites. As an additional control, a BP100-DsRed-tag54 plasmid was generated, similarly to DsRed-tag54 and including the BP100 antimicrobial sequence placed N-terminal to DsRed-tag54. The coding sequence was obtained by amplification of the pCbp100-dsred-tag54 DNA [8] with the same CHS_Spe and TagKDEL_Bam primers. PCR was in a final volume of 50 µl 1x reaction buffer with the appropriate concentrations of $MgCl_2$ and primers (Fisher Scientific SL, Madrid) (Table 1), 200 µM dNTPs and 1 unit Expand High Fidelity DNA polymerase (Roche Diagnostics Corporation). Reaction conditions were as follows: 3 min at 94°C; 10 cycles of 15 s at 94°C, 30 s at 49ºC and 1 min at 72°C; 20 cycles of 15 s at 94°C, 30 s at 58°C and 1 min, plus additional 5 s each successive cycle, elongation at 72°C; and a final extension of 10 min at 72°C.

On sequence verification (Macrogen, Seoul, Korea) using the CLCbio software (Aarhus, Denmark), the complete constructs, *bp100.2*, *bp100-dsred-tag54* and *dsred-tag54* plus promoter and terminator elements, were directionally inserted into the *Kpn*I and *Sbf*I sites of pCAMBIA1300. The resulting binary vectors were transferred into *A. tumefaciens* strain EHA105 by cold shock [26].

Production of transgenic rice plants

Commercial japonica rice (*Oryza sativa* L.) cv. Senia was transformed by *A. tumefaciens* to obtain transgenic rice lines expressing the chimeric proteins mentioned above, using hygromycin resistance as the selection trait. The control plasmid, pCambia1300 (transferring only the *hpt*II selection gene), was transformed in parallel. Embryonic calluses derived from mature embryos were transformed as previously described [27]. Hygromycin resistant T_0 plants were grown to maturity under standard greenhouse conditions to obtain the T_1 generation. Leaf samples of individual T_0 plants at the 5 leaf vegetative stage were used to extract genomic DNA and assess the presence and copy number of the transgene by real-time PCR (qPCR), targeting the coding region of every transgene as previously described [8]. The *actin* endogenous gene was used to normalize the Ct values. The number of fertile GM plants containing every plasmid was recorded to calculate the transformation efficiency compared to that of the pCambia 1300 plasmid.

Analysis of gene expression: plant material, RNA isolation and RT-PCR

Plant material: heat shock treatment

Wild type rice seeds (cv. Senia) were surface sterilized and germinated in vitro in sterile MS medium [28], including vitamins (2.2 g/L MS medium, 8 g/L agar and 30 g/L sucrose), under controlled conditions (28 ± 1ºC temperature and a 16 h light / 8 h dark photoperiod with fluorescent Sylvania Cool White lamps). After one week (2-leaf vegetative stage, V2), groups of 15 plants were treated at 42ºC for 0, 1, 2, 4, 8 and 16 h. For each treatment, three groups of five plants were collected as biological replicates, immediately frozen in liquid nitrogen and stored at -80ºC.

For each GM event, 15 T_1 seeds were surface sterilized and in vitro cultured, as mentioned above, up to the V2 stage. They were treated at 42ºC for 0 and 2 h and their leaves individually frozen in liquid nitrogen. The presence of the transgene was individually assessed in every plant using the Phire Plant Direct PCR Kit (Thermo Scientific, Lithuania) combined with the DsRed_for and Nos.te_rev (constructs with *dsred-tag54* and *bp100-dsred-tag54*) or the P82_for and Nos.te_rev (constructs with *bp100.2*) primer pairs (Table 1), according to the manufacturer's instructions. Samples giving positive PCR signal (i.e. harboring the transgene) were mixed for subsequent analyses.

Plant material: monitoring of the transformation process

The transformation to obtain transgenic rice was monitored using *A. tumefaciens* strain EHA105, with or without the basic pCambia1300 vector (with *hpt*II). Embryonic calluses derived from rice mature embryos were transformed and, when *hptII* was transformed, selected with hygromycin [27]. Samples of ten independent callus or events were taken at eight different stages for each transformation.

For embryo extraction, 120 hygromycin-resistant seeds (harboring *hptII*) and 80 non-transformed seeds were surface-sterilized and their embryos were immediately extracted and frozen, or germinated in 500 µL water or 500 µL water with 45 mg/L hygromycin B (40 seeds per treatment and embryo type). Germination was in a culture chamber (28 ± 1°C with a 16 h light / 8 h dark photoperiod, under fluorescent Sylvania Cool White lamps) for two and five days prior to embryo extraction and immediate freezing in liquid N_2. Untransformed seeds were not treated with hygromycin B.

RNA isolation and reverse transcription (RT) coupled to qPCR

Samples were homogenized in liquid nitrogen and 100 mg used to extract RNA with the Trizol reagent (Invitrogen Life Technologies) based protocol. After DNase I digestion (Ambion, Grand Island, NY), carried out according to the manufacturer's protocol, RNA concentration and quality was assessed by UV absorption at 260 and 280 nm using a NanoDrop ND1000 spectrophotometer (Nanodrop technologies, Wilmington, DE). RT-qPCR was carried out as previously described [7]. For each sample, cDNA was synthesized with random primers in duplicate and qPCR reactions targeting *bp100.2* and *dsred-tag54* transgenes, and *Os.hsp18* and *Os.hsp82* rice genes were carried out in triplicate. The qPCRs were in a final volume of 20 µl containing 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), the appropriate concentrations of primers (Fisher Scientific SL, Madrid) (Table 1) and 1 µl cDNA. The reaction conditions were as follows: 10 min at 95°C for initial denaturation; 50 cycles of 15 s at 95°C and 1 min at 60°C; and a final melting curve program of 60 to 95°C with a heating rate of 0.5°C/s. Melting curve analyses produced single peaks, with no primer-dimer peaks or artifacts, indicating the reactions were specific. All reactions had a linearity coefficient exceeding 0.995 and efficiency values above 0.95. The *ef-1α* gene was used for normalization, its suitability having been confirmed using the geNORM v3.4 statistical algorithm ([29]; *M* values below 0.5 in our samples). Triplicate biological samples, each containing at least five plants, were analyzed in each case.

Protein extraction and Western blot analyses

For each GM event, 15 T_1 seeds were surface sterilized and in vitro cultured as mentioned above, up to the V2 stage. Rice seedlings were treated at 42ºC for 2 h, then transferred to 28 ± 1°C with a 16 h light / 8 h dark photoperiod under fluorescent Sylvania Cool White lamps for two days, to allow recombinant protein accumulation and DsRed maturation, and then immediately frozen in liquid N_2. The Phire Plant Direct PCR Kit (Thermo Scientific, Lithuania) was used in combination with the SYDsRed_for and SYDsRed_rev primers (Table 1), according to the manufacturer's instructions, to individually identify transgenic T_1 plants. Transgenic seedlings were homogenized in liquid nitrogen and TSP extracted as previously described [8]. Protein concentration was determined using the Sigma Bradford Reagent and bovine serum albumin as standard. Twenty µg TSP were separated by PAGE, using 18% (w/v) SDS polyacrylamide gels, and electrotransferred to nitrocellulose membranes. Twenty-five to 1 pmol chemically synthesized controltag54 (GQNIRDGIIKAGPAVAVVGQATQIAKAGPAKDWEHLKDEL), mixed with 20 µg TSP extracted from untransformed rice seedlings, were included to allow quantification of the recombinant peptides. They were hybridized overnight with the mAb54k monoclonal antibody [25] (1:1500 dilution) at 4

°C, and with horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody (GE Healthcare Life sciences) (1:10000 dilution) for 1 h at room temperature. The hybridization signal was detected by ECL chemiluminescence (Luminata™ Forte HRP Chemiluminescence Detection Reagents, Millipore – Darmstadt, Germany).

Phenotype evaluation

T_1 seeds carrying pHsp82::*bp100.2*, pHsp82::*dsred-tag54* (3 independent events per construct) or *hptII*, and conventional Senia seeds, were surface sterilized and in vitro cultured as mentioned above, up to the V2 stage (height, 6±1 cm). They were all treated at 42ºC for 2h and allowed to recover for three days under the same conditions. Plant growth was monitored daily by measuring the height of the aerial part. Transgenic plants were identified using the Phire Plant Direct PCR Kit (Thermo Scientific, Lithuania) as mentioned above, and only those harboring the transgene (at least six plants per event) were included in statistical analyses. As an additional control, six untransformed Senia plants were grown in parallel and not subjected to heat shock.

Confocal microscopy

Transgenic rice seedlings, obtained as mentioned above, were treated at 42ºC for 2 h and transferred to the standard conditions for two days to allow accumulation of the recombinant protein and DsRed maturation. Radicles were observed under an FV1000 Olympus confocal microscope and red fluorescent images collected with 543 nm excitation using a 550-600 nm emission window. The ImageJ software (http://rsb.info.nih.gov/ij/) was used to calculate the number and size of fluorescent spots and quantify the DsRed fluorescence.

Results

Rationale of the approach and selection of suitable promoters

With the increasingly abundant transcriptomics analytical tools and datasets available, we used an in silico approach to initially select a small number of candidate heat shock promoters. The EMBL-EBI Array Express website was searched for datasets on transcription profiling of the *Oryza sativa* response to heat shock. A single microarray experiment was found (E-GEOD-14275, [10]) that questioned 14-day old seedlings, grown at 28-30ºC, and with or without heat stress at 42ºC. Expression data was available for a total of 22,905 sequences. Those with the highest expression

levels had fluorescence intensities up to 13,800, and 16,300 units in microarrays hybridized with RNA from control and heat-shock treated seedlings. Only 15 and 17 sequences, respectively, had values above 10,000 fluorescence units. Up to 19 sequences, corresponding to 16 genes, had fluorescence intensities more than 5,500 units higher in heat-shock treated than in control seedlings (Table 2). This included three sequences with very high expression values under induction conditions (above 10,000 fluorescence units) and sequences with the greatest changes in expression in response to heat stress treatment (up to 615-fold). These sequences were initially selected to drive the expression of phytotoxic peptides in rice.

Table 2. Sequences with the greatest expression changes in response to heat stress in rice, extracted from publically available microarray hybridization data. Details on the sequence (representative public ID, Affymetrix code and description) and the mRNA expression in response to treatment at 42ºC for 3 h (normalized fluorescence units in rice seedlings under control [control] and heat-shock [heat-shock] conditions; fold change [fold] and difference [HS-C] of normalized fluorescence intensities in the two conditions are also indicated). Note that the sequences with the same Affymetrix number correspond to the same gene. Dark to light scale of shading represents high to low expression level. The promoters of the two sequences indicated in bold were selected to produce transgenic plants.

SEQUENCE				mRNA EXPRESSION			
gene	ID	Affymetrix code	description	control	heat-shock	fold	HS-C
Os.hsp18	**AK071240.1**	**Os.519.1.S1_at**	**17.5 kDa class II heat shock protein, putative**	**460**	**10547**	**23**	**10087**
	AK071613.1	Os.165.1.S1_at	Probenazole-inducible protein Pbz1	2674	11310	4	8636
	AB110191.1	Os.12244.1.S1_at	17.4 kDa class I heat shock protein, putative	366	7763	21	7397
	AU165294	Os.37773.1.S1_x_at	17.4 kDa class I heat shock protein, putative	85	7463	88	7378
	AK069860.1	Os.170.3.S1_at	Chitinase	354	7322	21	6969
	D30763.1	Os.12393.2.S1_at	Ferredoxin	3120	9810	3	6690
	AK064065.1	Os.51848.1.S1_x_at	Transposon protein, putative, CACTA, En/Spm sub-class	1438	8098	6	6660
	AU165294	Os.37773.1.S1_at	17.4 kDa class I heat shock protein, putative	71	6543	92	6472
	D30763.1	Os.12393.2.S1_x_at	Ferredoxin	3399	9764	3	6365
	X60820.1	Os.4775.2.S1_at	16.9 kDa class I heat shock protein	72	6298	87	6226
	AK069098.1	Os.37869.2.S1_a_at	Unnamed protein product	2883	9101	3	6218
	AB098712.1	Os.37729.1.S1_s_at	Bowman-Birk type bran trypsin inhibitor precursor	140	6226	44	6086
	AK072547.1	Os.13478.1.S1_at	cDNA clone, full insert sequence Oryza sativa (japonica cultivar-group)	1008	7051	7	6044
	D30763.1	Os.12393.2.S1_s_at	Ferredoxin	3405	9438	3	6033
	AJ302060.1	Os.46902.1.S1_at	Glycine rich RNA binding protein (grp5 gene)	1924	7704	4	5780
Os.hsp82	**AK063751.1**	**Os.11039.1.S1_s_at**	**Heat shock protein 82, putative**	**9**	**5786**	**615**	**5777**
	AY074786.1	Os.12706.1.S1_at	Dehydroascorbate reductase (DHAR)	4284	9961	2	5677
	CB633557	Os.46024.1.S1_at	Heat shock cognate 70 kDa protein, putative	762	6371	8	5608
	CB621753	Os.11573.2.A2_a_at	RuBisCO activase large isoform precursor	4651	10221	2	5570

The transcription patterns of these 16 genes were subsequently compared with the Collection of Rice Expression Profiles (CREP) database (http://crep.ncpgr.cn/crep-cgi/home.pl), a compilation of quantitative transcriptomes of 39 tissues of various rice genotypes, from the Indica and Japonica groups, obtained using the commercial Affymetrix Rice GeneChip microarray. We specifically focused on the tissues and developmental stages with special relevance in transformation and regeneration to obtain GM plants. They included callus at different steps of agrotransformation and hygromycin based selection, and imbibed seeds and seedlings at various developmental stages. Multiple gene expression profiles were retrieved using the Multi-genes Chronologer tool (Figure 1). The Os.519.1.S1_at sequence combined low expression levels in all 14 analyzed tissues considered relevant for transformation and very high expression in heat-shock treated seedlings (0.073 and 1.190 normalized fluorescence units). Although it had some expression in control seedlings (0.052

normalized fluorescence units), this promoter was chosen to drive the expression of phytotoxic peptides in plants. A second selected sequence, *Os.11039.1.S1_s_at*, had extremely low expression values in the 14 tissues and control seedlings but only moderate expression in response to high temperature treatment (0.033, 0.001 and 0.650 normalized fluorescence units, respectively). There was no induction of *Os.519.1.S1_at* or *Os.11039.1.S1_s_at* upon cold, salt or drought stresses, as assessed in silico using profiling datasets with GSE6901 as the reference, reporting transcriptome analyses of 7-day old seedlings of Indica rice variety IR64 subjected to 4±1ºC, 200 mM NaCl or insufficient moisture in their roots for 3 h (Supplementary Figure 1).

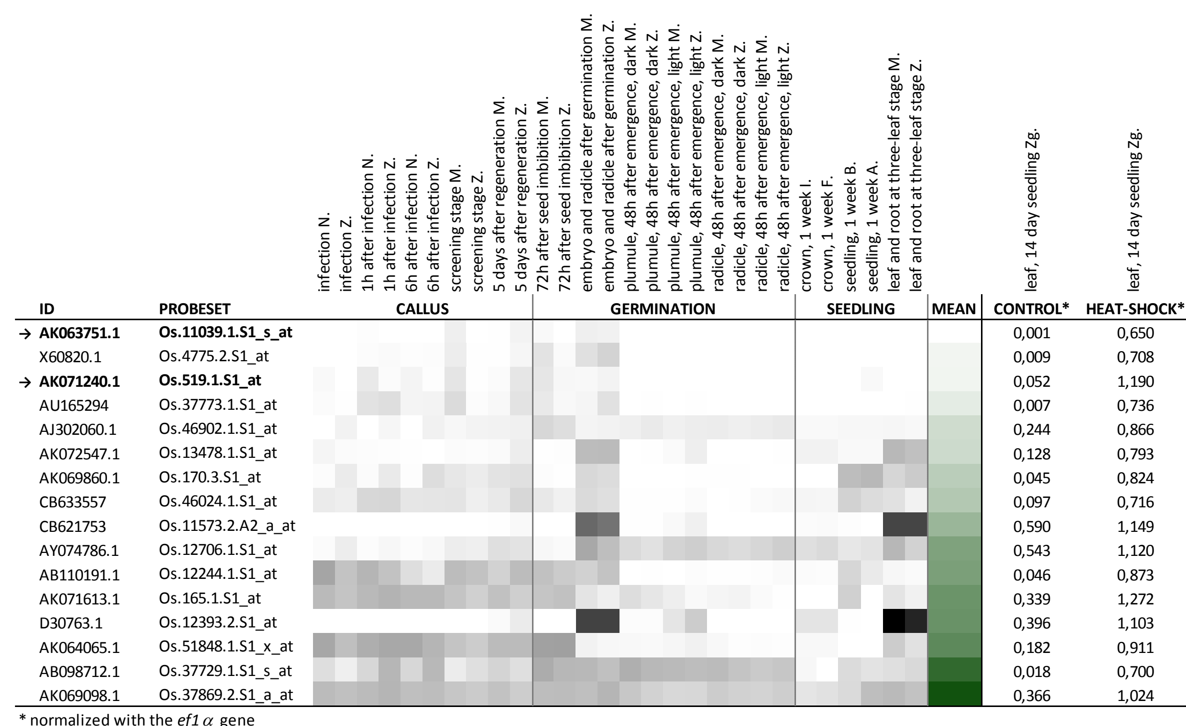

Figure 1. A summary of the expression profiles of a selection of sequences induced by high temperature, in callus and seedlings of eight rice genotypes (Bala [B], FL478 [F], IR29 [I], Minghui 63 [M], Zhenshan 97 [Z], Indica group, and Azucena [A], Nipponbare [N] and ZhongHua 11 [Zg], Japonica group), from published in silico data. Cluster analysis of 16 genes with the highest expression or induction in response to heat-shock, in a total of 14 tissues considered relevant to the process of obtaining transgenic plants (grey scale). The overall mRNA levels in these tissues and developmental stages were estimated using the mean expression values (green scale). Dark to light color scale represents high to low expression levels, the darkest color corresponding to 90162 (grey scale) and 19788 (green scale) normalized fluorescence units. Arrows indicate the two sequences whose promoters were selected to produce transgenic plants.

Expression patterns of the two selected genes in response to heat shock and during the process of *Agrobacterium tumefaciens* mediated stable transformation of rice using the *hpt*II selection gene

Specific real-time PCR (qPCR) assays were developed to target the coding sequences of *Os.hsp18* and *Os.hsp82* and used, coupled to reverse transcription, to experimentally assess the expression patterns of the selected genes in a range of developmental stages and conditions. Treatment of rice

plants with high temperature resulted in increased expression of the two genes (Figure 2). *Os.hsp18* and *Os.hsp82* were highly induced by exposure to 42ºC for 2 to 4h, reaching expression levels of 294 and 516-fold that of *ef-1α* in leaves and 326 and 796-fold in roots. There was a decrease in gene expression after these time points.

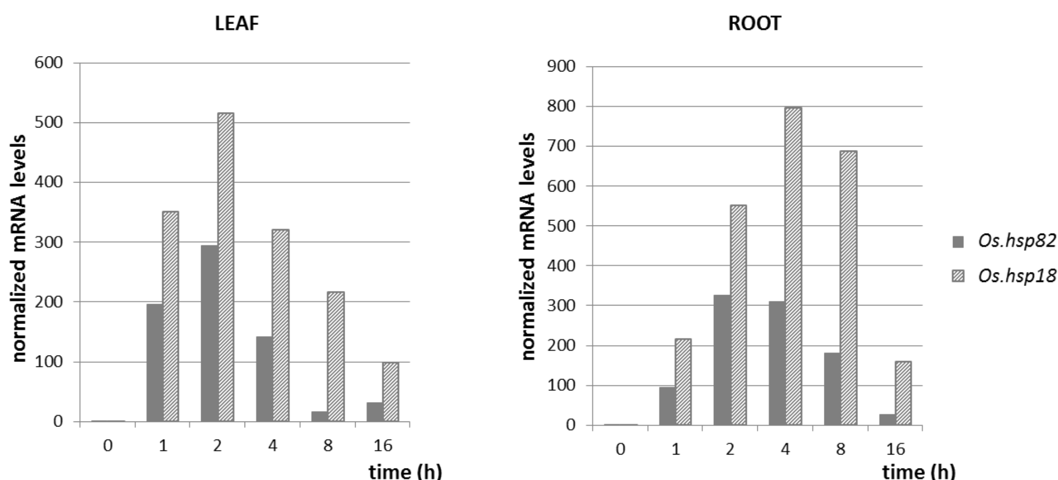

Figure 2. Transcriptional response to high temperature of *Os.hsp18* and *Os.hsp82* in rice var. Senia. Plants at the two leaf vegetative stage were exposed to 42ºC for 0, 1, 2, 4, 8 and 16 h, then the mRNA levels of the selected sequences, from leaf and root samples, were analyzed by RT-qPCR. Three biological replicates, each of five plants, with two experimental replicates, were analyzed per treatment. Relative standard deviation was consistently below 10%. The *ef-1α* reference gene (gNORM M value <0.5 in these samples) was used for normalization and the given mRNA values correspond to fold expression vs. the reference gene.

Additionally, their expression levels were determined during *A. tumefaciens*-mediated transformation and selection using the hygromycin resistance phenotype (Figure 3). Senia wild type seeds were induced to generate primary callus and the standard transformation procedure was followed, using *A. tumefaciens* carrying pCAMBIA1300 (with the *hpt*II selection gene) to produce transgenic plants. *A. tumefaciens* not transformed with the binary plasmid was used as control, without hygromycin as a selection agent. Mature and imbibed embryos from untransformed and transgenic S-hptII plants were also analyzed. *Os.hsp18* was clearly expressed at higher levels than *Os.Hsp82* in all tested tissues and stages. Expression was up to 28-fold that of the reference gene in transformed callus incubated for two weeks in hygromycin selection medium and that of mature embryos, and up to 15-fold that of *ef1a* upon *A. tumefaciens* infection. Seed imbibition rapidly resulted in mRNA decline. *Os.hsp82* had a similar expression pattern, although the level was less than that of *ef1a* in virtually all tested samples. As a control, we measured the mRNA levels of the *hptII* transgene, regulated by the CaMV 35S strong constitutive promoter. They were substantially higher than those of *Os.hsp18* and especially *Os.hsp82* at all stages in which there were uniquely transgenic cells (i.e. after hygromycin selection).

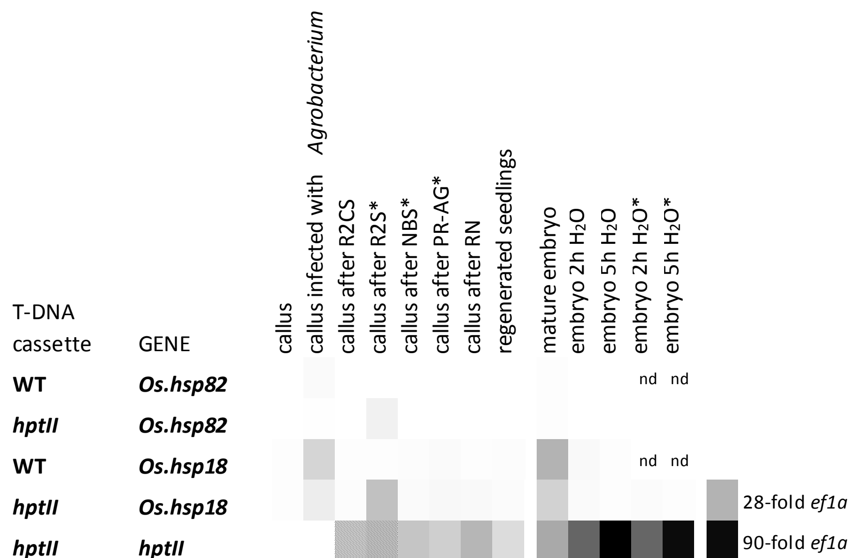

Figure 3. mRNA expression profiles of *Os.hsp18*, *Os.hsp82* and the *hptII* transgene, driven by the CaMV 35S promoter, in rice var. Senia. Different stages during transformation with *A. tumefaciens*, either carrying pCAMBIA1300 (*hptII*) or no T-DNA (WT), and expression in mature and imbibed embryos, are shown. The following stages of callus are shown: 6 weeks after induction of embryogenic callus; immediately after 15 min infection with *A. tumefaciens*; after a 3-day incubation in R2CS medium; after a 2-week incubation in R2S selection medium; 3 weeks after transfer to NBS selection medium; after an 8 to 10-day incubation in PR-AG selection medium; 3-4 weeks after induction of regeneration in RN medium; and two weeks after seedling culture in P medium. Embryos extracted from mature seeds, either before or after imbibition in water for two or five hours, were also analyzed. When the *hptII* DNA cassette was transformed, transgenic cells were selected using hygromycin (asterisk). Three biological replicates, each of five calluses, with two experimental replicates, were analyzed at each stage. The *ef-1α* reference gene (M values <0.5 in these samples) was used for normalization. Dark to light color scale represents high to low expression level. All values were in the 90 to 0.01-fold range. nd: not determined. Striped: underestimated values due to callus samples containing a mixture of transgenic and non-transgenic cells.

Os.hsp18 and *Os.hsp82* promoter fragments regulate the expression of transgenes in response to heat treatment

The sequences corresponding to the promoters of *Os.hsp18* and *Os.hsp82* were retrieved from the Plant Promoter Database, and 553 and 1016 bp promoter fragments of *Os.hsp18* and *Os.hsp82*, respectively, were PCR amplified from rice var. Senia genomic DNA. Their 3’ end was at position -1 relative to the ATG translation start codon. *Os.hsp18* and *Os.hsp82* promoter fragments, pHsp18 and pHsp82, were cloned and sequence verified. The activity of these promoter fragments was experimentally assessed using the *Discosoma* spp. red fluorescent reporter protein, DsRed [24], and the epitope tag54 sequence to detect recombinant proteins through the specific mAb54 antibody [25]. The sequence encoding the DsRed-tag54-KDEL reporter (hereafter, DsRed-tag54) was placed in-frame with the sequence encoding the signal peptide of murine monoclonal antibody mAb24 under the control of the *Os.hsp18*, *Os.hsp82* or *Zm.ubi* [30] promoters and *nos* terminator. All constructs were sequence verified, introduced in *A. tumefaciens* and used to transform rice plants,

along with the *hpt*II marker for hygromycin selection. All constructs yielded hygromycin-resistant plants and those derived from different calluses were considered independent events.

The transgene copy number and expression profile were assessed in three independent events representing each promoter. The ratio of *dsred-tag54* to the *actin* reference gene sequence was close to 0.5 (mean and SD, 0.63 ± 0.27), as determined by qPCR using leaf genomic DNA from T_0 plants, suggesting single-copy insertions. Transgene expression was quantified by RT-qPCR in leaf samples of transgenic T_1 plants at the two leaf vegetative stage (V2) with or without exposure to 42ºC for 2 h (Figure 4). Significant amounts of *dsred-tag54* mRNA were only observed when plants were kept under control temperature conditions, where the transgene was driven by the constitutive pUbi promoter. The expression values, normalized with the *ef1a* constitutive gene, were 0.47 to 2.5, 0.73 to 18.84 and 603 to 904 for pHsp82, pHsp18 and pUbi, respectively. One-way ANOVA, *P*=0.000, gave two groups in Tukey's b posttest with α<0.05. Upon heat treatment, the phsp18 promoter achieved transgene mRNA levels from 214 to 1,117-fold that of *ef1a*, similar to those reached with the pUbi promoter. The levels of expression of the transgene was slightly lower with the pHsp82 promoter, 208 to 636-fold that of *ef1a*, but statistically similar (one-way ANOVA *P*=0.279). As expected, no mRNA encoding DsRed-tag54 was detected in untransformed rice plants. This proved the capacity of the pHsp82, and especially the pHsp18, promoter fragments to drive the expression of a reporter gene after exposure to heat stress up to levels similar to those achieved by a frequently used strong constitutive promoter.

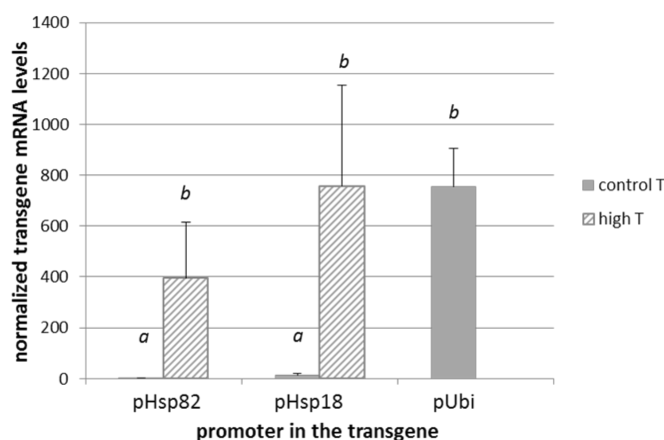

Figure 4. Transgene mRNA expression levels in leaves of GM rice plants in which the reporter *dsred-tag54* was regulated by three different promoters, pHsp82, pHsp18 and pUbi. Three independent events were analyzed carrying every construct. For each event, T_1 seeds were germinated under control conditions and sets of eight plants at the two leaf vegetative stage were either subjected to 42ºC for 2 h, or not, and their leaves were individually sampled. Sets of five plants harboring the transgene (i.e. giving positive transgene signal using the Phire Plant kit) were collectively analyzed by RT-qPCR targeting the sequence encoding DsRed-tag54, and normalized against *ef1a*. Mean and SD values corresponding to each promoter are shown. Letters indicate statistically different transgene mRNA values (One-way ANOVA, Tukey's b posttest α<0.05).

Use of *Os.hsp18* and *Os.hsp82* promoters to drive the expression of phytotoxic BP100 derivatives in rice

The capacity of pHsp18 and pHsp82 promoter fragments to drive the expression of phytotoxic cationic α-helical antimicrobial peptides in stably transgenic plants was evaluated using BP100.2. The antimicrobial peptide has high phytotoxic and hemolytic activities and its constitutive expression in transgenic rice is incompatible with the survival of GM plantlets [7]. The sequence encoding BP100.2 was placed in-frame with the sequence encoding the signal peptide of the *N. tabacum* pathogenesis related protein PR1a, under the control of the pHsp18 and pHsp82 promoters. After sequence verification, the constructs were introduced in *A. tumefaciens* and used to transform rice plants along with the *hpt*II marker. The empty vector, with only the *hpt*II selection gene, was transformed in parallel. Joint analysis of the transformation efficiencies achieved for constructs encoding DsRed-tag54 and BP100.2 under the control of pHsp18, pHsp82 and pUbi promoters showed that four out of five constructs yielded hygromycin-resistant plants with efficiencies similar to that of the empty vector (mean efficiency and SD, 97±18%, Table 3). As expected, this included all vectors encoding the non-phytotoxic DsRed-tag54 reporter, irrespective of the specific promoter regulating its expression (constitutive pUbi or and heat-shock inducible pHsp18 and pHsp82).

Remarkably, the vector encoding BP100.2 under the control of the high temperature inducible promoter pHsp82 produced fertile transgenic rice plants with the same efficiency. The presence of the *bp100.2* or *dsred-tag54* transgene was confirmed in all T_0 events using qPCR. Conversely, no GM plants were obtained with the construct encoding the highly phytotoxic BP100.2 antimicrobial peptide under the control of the pHsp18 promoter. The efficiency of this transformation was at least 30-fold below that of the control plasmid, which can be considered not workable. This is in agreement with the higher activity of the pHsp18 promoter, compared to pHsp82, during transformation.

Table 3. Transformation efficiencies of the different constructs encoding the DsRed-tag54 reporter or the phytotoxic BP100.2 AMP, under the control of a rice heat-shock (pHsp18 and pHsp82) or a maize constitutive (pUbi) promoter.

Promoter	Coding sequence	Transformation efficiency (%)*
pHsp18	*dsred-tag54*	117
pHsp18	*bp100.2*	<3
pHsp82	*dsred-tag54*	108
pHsp82	*bp100.2*	80
pUbi	*dsred-tag54*	84

*Normalized with pCambia1300 plasmid harboring only *hpt* II

Transgene expression profiles were assessed in three independent events carrying sequences encoding BP100.2 under the control of pHsp82 (pHsp82::*bp100.2*), using transgenic plants encoding DsRed-tag54 regulated by the same promoter (pHsp82::*dsred-tag54*) as the control. Hygromycin-resistant plants derived from different calluses were considered independent events. The ratio of *bp100.2* to the reference gene sequence was close to 0.5 in these events (mean and SD, 0.53 ± 0.29) as determined by qPCR using leaf genomic DNA from T_0 plants, suggesting single-copy insertions. Transcript levels of the *bp100.2* transgene were, prior to heat shock, 2.18 to 5.70-fold the level that of *ef1a* (Figure 5), i.e. similar to those of *dsred-tag54* (One-way ANOVA *P*=0.161). Upon heat shock, pHsp82 drove transcription to similar levels as the phytotoxic *bp100.2* (275 to 357-fold the level of *ef1a*) and the control *dsred-tag54* (One-way ANOVA *P*=0.592). Thus, pHsp82 promoter is a suitable tool to achieve transcription of transgenes encoding phytotoxic recombinant peptides in stably GM plants.

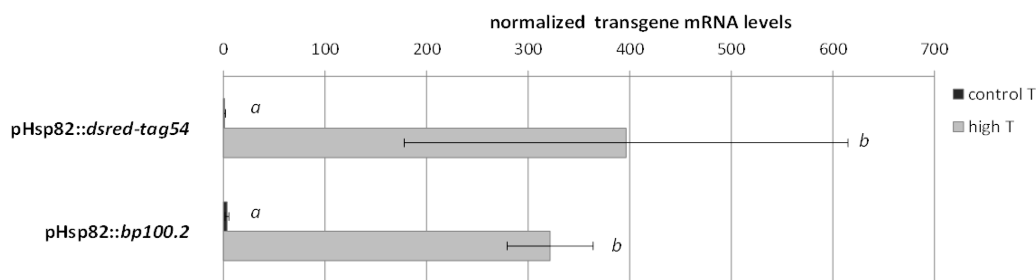

Figure 5. Transgene mRNA expression levels in leaves of GM rice plants harboring pHsp82::*bp100.2* or pHsp82::*dsred-tag54* constructs. Three independent events of each construct were analyzed. For each event, T_1 seeds were germinated under control conditions and sets of eight plants at the two leaf vegetative stage were either subjected to 42ºC for 2 h (black bars) or not (grey bars), and their leaves individually sampled. Sets of five plants harboring the transgene (i.e. giving positive transgene signal using the Phire Plant kit) were collectively analyzed by RT-qPCR targeting the sequence encoding DsRed-tag54 or BP100.2, and normalized against *ef1a*. Mean and SD values corresponding to every transgene are shown. Different letters indicate statistically different transgene mRNA values (One-way ANOVA, Tukey's b posttest $\alpha<0.05$).

Recombinant BP100.2 is difficult to detect due to its low extinction coefficient (low content of aromatic amino acids) and lack of immunogenicity [8]. We used its phytotoxic character to indirectly confirm the synthesis of BP100.2 in GM plants in response to heat treatment. We predicted that, in the presence of the phytotoxic recombinant BP100.2, transgenic plants would show altered phenotype after heat shock as compared to control plants. As shown in Figure 6, untransformed plants not subjected to heat treatment were taller than any temperature-stressed groups. Three days after heat shock, plants expressing *bp100.2* were shorter than those either untransformed or expressing *dsred-tag54* or *hptII* (One-way ANOVA P=0.000, three groups after Tukey b posttest with $\alpha<0.05$). This strongly suggested that phytotoxic recombinant BP100.2 was produced in these plants.

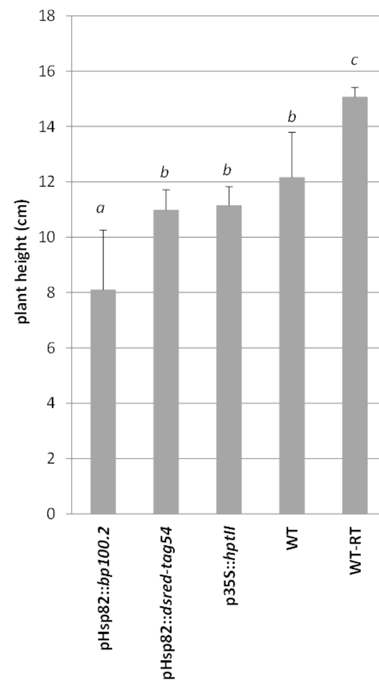

Figure 6. Growth of transgenic rice plants carrying pHsp82::*bp100.2* after heat shock. T_1 seeds of rice plants carrying pHsp82::*bp100.2*, pHsp82::*dsred-tag54* (three independent events per construct) and p35S::*hptII* were germinated for 1 day in the presence of hygromycin, and six plants per event were then in vitro cultured, without the selection agent, up to the V2 stage (height, 6±1 cm). Six untransformed Senia plants were grown in parallel (WT). They were all treated at 42ºC for 2h and the length of their aerial parts was measured after three days incubation under standard conditions. Six untransformed plants were not subjected to heat stress, as an additional control (WT-RT). Means and SD of the height of the different groups are shown. Letters indicate statistically significant differences (One-way ANOVA, Tukey b posttest with α value <0.05).

Production of recombinant reporter proteins in GM rice plants using high temperature inducible promoters

Phytotoxic recombinant BP100 derivatives in transgenic plants were quantified by fusion of BP100 to a reporter moiety encompassing the fluorescent DsRed and the tag54 epitope. A construct was obtained in which the pHsp82 promoter drove the expression of BP100-DsRed-tag54-KDEL (hereafter, BP100-DsRed-tag54), with the same signal peptide and regulatory elements as the pHsp82::*bp100.2* construct. It was sequence verified and used to transform rice plants using the same *Agrobacterium*-based strategy, achieving similar transformation efficiency as the control (109%). Synthesis of *bp100-dsred-tag54* mRNA was assessed by RT-qPCR in three independent events with single copy insertions (as determined by qPCR of T_0 leaves using *ef1a* as reference, 0.52 ± 0.15). Residual mRNA levels detected in leaves of V2 plants grown under control temperature were in the range of 0.07 to 0.3-fold *actin* levels, but increased to 142 to 275-fold after a 2 h exposure to heat shock. These values were not statistically distinguishable from those of pHsp82-

regulated transgenes expressing *bp100.2* and *dsred-tag54* in the same conditions (One-way ANOVA P=0.524 and P=0.205 for control and heat treated plants, respectively).

Transgenic plants from three independent events carrying pHsp82::*bp100-dsred-tag54* were grown to the V2 stage under controlled conditions, heat-induced for 2 h at 42ºC and allowed to accumulate recombinant BP100-DsRed-tag54 for an additional two days. Three events of transgenic plants expressing *dsred-tag54* under the regulation of the pHsp82 and pHsp18 promoters were used as control. Plants with the constitutive pUbi promoter driving the expression of the same recombinant protein were included in the assay, omitting the high temperature induction step. As shown in Figure 7A, TSP extracts from all transgenic plants expressing *dsred-tag54* produced a major band of 29.9 kDa in western blot assays, which is similar to the DsRed-tag54 anticipated size. There was an additional secondary band of 22.1 kDa that probably corresponds to a cleavage product originated by boiling TSP before SDS-PAGE [31]. TSP extracted from all three samples expressing *bp100-dsred-tag54* under the control of pHsp82 produced a faint band of the expected size (31.5 kDa), although most anti-tag54 reactive protein had an apparent MW of 37.9 kDa (Figure 7B). This shift could be the result of partial unfolding or interaction of different molecules, which is in agreement with a similar-sized minor band detectable in extracts of plants producing recombinant DsRed-tag54. As expected, no immune-reactive bands were observed upon analysis of TSP from untransformed plants, those uniquely expressing the hygromycin resistance selection marker, and those transformed with pHsp82::*dsred-tag54* and not subjected to high temperature (Figure 7A). Thus, phytotoxic recombinant BP100 derivatives were produced and accumulated after heat induction in transgenic rice using the pHsp82 heat inducible promoter. Quantification of the immune-reactive bands (Figure 7C) showed that the pUbi promoter led to approximately 0.9% TSP of DsRed-tag54, while promoters derived from *Os.hsp18* and *Os.hsp82* led to recombinant DsRed-tag54 levels up to 0.3 and 0.15% TSP, respectively, after heat shock. Finally, use of the same pHsp82 promoter to drive the expression of the phytotoxic BP100 fusion protein led to slightly lower accumulation levels, up to 0.07% TSP in these conditions.

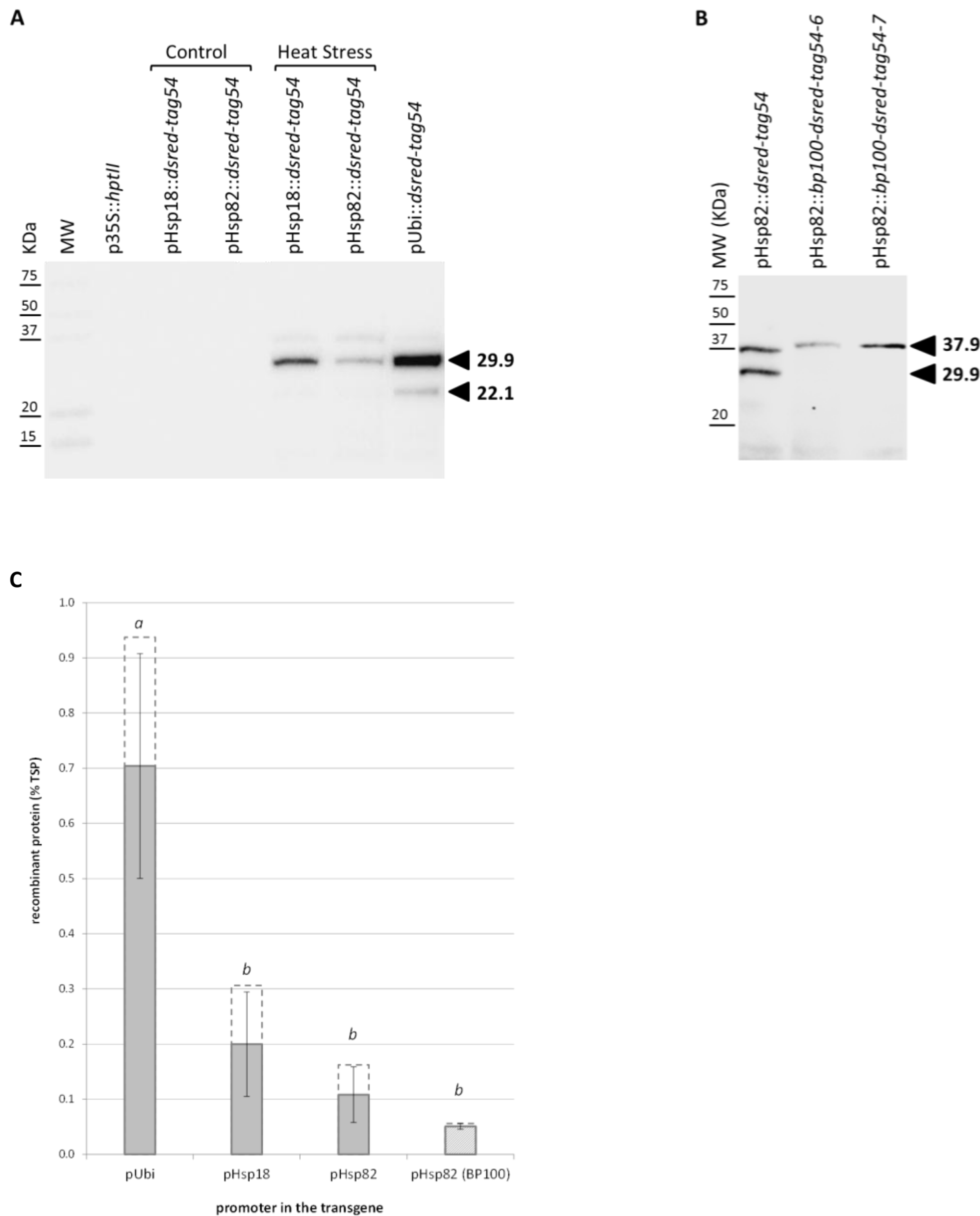

Figure 7. Recombinant DsRed-tag54 and BP100-DsRed-tag54 accumulation in transgenic rice seedlings.
Western blot analysis of proteins from GM plants carrying: **(A)** p35S::*hptII*, pUbi::*dsred-tag54*, pHsp18::*dsred-tag54* and pHsp82::*dsred-tag54*, either treated at 42ºC (heat shock) or not (control); and **(B)** pHsp82::*dsred-tag54* and pHsp82::*bp100-dsred-tag54* (two independent events) treated at 42ºC. **(C)** Recombinant protein accumulation values. Means and SD of three independent events per construct are shown in filled boxes; letters indicate statistically different values; values corresponding to the highest producer event are shown in dashed boxes. Recombinant protein in TSP was extracted from rice seedlings (five plants per event) and 20 µg of TSP per lane was boiled for five minutes and separated by SDS-PAGE before transfer to nitrocellulose filters. Recombinant proteins were detected using the mAb54k antibody (diluted 1:1500) and the horseradish peroxidase-labeled anti-mouse IgG secondary antibody (diluted 1:10000) followed by ECL chemiluminescent detection. Five to 20 pmol of chemically synthesized controltag54 mixed with 20 µg TSP from wild type rice was used as standards for quantification. Untransformed rice var. Senia (WT) and GM rice carrying p35S::*hptII* were used as controls.

One week after germination, pHsp82::*bp100-dsred-tag54* and control pHsp82::*dsred-tag54* transgenic plantlets from three different events per construct were induced by heat shock, and the recombinant chimeric protein allowed to accumulate and mature for three days prior to confocal

microscopy of their radicles. All analyzed transgenic plants displayed red fluorescence, further confirming the accumulation of the recombinant proteins. Fluorescence intensities were measured in four fields per radicle. The p82::*bp100-dsred-tag54* and control p82::*dsred-tag54* plants expressed similar levels of recombinant proteins, with mean and SD values of 1258±420 and 981±286 fluorescence units per field, respectively (One-way ANOVA P=0.135). This demonstrated that recombinant phytotoxic BP100-derived peptides could be accumulated to levels similar to those of the DsRed-tag54 reporter in stable GM plants constructed with the pHsp82 temperature inducible promoter. The DsRed-tag54 fluorescence showed a typical ER reticular pattern accompanied by a few spherical structures, attributed to the known effects of high temperatures on the internal organization of the cell such as fragmentation of the Golgi system and the ER [32]. In contrast, fluorescence in pHsp82::*bp100-dsred-tag54* radicles was essentially in numerous and widely distributed vesicles, obscuring the ER network (Supplementary Figure 2), in agreement with BP100 inducing ER-derived protein bodies [8].

Discussion

Biotechnological production of cationic α-helical antimicrobial peptides that have potent activities against pathogenic microorganisms is of great interest [33], but their constitutive expression is not compatible with the viability and fertility of host plants [7]. This problem has been previously approached by expression of peptides (i) with suboptimal antimicrobial activity linked to lower phytotoxicity [34], and (ii) with rational sequence modification to achieve a targeted reduction in phytotoxicity without affecting antimicrobial potency [8]. Modifications often alter the properties of the peptides not only in terms of antibacterial activity but also the range of target bacterial species [3,5,35]. This makes the design of a peptide with optimal properties difficult [36]. A tool to produce highly active peptides of interest in plants, irrespective of their phytotoxic character, is desirable. Inducible promoters could potentially reduce the negative impact of toxic recombinant peptides on the transgenic plant by forcefully restraining the presence of the peptide during plant development, while allowing high expression upon induction. We predicted that it would only be possible to successfully generate transgenic plants carrying transgenes that encoded phytotoxic peptides by using promoters extremely inactive throughout transformation and regeneration, due to the vulnerability of the plant material along this process. Due to the practicality and low cost of using high temperature as inducing agent in plants, and the extensive literature on the plant response to heat shock [37–42], we focused on heat shock promoters to assess the feasibility of obtaining

recombinant phytotoxic antimicrobial peptides in rice, using as model, highly active and phytotoxic peptides derived from BP100.

Profiling technologies such as microarray hybridization have been widely used to systematically investigate transcriptomic changes during plant development, in different tissues and in response to diverse environmental conditions, including heat shock [43–45]. We selected candidate promoters in silico on the unique basis of the massive transcriptome information gathered on publically available web sites (MIAMExpress, CREP), specifically focusing on studies on prolonged heat shock transcriptional response [10]. A similar strategy has recently been used to identify abiotic stress-inducible promoters intended to drive the overexpression of genes encoding nontoxic, stress resistance proteins in transgenic plants, with the aim of reducing the stunted growth and decrease in yield that may be caused by constitutive overexpression of these transgenes [46]. In contrast, here we selected candidate promoters for the expression of phytotoxic recombinant peptides in plants, to drive high gene expression after heat treatment with minimal gene expression under any other condition. This minimal expression especially focuses on callus at various stages during agrotransformation and different tissues in the germination and seedling developmental stages.

The four sequences which best fulfilled these requirements [AK063751.1 (*Os.hsp82*), X60820.1, AU165294 and AK071240 (*Os.hsp18*), Figure 1] encoded heat shock proteins belonging to the Hsp90 (*Os.hsp82*) and the small Hsp families (X60820.1, AU165294 and *Os.hsp18,* UniProt database). Heat shock proteins are found in every organism [47] and play important roles in cell protection against deleterious effects of stress e.g. acting as molecular chaperones [47–53] and in cellular functions related to growth and development [47,54]. Small Hsp (sHsp) are the most abundant, heterogeneous and stress-responsive group of Hsp in higher plants [55–58]. The genes for more than 20 sHsp have been identified in rice, with specific spatial and temporal regulation under stress and in developmental stages [40,59]. Specifically, the *Os.hsp18* gene, encoding the cytosolic class II Hsp18.0, had very high expression upon induction, although our microarray analysis showed that it retained some activity in developmental stages and conditions that are fundamental to the production of GM plants. Hsp90 are, in general, constitutively expressed and among the most abundant proteins in cells, although their expression increases in response to stress [37]. *Os.hsp82* had extremely low expression in control tissues and reached high levels of expression in response to heat, but *Os.hsp18* and especially *Os.hsp82* were not significantly induced by other stress conditions such as cold, NaCl and drought, in agreement with previous publications [10,60,61]. We therefore considered that *Os.hsp82*, and perhaps *Os.hsp18*, promoters would be able to drive the expression of transgenes encoding phytotoxic peptides in rice, possibly the latter with higher yields, and allow

survival of GM plants. Their moderate mRNA levels in seeds, as determined using the CREP platform, would most probably allow fertility of the plants.

Experimental assessment of the expression patterns of these two genes in the rice genotype of interest, Senia, including additional tissues and conditions relevant to transformation and in vitro culture, allowed ratification of the *Os.hsp18* and *Os.hsp82* candidate sequences. They were both induced in leaves and root of the Senia rice genotype by 1 to 16 hours exposure to high temperature (42ºC), reaching mRNA values up to 500 and 800 (*Os.hsp18*, leaves and root, respectively) and 300-fold (*Os.hsp82*) those of the reference gene after 2-4 hours treatment. These results were in agreement with those obtained in silico and previous experimental data [10,60,62]. In a thorough analysis of *A. tumefaciens*-mediated transformation of Senia rice and selection using the hygromycin resistance trait, *Os.hsp18* and *Os.hsp82* mRNA levels were consistently less than 20 and 60-fold lower (for *Os.hsp18* and *Os.hsp82*, respectively) than in leaves under induction conditions; the highest values were a transient response to *Agrobacterium* infection and hygromycin treatment. As expected, the *hptII* transgene regulated by the p35S constitutive promoter was expressed about 10 and 300-fold higher than those of the *Os.hsp18* and *Os.hsp82* genes, respectively, in the same tissues and stages. Thus, the *Os.hsp18* and *Os.hsp82* promoters were suitable for experimentally assessing our approach.

Five hundred to 1,000 base pair promoter fragments of *Os.hsp18* and *Os.hsp82*, respectively, regulated the expression of a reporter sequence in leaves of transgenic rice plants, in a similar manner as the endogenous genes. The low transgene mRNA levels measured prior to heat shock in GM plants carrying pHsp18::*dsred-tag54* and pHsp82::*dsred-tag54* (about 10 and 1-fold those of the reference gene, respectively) increased to approximately 700 and 400-fold that of the reference gene 2 hours after heat treatment. Even though different GM events had different transgene mRNA levels, globally the expression of endogenous *Os.hsp18* and *Os.hsp82* genes was similar to, respectively, that of the *dsred-tag54* transgene driven by the pHsp18 and pHsp82 promoters (One-way ANOVA P=0.465 and P=0.463, respectively). In addition, western blot analyses and confocal microscopy showed accumulation of recombinant DsRed-tag54 specifically upon heat shock in leaves and roots carrying pHsp18::*dsred-tag54* (data not shown) and pHsp82::*dsred-tag54*. The selected pHsp18 and pHsp82 promoter fragments retained the essential elements to drive heat shock induction in these tissues, and they performed as expected in GM plants. This is in agreement with previous publications where regulatory elements including the TATA box were in silico predicted in the two 5’ proximal regions [63,64].

Use of pHsp18 and pHsp82 heat-inducible promoter fragments to drive the expression of the phytotoxic peptide BP100.2 in rice showed that, although their levels under non heat-stressed conditions were very low, pHsp18 did not allow survival of fertile transgenic plants. Remarkably, transgenic plants producing BP100.2 and BP100-DsRed-tag54, also phytotoxic [8], were obtained with pHsp82, with the same efficiency as those expressing a non-toxic recombinant protein such as the reporter DsRed-tag54. After heat treatment, leaves of plants carrying pHsp82::*bp100.2* and pHsp82::*bp100-dsred-tag54* produced levels of transgene mRNA similar to those harboring the *dsred-tag54* reporter under the control of the same pHsp82 promoter, indicating that these transgenes were fully functional. As BP100 is difficult to detect due to lack of antigenicity and a low extinction coefficient [8], a phenotypic approach was indirectly used to prove the synthesis of BP100.2 in these cells. High temperatures are known to cause a significant decline in relative growth rate [65]. Growth of pHsp82::*bp100.2* plants after a high temperature shock was slower than that of untransformed or transgenic control plants, which supported the production of the phytotoxic recombinant BP100.2 peptide in these cells.

In view of the somewhat higher expression of *Os.hsp18* than *Os.hsp82* during transformation and regeneration (about 5-10 fold), we hypothesized that using the *Os.hsp82* to drive the expression of toxic peptides in plants would be more successful than using the *Os.hsp18* promoter. We found that pHsp82, but not pHsp18, was able to drive the expression of phytotoxic recombinant peptides in plants. This supports our initial hypothesis, and experimental approach, that it is essential that promoter sequences to regulate the expression of phytotoxic transgenes in plants are chosen on the basis of their extremely low basal activity in the tissues, the developmental stages and under the conditions for production of GM plants and germination. No viable transgenic plants were obtained that expressed BP100.2 under the control of 1000-bp promoter fragments of the *Os.37773.1.s1_at* (AU165294) and *Os.165.1.s1_at* genes, with mRNA levels above those of *Os.hsp18* during the transformation process (Figure 1). These results place the threshold of basal expression of a phytotoxic transgene during production of the GM plant at very low levels, similar to those of the *Os*.*hsp82* gene. We then re-evaluated the initially selected heat stress-responsive genes on the basis of this threshold. Five additional sequences (AB110191.1, AU165294, AK069860.1, X60820.1 and AB098712.1, Table 2) had higher expression than *Os.hsp82* after heat shock, and mRNA levels below *Os.hsp18* in control conditions. Four of them were expressed at levels above the *Os.hsp18* threshold during transformation (Figure 1), so their promoters would not be suitable for producing GM plants by driving the expression of phytotoxic components. As X60820.1 had expression patterns similar to *Os.hsp82*, its promoter most probably would be suitable to drive this expression.

However, it is unlikely that yields would be substantially above those of pHsp82, as deduced from its moderate transcriptional response to heat shock.

Recombinant BP100 derivatives were produced in transgenic plants in response to heat treatment through regulation of the pHsp82 promoter. Three days after induction, BP100-DsRed-tag54 represented 0.07% of seedling TSP, determined by western blot using the tag54 epitope. Using the same promoter to drive the expression of DsRed-tag54 yielded up to 0.15% TSP under the same conditions. After the same treatment, BP100-DsRed-tag54 was accumulated at statistically similar levels to the reporter DsRed-tag54, as determined by quantification of the fluorescence signal in confocal micrographs. This discrepancy could be explained by the unusual physicochemical properties of BP100 (highly cationic, pI=11.5), known to result in interactions with many components of the cell and reduce the efficiency of isolation from complex matrices [8]. In agreement, although an anti-tag54 reactive band of the anticipated size was clearly visible in TSP from heat-treated pHsp82::*bp100-dsred-tag54* leafs, the major band had slower mobility, which is compatible with BP100 driven molecular interactions. We have previously described [8] how the BP100 sequence induces the formation of ER-derived vesicles or protein bodies (PB), functioning similarly to the tandemly repeated VPGXG elastin-like motif [66], hydrophobins [67] and the �-zein derived Zera polypeptide [68]. Recombinant BP100-DsRed-tag54 accumulates in these vesicles, together with other luminal ER proteins, in transiently transformed *N. benthamiana* leaves [8]. Here we observed that recombinant BP100-DsRed-tag54 had a similar vesicular pattern in radicles of transgenic rice after high temperature induction, further establishing that recombinant BP100 derivatives accumulate in newly formed ER-derived vesicles in different host plant species.

It is therefore clear that phytotoxic compounds such as BP100 can be expressed in stably transformed plants using a strategy based on inducible promoters. Constitutive expression of this type of transgene has only previously been achieved in transient systems, the harmful effects of the recombinant proteins strongly interfering with normal growth and development of the transgenic plants. With the pHsp82 promoter, we estimate a yield of about 0.1% TSP in our initial induction conditions. Larkindale and Vierling [69] have showed that different acclimation schemes resulted in different yields of Hsp in *Arabidopsis*. Notably, a gradual temperature increase led to higher transcript levels than heat shock without acclimation. Here, use of an acclimation treatment (6 h gradual increase prior to 2 h at 42ºC) increased the recombinant protein yield 3 fold (Supplementary Figure 3). Thus, this approach allowed obtaining up to about 0.3% TSP phytotoxic recombinant proteins. This is about half the yields of the BP100.gtag peptide, specifically designed to display extremely low toxicity, achieved using the constitutive maize pUbi promoter in the same

rice system [8]. Recombinant Cry1B has been produced in rice at similar yields (0.2% TSP) under the control of the maize proteinase inhibitor (Mpi) wound-inducible promoter [70]. Higher yields of recombinant *Acidothermus cellulolyticus* endoglucanase E1, 1.3% TSP, have been achieved in transgenic tobacco using the tomato Rubisco small subunit (RbcS-3C) light-inducible promoter [71]. Note that this type of highly inducible promoter does not allow production of phytotoxic compounds.

In a prospective exercise, our experimental approach was used to identify alternative promoter sequences with increased performance to drive the expression of phytotoxic peptides in plants. To err on the safe side, we looked for genes displaying expression levels similar or below those of *Os.hsp82* during production of transgenic plants, coupled to the highest possible mRNA levels under induction conditions (similar or above those of *Os.hsp18*). Transcriptome-wide datasets are available for rice seedlings under control and a range of abiotic stress conditions, elicitor and hormone treatments, and were analyzed according to the above mentioned criteria. These included salinity, desiccation and suboptimal temperature (4ºC) (GEO Accession Series GSE6901, [72–74]); submergence (GEO Acc. Series GSE41103); phosphorous (P), iron (Fe) (GEO Acc. Series GSE17245, [75]), chrome VI (Cr), cadmium (Cd), lead (Pb) and arsenic (As) (GEO Acc. Series GSE25206, [76]); and methanol (GEO Acc. Series GSE26387, [77]) indole-3-acetic acid (auxin, IAA), benzyl aminopurine (cytokinin, BAP), abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ethylene derivative, ACC), salicylic acid (SA) and jasmonic acid (JA) (GEO Acc. Series GSE37557). In a first step, a total of 23 sequences were identified with high expression under induction conditions (above that of *Os.hsp82*) and minimal expression levels in control conditions (below that of *Os.hsp18*) (Figure 8). In a second step, the selected sequences were filtered on the basis of their expression patterns in callus and during the transformation process and production of transgenic plants (taken as the mean of the microarray normalized data in these tissues).

There were only four sequences with mRNA levels below *Os.hsp82* in the analyzed tissues and developmental stages, strongly suggesting that their promoters would serve to drive the expression of phytotoxic proteins in transgenic plants. They were induced by drought, NaCl, phosphorus and the heavy metals Cr(VI) and As. Only a single sequence, Os.49245.1.S1_at, combined such very low basal levels and high levels after induction (similar to *Os.hsp18* after heat shock). It encodes a homeobox leucine zipper protein and is highly induced both by drought and NaCl. Its promoter would therefore be a good candidate to drive expression of phytotoxic recombinant proteins at levels comparable to those achieved with the pHsp18 promoter, in response to drought or NaCl. Note that five additional sequences had mRNA levels below *Os.hsp18* but above *Os.hsp82* in the

analyzed tissues and developmental stages. Their promoters might also be suitable to drive the expression of toxic compounds in GM plants, but their expression levels upon exposure to the induction conditions were below that of *Os.hsp18*. The best use of our promoter selection strategy would most presumably be achieved by using longer-term expression data to select sequences with the longest span of transcriptional response to the stimulus, which we could speculate would result in higher accumulation of the recombinant proteins.

In conclusion, we produced phytotoxic α-helical antimicrobial peptides derived from BP100 in plants on the basis of strict regulation of transgene transcription. A requirement was that transcription was below a rigorous threshold in specific plant tissues and developmental stages, particularly during transformation and regeneration of GM plants. The heat shock induced promoter, pHsp82, fulfilled this condition and was capable of driving production of phytotoxic recombinant peptides in plants, although at yields considered to be rather low for most commercial applications. In addition, we demonstrate that, thanks to the increasing information available, in silico analysis of transcriptome profiles is a suitable and inexpensive approach to select promoters with specific activity patterns.

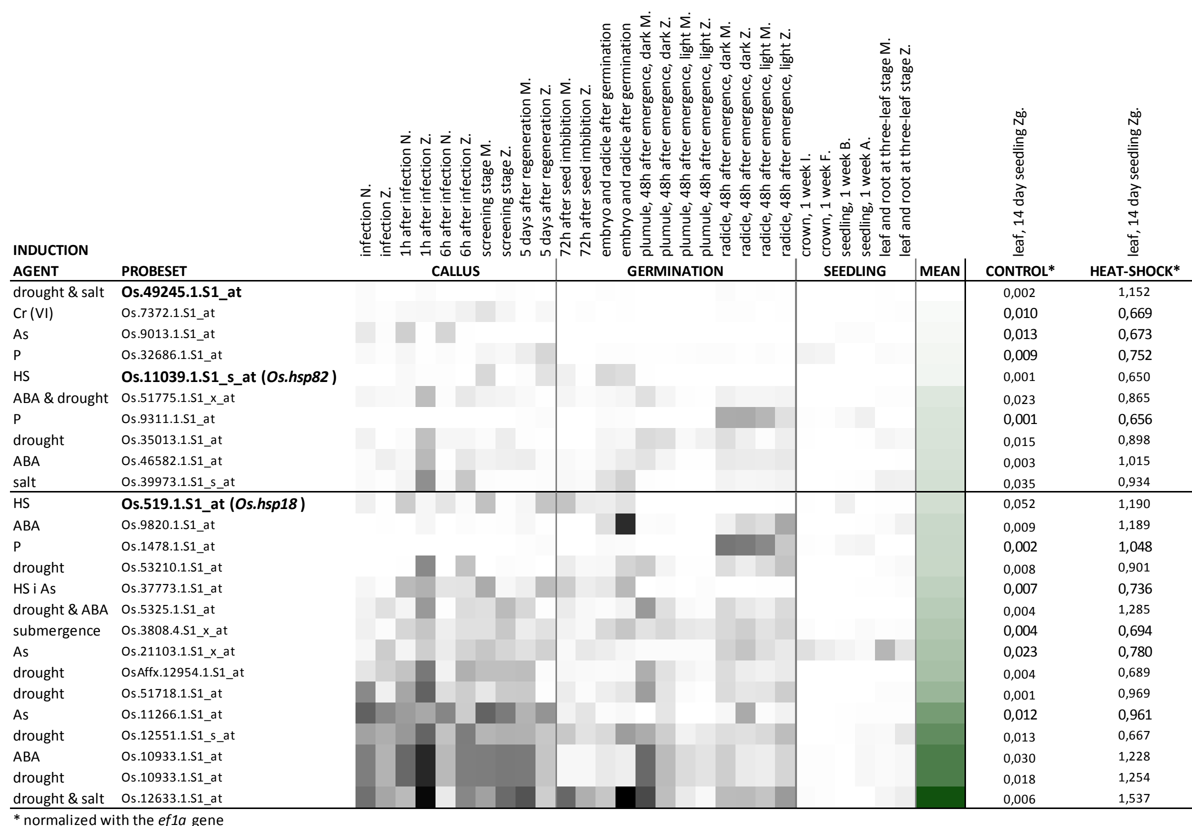

Figure 8. A summary of the expression profiles of a selection of sequences induced by temperature, drought and NaCl stress, and hormone treatment, in callus and seedlings of rice, based on in silico published data. Genes with mRNA levels below that of *Os.hsp18* in control seedlings (control) and above that of *Os.hsp82* after induction (induced) are shown. Normalized expression levels in 28 tissues, relevant to the process of obtaining transgenic plants, of three rice genotypes: Minghui 63 (M, Indica), Zhenshan 97 (Z, Indica) and Nipponbare (N, Japonica) (grey scale). The mRNA levels in these tissues and developmental stages were estimated using the mean expression values (green scale). Dark to light color scale represents high to low expression levels, the darkest corresponding to 37,405 (grey scale) and 12,480 (green scale) normalized fluorescence units.

Acknowledgments

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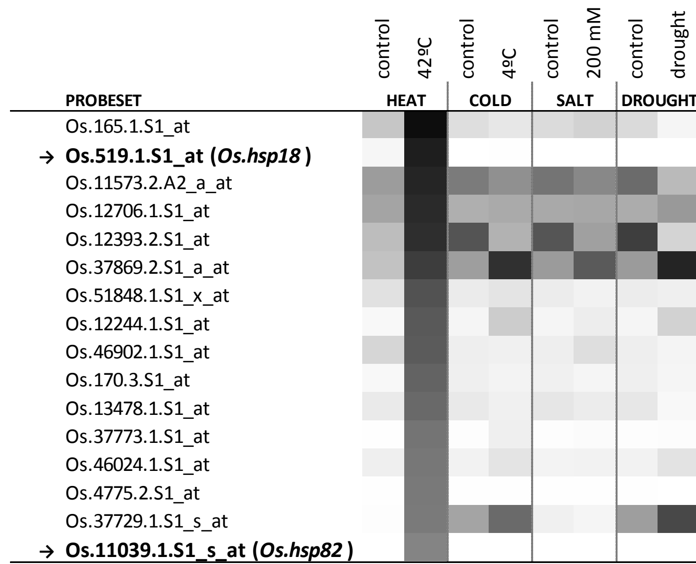
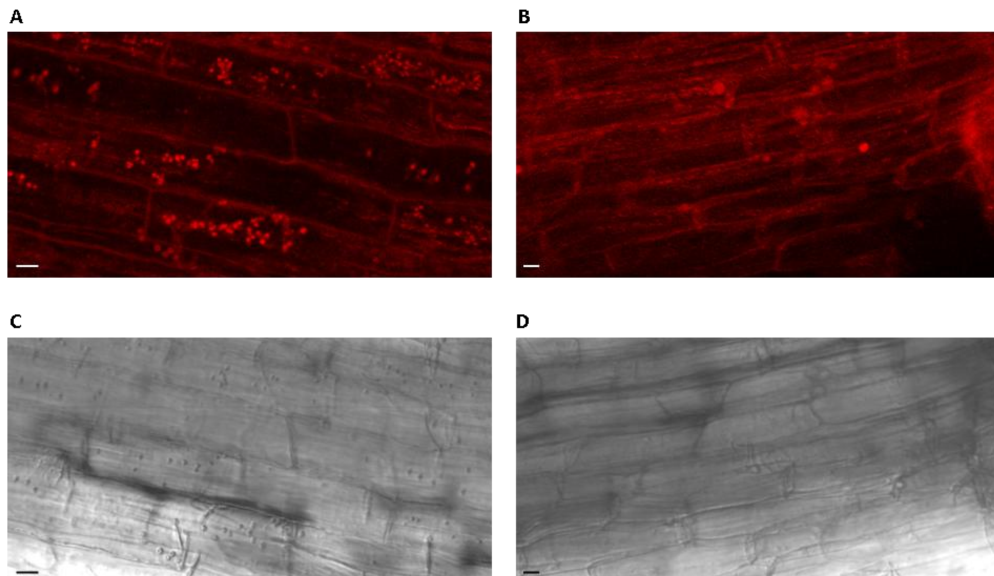
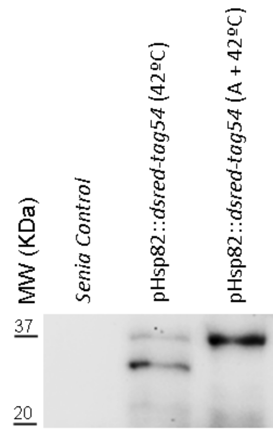
Supplementary Figures



Supplementary Figure 1. A summary of the expression profiles of 16 genes selected as having the highest expression or induction in response to heat-shock in 7-day IR64 (Indica) seedlings subjected to cold, salt and drought stress for 3 h. Data on 14-day ZhongHua 11 (Japonica) seedlings subjected to heat shock for 3 h are also shown. Dark to light color scale represents high to low expression levels, black corresponding to 11,309 normalized fluorescence units. Arrows indicate the two sequences whose promoters were selected to produce transgenic plants.



Supplementary Figure 2. Confocal micrographs of rice radicles from transgenic plantlets carrying pHsp82::*bp100-dsred-tag54* (A and C) and control pHsp82::*dsred-tag54* (B and D), at the V2 developmental stage, subjected to 42ºC for 2 hours and further incubated under control growth conditions for three days in a culture chamber. (A and B), DsRed fluorescence; (C and D), bright field. Scale bars: 0.5 µm.



Supplementary Figure 3. Recombinant DsRed-tag54 accumulation in transgenic rice seedlings after exposure to gradually increasing temperature.

Western blot analysis of proteins from GM plants of a single event carrying pHsp82::*dsred-tag54*, either directly subjected to 42ºC or with a 6 h initial step of gradual temperature increase up to 42ºC (A + 42ºC). Recombinant protein in TSP was extracted from rice seedlings (five plants per event) and 20 µg of TSP per lane was boiled for five minutes and separated by SDS-PAGE before transfer to nitrocellulose filters. Recombinant proteins were detected using the mAb54k antibody (diluted 1:1500) and the horseradish peroxidase-labeled anti-mouse IgG secondary antibody (diluted 1:10000) followed by ECL chemiluminescent detection. Untransformed rice var. Senia (WT) was used as control.

CHAPTER VI

GENERAL DISCUSSION

Phytopathogens are responsible for the decrease in the quality and security of crops, generating important economic losses in worldwide food production. Traditionally, protection of crops against viruses, bacteria and fungi has been based on highly effective chemical pesticides. However, their use can have a negative environmental impact: pathogens may develop resistance; and they possess intrinsic toxicity (McManus et al., 2002; Vidaver, 2002). As a consequence, these products are subjected to strong restrictions and regulatory requirements in many countries. In the European Union, the use of pesticides is regulated by Directives 91/414/CEE and 2009/128/CE. Under these regulations, pesticides such as streptomycin, oxytetracycline and kasugamycin cannot be used (http://ec.europa.eu/sanco_pesticides). Some copper compounds are permitted as pesticides; however, its use is limited due to its toxicity and the risk of unfavourable effects either on the environment or on human and animal health. Consequently, there is a lack of effective compounds to protect some important crops with high economic impact from specific diseases like fire blight, an important contagious disease affecting principally apples and pears. These are the reasons why there is a need to develop new phytosanitary products that adhere to the present legal framework: wide activity range, pathogen selectivity, low toxicity to non-pathogen organisms, no development of resistance phenotypes in pathogens, and minimal impact to the environment. In this context, AMPs have great potential as alternative antimicrobial agents to control plant crop diseases (Montesinos, 2007). Natural AMPs produced in a variety of living organisms are good candidates, but they are produced in low quantities and they tend to have low activity. Moreover, their extraction and purification are usually complex and costly. Synthetic AMPs have been designed to improve the properties of natural AMPs and more effective molecules have been described (Kamysz et al., 2005; Dawson and Liu, 2011; Wiradharma et al., 2011). In particular, the synthetic cationic α-helical undecapeptide BP100 (KKLFKKILKYL-NH_2), designed at the University of Girona on the basis of a Cecropin A - Melittin hybrid, displays potent bactericidal activity (Ferré et al., 2006; Badosa et al., 2007) against *Ea* with MICs assessed *in vitro* 2 - 5 µM; and 50% growth inhibition concentration ED_{50}, 2.5 µM, *Pss* (MIC, 2 - 5 µM; ED_{50}, 2.1 µM) and *Xav* (MIC, 2 - 5 µM; ED_{50}, 1.9 µM). These values are in the same order of magnitude as that of standard antibiotics and antifungals such as penicillins, aminoglycosides or ketoconazole. BP100 has also been tested *in vivo* by evaluating their preventive effect of inhibition of *Ea* infection in detached apple and pear flowers. The peptide showed efficacies in flowers of 63–76% at 100 mM, being more potent than other synthetic peptides (Badosa et al., 2007). Moreover, BP100 shows very high biocompatibility, with acute oral toxicity, determined as the LD_{50}, higher than 2,000 mg/kg of body weight in mice, thus it can be considered to exhibit very low toxicity (Montesinos and Bardaji, 2008). Because of these characteristics, coupled to the urgent need of new compounds to control bacterial diseases in plants, BP100 has high potential for commercialisation in the phytosanitary field as a promising

alternative tool to combat some bacterial plant diseases such as fire blight in pear and apple caused by *Ea*. However, although synthetic peptides have been described as effective molecules, their major drawback is the economic cost of large-scale chemical synthesis, being economically viable only for peptides below 6 amino acids and for applications with very high added value, such as those in the pharmaceutical field. As an alternative, heterologous production of AMPs in living systems used as biofactories can potentially lead to high amounts of product in a sustainable way. Accordingly, efforts are in progress to produce these peptides in bacteria, fungi and plants (Parachin et al., 2012).

In this work, rice has been used as a model to produce AMPs. Resources for rice research have developed rapidly in recent years, including a high-quality genome sequence (Matsumoto et al., 2005) (one of the best-sequenced genomes among all multicellular eukaryotes), full-length cDNA collections (Xie et al., 2005; Lu et al., 2008c), gene expression atlas (Fujita et al., 2010; Wang et al., 2010; Ohnishi et al., 2011) and libraries of mutants (Jeon et al., 2000; Nakamura et al., 2007; Krishnan et al., 2009; Sakurai et al., 2011). Rice has been largely studied for various reasons: (i) it is a staple food for a large fraction of the world's population, which has encouraged molecular engineering aiming at the production of varieties with pathogen resistance and abiotic stress tolerance, in order to maintain or increase worldwide food supply (see as examples [Xu et al., 2011b; Lee et al., 2013]); (ii) it has the smallest genome in the *Gramineae* family (~430 Mb) and it has similarity and co-linearity with other important plant genomes as wheat or maize, which allows the extrapolation of many gene functions to these species; and (iii) in comparison to other grass species, rice can be easily transformed through *Agrobacterium* based protocols (Hiei et al., 1994), so transgenic rice is routinely used for a variety of research purposes. These advances and the combination of sequence and genetic resources provide a valuable foundation to consider rice as a valuable model plant. Furthermore, rice is a suitable platform to produce recombinant proteins because it has been qualified as GRAS (generally recognized as safe), it possesses high grain yield, it is easy to scale-up because it does not have any toxic compounds (such as alkaloids which should have to be removed during the downstream process) and is a self-pollinating plant (which implies very reduced risk of unintended gene flow in the environment). Because of these advantages, we employed this cereal as a model to study into detail the production of AMPs in plant biofactories. Other plant species have proved suitable to deploy molecular farming. Among the preferred ones, *Nicotiana benthamiana* and *N. tabacum* have been used to produce recombinant proteins such as antibodies, vaccines or cytokines (De Muynck et al., 2010; Tremblay et al., 2010; Bortesi et al., 2012). In addition to being highly amenable to transform and regenerate, tobacco also combines many economic advantages, such us high biomass yield, high scale-up capacity, well-established

transformation protocols and availability of large scale infrastructures for processing (Schillberg et al., 2005). However, the majority of tobacco cultivars contain high amounts of toxic compounds such as nicotine and other alkaloids, which have to be removed during the purification process, and as a result, the costs of downstream processing remain high. Many efforts are currently being carried out to optimize a strategy to remove these host cell compounds from tobacco (Buyel et al., 2013; Buyel and Fischer, 2014a; Buyel and Fischer, 2014b).

The present work approaches the production of synthetic and strongly cationic α-helical peptides (BP100 and its derivatives, BP100ders) with powerful activities against relevant phytopatogenic bacterial species, in rice. This was initially approached by constitutive expression of BP100ders designed to accumulate in the ER, both to facilitate the accumulation of large amounts of recombinant peptides (Pelham, 1990; Gomord et al., 1997; Benchabane et al., 2008) and to protect the host cell from the possible toxicity of high amounts of AMPs. This strategy was insufficient to protect the plant cell and thus, two alternative approaches were explored: (i) design of new BP100ders with at least the same antimicrobial activity as BP100 and reduced phytotoxicity; and (ii) restrict the expression of phytotoxic BP100ders to certain tissues through heat shock inducible promoters. We additionally defined certain roles to assist the design of BP100ders with low phytotoxic activity and the best experimental test to rapidly assess it; and developed and validated an approach to select promoter sequences suitable to drive the expression of phytotoxic peptides in plants.

Design of BP100ders to be produced in GM plants

Due to the difficulty in producing small peptides in plants (Streatfield, 2007), the BP100 sequence was modified to increase its length up to 15 to 48 amino acids, including the four amino acids KDEL ER retention motif. It has been demonstrated that modification of AMPs usually has an effect on their antimicrobial and also haemolytic activities (Cavallarín et al., 1998; Osusky et al., 2000; Papo et al., 2002; Lee et al., 2004; Kamysz et al., 2005; Jiang et al., 2008). The haemolytic activity has been considered as an indicator of toxicity towards mammalian cells. The KDEL C-terminal motif proved not to affect the antimicrobial activity of the peptides as demonstrated by *in vitro* bacterial growth studies in which up to 8 pairs of identical BP100ders with the only exception of either including or not a KDEL C-terminal motif were compared (Pearson correlation P=0.7 with bilateral signification 0.05); nor did it have an effect on the haemolytic activity as determined using the same peptides. This made our approach based on targeting active recombinant AMPs to the ER feasible. Peptide length was increased either by tandemly repeating the BP100 unit (two or three copies in sense or

in antisense orientation), linked by structurally flexible AGPA sequences, ([Montero, 2012]; this Thesis [Nadal et al., 2012]); or through BP100 elongation with sequences from natural AMPs (Melittin, Magainin or Cecropin A) (this Thesis [Nadal et al., 2012; Company et al., 2014a]). Similar to the study of the KDEL sequence, comparison of 8 pairs of identical sequences with the only exception of either carrying or not the AGPA hinge showed that it had no effect on the antibacterial activity of the BP100ders but AGPA tended to decrease the haemolytic activity of peptides (Pearson correlation P=0.5 with bilateral signification 0.25). This is in agreement with recently published results obtained with a larger collection of BP100ders (Badosa et al., 2013).

BP100 modifications tended to somehow alter the *in vitro* growth inhibition of *Ea*, *Xav* and *Pss*, compared to BP100. In particular, BP100 tandem copies (multimerization strategy) did not result in a proportional improvement of the antibacterial activity (as it could be expected), but especially BP100.3 decreased it (Montero, 2012). Moreover, this was accompanied by a strong increase in the haemolytic activity of BP100der. This is in contrast to previous reports associating drendrimeric peptides to improve the antimicrobial potency and reduce the haemolytic activity respect to monomeric peptides (Tam et al., 2002; Pini et al., 2005). Placement of two BP100 units in inverted orientation resulted in similar (compare BP100.2i and BP100.1) or slightly improved (compare BP100.2mi and BP100.m) antimicrobial activity but still increased the haemolytic activity. In contrast, peptides elongated with portions of the natural AMPs Magainin and Cecropin A (BP100.g, BP100.g2 and BP100.c) had strongly reduced haemolytic activities without any significant loss of antibacterial activity (Table 5) (this Thesis [Nadal et al., 2012; Company et al., 2014a]; [Badosa et al., 2013]). Thus, the latter seems the better strategy.

Table 5. Sequence and antibacterial and haemolytic activities of BP100ders.

			CMI (µM)			
BP100der	**Derivative type**	**Sequence**	***Ea***	***Pss***	***Xav***	**HA***
BP100	control	**KKLFKKILKYL**				
BP100.1	multimerization	**KKLFKKILKYL**KDEL				
BP100.2	multimerization	**KKLFKKILKYL**AGPA**KKLFKKILKYL**KDEL				
BP100.3	multimerization	**KKLFKKILKYL**AGPA**KKLFKKILKYL**AGPA**KKLFKKILKYL**KDEL				
BP100.2i	multimerization inverted	**KKLFKKILKYL**AGPA**LYKLIKKFLKK**KDEL				
BP100.m	elongation mellitin	**KKLFKKILKYL**AGPATTGLPALISWKDEL				
BP100.2mi	multimerization inverted	**KKLFKKILKYL**TTGLPALISAGPASILAPLGTT**LYKLIKKFLKK**KDEL				
BP100.c	elongation cecropin A	**KKLFKKILKYL**AGPAVAVVGQATQIAKKDEL				
BP100.g	elongation magainin	**KKLFKKILKYL**AGPAKFLHSAKKDEL				
BP100.g2	elongation magainin	**KKLFKKILKYL**AGPAGIGKFLHSAKKDEL				
BP100.gtag	elongation magainin	**KKLFKKILKYL**AGPAKFLHSAKAGPAKDWEHLKDWEHLKDWEHLKDEL				

*** hemolytic activity (150 mM) vs. BP100**

The BP100 sequence is highlighted in bold. Antibacterial activity was determined against *Ea, Pss* and *Xav*. Dark to light green scale represents high to low antibacterial activity. Haemolytic activity is shown as the ratio between each peptide and the reference peptide BP100, calculated at 150 µM. Dark to light orange scale represents high to low haemolytic activity.

Recombinant BP100der can be toxic to the host plant upon accumulation at high levels

Constructs encoding ten different BP100ders under the control of the maize Ubiquitin constitutive promoter and targeted to the ER were introduced into *Agrobacterium* and used to transform rice callus. These included BP100.1, BP100.2, BP100.3, BP100.2i, BP100.2mi, BP100.m, BP100.c, BP100.g, BP100.g2 and BP100.gtag and, with the exception of BP100.3 (and BP100.2), had strong activity against the phytopathogen strains of interest. There were important differences in the transformation efficiencies of these transgenes, which were not directly linked to the antibacterial activity of the encoded peptides. As an example, transgenes encoding BP100.2 and BP100.3, with somehow low and very low antibacterial activity, respectively, had transformation efficiencies below 1% that of the control plasmid, carrying the selection gene alone (*hptII*) (this Thesis [Nadal et al., 2012]). In contrast, transgenes encoding BP100ders with higher antibacterial activity, such as BP100.gtag and BP100.c, had transformation efficiencies about 35-50% that of the control plasmid (this Thesis [Company et al., 2014a]).

We then hypothesized that this was caused by the toxicity of high levels of recombinant BP100ders accumulated in host plant cells. The constitutive promoter drives the expression of *bp100der* transgenes in all GM cells, including the initial callus and plantlet stages. Although BP100 has been demonstrated to be innocuous to non-target organisms at concentrations largely above their MICs (Montesinos and Bardaji, 2008; Montesinos et al., 2012), the presence of too high amounts of BP100ders in plant GM cells along the transformation process could result in their lack of capacity to divide and produce a viable transgenic plant. This hypothesis was subsequently tested by demonstrating that high levels of chemically synthesized BP100der (i.e. 100 fold higher than their MICs) were toxic to plant cells and tissues (this Thesis [Nadal et al., 2012]). On the one hand, inoculation of BP100ders in tobacco leaves induced lesions, which diameters depended on the peptide doses. On the other hand, incubation of rice seeds with different concentrations of BP100 and BP100ders resulted in inhibition of germination, mainly at the shoot level, in a dose dependent manner, demonstrating the toxicity of the BP100 and its derivatives against rice. Melittin, a highly cytotoxic peptide (Raghuraman and Chattopadhyay, 2007; Walsh et al., 2011), also affected rice germination in a negative way, although specifically inhibiting radicle development, which indicates different mechanisms compared to BP100ders.

Additional results obtained in this work further evidence the toxicity of high doses of BP100ders against plant cells. Short-term incubation of rice plantlets (2 leaf vegetative stage) with high doses of BP100.gtag had strong effects on the ultrastructure of their cells. Specifically, their ER was visibly altered and numerous vesicles with electron-dense granules appeared; in some cases mitochondria

were affected and plasmolysis occurred. As it will be discussed later, these alterations are similar to those described in response to high doses or long exposures to toxic substances (Liu et al., 2009; Jiang and Liu, 2010; Fan et al., 2011). This demonstrated that accumulation of high amounts of recombinant BP100ders was toxic to the host cell.

Development of tools to predict phytotoxicity and allow constitutive production of active BP100ders in transgenic plants

From our transformation results it was also clear that accumulation of different BP100ders entailed different degrees of phytotoxicity (compare e.g. the transformation efficiencies of transgenes encoding BP100.3, BP100.2mi, BP100.c and BP100.g, i.e. <1%, ~3%, ~35% and ~50%, respectively). Moreover, not only the efficiency of transformation was, for certain peptides (e.g. BP100.2mi), below the levels that can be considered as workable, but the scarce events obtained that constitutively expressed certain BP100ders had extremely reduced transgene mRNA levels, far below those normally achieved for other transgenes. This suggests that only events in which the transgene is expressed at very low levels will survive; and these events will have extremely low yields of recombinant protein and will have no interest as biofactories. Such low expression rates are probably linked to positional effects of the transgene in the genome, (Matzke and Matzke, 1998; Gelvin and Kim, 2007) (we systematically analysed events with single or very low transgene copy numbers to avoid gene silencing), (Linn et al., 1990; Hobbs et al., 1993; Stam et al., 1997). Note that events with reduced transgene expression can be suitable with a completely different aim, i.e. obtaining GM plants with resistant phenotypes (Coca et al., 2006; Jung, 2013; Sadumpati et al., 2013; Wu et al., 2013) as we have shown in this work (see below). Therefore, it was important to develop a tool for rapid and accurate prediction of the phytotoxicity of a given peptide in order to estimate the likelihood of obtaining transgenic plants for use as biofactories of this particular peptide. Such tool would allow screening a number of candidate peptides and selecting those with the highest probability of being produced with moderate or high yields in transgenic plants. This is especially interesting since the whole process of producing GM plants (i.e. from the cloning to the establishment of optimal homozygous lines) is highly laborious and time-consuming. Joint analysis of the transformation efficiencies and toxicity to plant cells (both in the rice germination and the tobacco leaf infiltration tests) (Figure 4) showed that, at least with the BP100ders here studied, there was no significant correlation (Pearson coefficient of -0.488 and 0.586, respectively). We thus concluded that the possibility of obtaining transgenic rice constitutively expressing transgenes

encoding BP100ders cannot be directly inferred from the quantification of the capacity of these peptides to damage tobacco leaves or inhibit the correct seedling development.

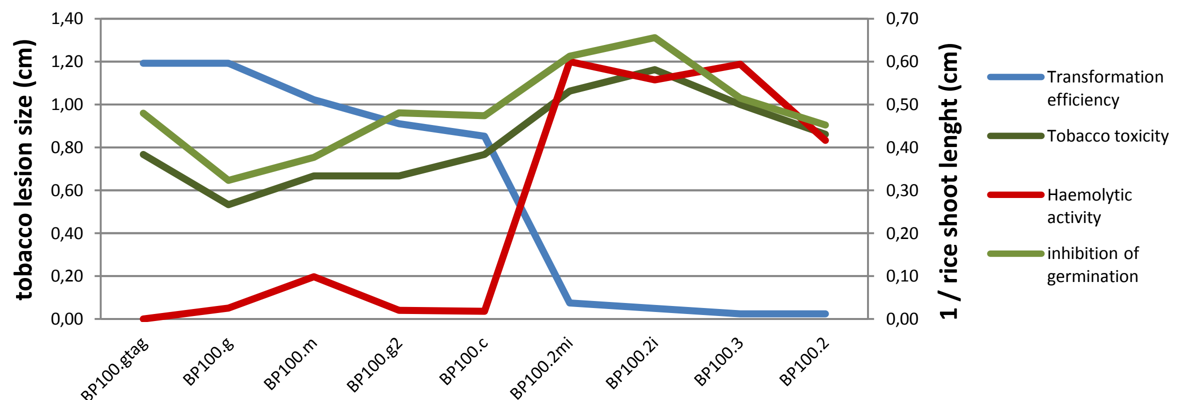


Figure 4. Phytotoxicity of chemically synthesized BP100ders using *N. benthamiana* leaf inoculation and rice germination assays. Haemolytic activity of BP100ders and transformation efficiencies are also represented. Dark green: tobacco infiltration test (left axis, diameter [cm] of the lesions in tobacco leafs); light green: inhibition of rice shoot growth (right axis, 1/shoot length in cm); red: haemolytic activity test (right axis, % haemolytic activity at 150 µM [confidence intervals for α=0.05 ≤10%]); blue: transformation efficiency (left axis, % transformation efficiency normalized with that of the selection gene *hptII*).

Along basic characterization of newly designed peptides, not only the antimicrobial activity of chemically synthesized molecules is determined using *in vitro* tests but also its haemolytic activity as an indicator of toxicity against animal eukaryotic cells. BP100 and many similar undecapeptides in the CECMEL11 library (Montesinos and Bardaji, 2008), and as well most BP100ders assayed in this work and by others (Badosa et al., 2013), display haemolytic activities that indicate very low toxicity to non-target cells. However, we observed an inverse correlation between the haemolytic activities of our BP100ders and the transformation efficiency of *bp100der* constructs encoding it for constitutive expression in plants (Figure 4). This allowed defining a threshold at about 15% haemolytic activity at 150µM which separated (i) below the threshold, BP100ders that could be expressed in plants with transformation efficiencies above 30% that of the control and adequate transgene expression levels; and (ii) above the threshold (in this Thesis considered more than 68%), BP100ders with extremely reduced probability of obtaining transgenic plants that produce sufficient yields of recombinant peptides as to be useful as biofactories. Therefore, the haemolytic activity can be considered as a suitable phytotoxicity marker, here measured as the likelihood of obtaining transgenic plants for constitutive expression and ER accumulation of BP100ders, with reasonable yields.

Our phytotoxicity tests carried out using plants (i.e. tobacco leaf infiltration and rice germination assays) demonstrated that high doses of BP100ders are toxic to plant cells. However, they failed in

predicting their toxicity upon accumulation as recombinant peptides. On the contrary, the hemolytic activity of chemically synthesized BP100ders proved to be a good tool to predict the toxicity of recombinant peptides. Taking into account the mode of action of AMPs, this might be explained on the basis of the presence of the cell wall in plant cells, which can possibly interfere with the activity of exogenously applied BP100ders at the level of the cell membrane and cytoplasm. On the other hand, mammalian cells lack the cell wall, thus the membrane is more accessible to AMPs. The structure and composition of the membranes of the target cells may also have an influence on AMP activity.

BP100ders with high antibacterial activity and low haemolytic activity can be produced in transgenic plants by constitutive expression and accumulation in the ER

Some authors have previously expressed in plants transgenes encoding AMPs that were specifically selected to display only modest antimicrobial activity (Osusky et al., 2000; Osusky et al., 2004). This strategy was taken to avoid the deleterious effects of expressing highly active peptides on the morphology and productivity of the transgenic plant. In this work we demonstrated that an alternative strategy is possible, based on modification of the BP100 sequence and selection of BP100ders with potent antimicrobial activities and haemolytic activities below a threshold: these peptides can be constitutively expressed in transgenic plants, at least upon accumulation in the ER (this Thesis [Nadal et al., 2012; Company et al., 2014a]).

Transgenic rice lines expressing *bp100.2i* and *bp100.2mi*, encoding moderately phytotoxic peptides and only achieving very low mRNA levels, had agronomic performance somehow below that of untransformed plants, e.g. with yields of 60% and 70%, respectively; and more severe ultrastuctural changes, similar to those observed upon short exposure of plant cells to toxic compounds (see below). Such toxic effects were further confirmed in a different plant species, *Arabidopsis thaliana*, using the *bp100.dsred.tag54* construct encoding the phytotoxic BP100 fused to the fluorescent reporter DsRed and the tag54 antigen to facilitate detection, for accumulation in the ER. Although with very low transformation efficiency, a few transgenic *A. thaliana* seeds were identified that expressed recombinant BP100-DsRed-tag54 (as demonstrated by DsRed fluorescence detected by confocal microscopy) but did not survive ulterior developmental stages and fertile transgenic plants were never obtained. Even if our approach included confining recombinant peptides in the ER (and ER derived vesicles, see below), this was insufficient to protect the host plant from the toxic effects of these BP100ders, long-term exposure to these peptides displaying a detrimental effect that

interfered normal growth and development of the host plant. Taking this into account, it would not be advisable to produce highly phytotoxic (identified as highly haemolytic) peptides in plant biofactories using this approach. Remarkably, BP100 and phytotoxic BP100ders would be compatible with short-term applications such as transient expression and local delivery. Recently, BP100 has attracted considerable attention as a cell-penetrating peptide (CPP) due to its ability to deliver functionally active cargoes (such as RNA, DNA, drugs, antibodies and nanoparticles) into cells in a nondestructive manner. Eggenberger and co-workers (Eggenberger et al., 2011) used BP100 as a fast and efficient cell-penetrating agent to deliver the actin-binding Lifeact peptide (MGVADLIKKFESISKEE) into the cytosol of tobacco cells. Similarly, Lakshmanan and colleagues (Lakshmanan et al., 2013) developed a novel method for plant transient transformation based on fusion to BP100, which acts as gene delivery agent.

In contrast, rice lines expressing transgenes that encode the selected BP100ders (i.e. with haemolytic activity below a threshold) had overall phenotypic characteristics similar to that of untransformed plants. As an example, the ultrastructure of transgenic rice cells which accumulated BP100.gtag only showed dictyosome and ER alterations similar to those associated with a highly active endomembrane system (besides the newly formed protein bodies, see below), as it could be expected due to recombinant peptide targeting to the ER.

Expression of transgenes encoding BP100ders proved to confer the host plant resistance to target pathogens (e.g. *Dickeya chrysanthemi*, closely related to *Ea*) and tolerance to reactive oxygen species, rapidly generated upon biotic stresses and involved both in the stress response and the damaging effects of various stresses (Mittler et al., 2004). Moreover, analysis of the resistance phenotypes of the different events, in combination with the corresponding transgene expression levels and the phytotoxicity of the different BP100ders seemed to indicate that there is a complex equilibrium between recombinant BP100ders phytotoxicity and antimicrobial activity. In conclusion, it should be possible to generate transgenic plants with pathogen (or stress) resistance on the basis of low expression of highly antimicrobial BP100ders, with no important impact on their fitness. On the other hand, elongation of the BP100 sequence with portions of natural AMPs (mainly Magainin and Cecropin A) and inclusion of reporter moieties seem to be the best approach to design BP100ders with high antimicrobial activities and compatible with production in plant biofactories.

Expression of transgenes encoding BP100ders with high antibacterial activity and high phytotoxicity through control of transgene expression

Some cationic α-helical antimicrobial peptides display very interesting properties as novel therapeutics and preservatives, but they are combined to high haemolytic activity and thus, they cannot be produced in plant biofactories (i.e. their constitutive expression is not compatible with the viability and fertility of host plants producing reasonable yields) (this Thesis [Nadal et al., 2012]).

In recent years, alternative transformation technologies have been developed for *Arabidopsis* based on *in planta* ovule transformation (Clough and Bent, 1998; Bent, 2006) and thus, not requiring long *in vitro* cell culture steps such as callus formation, regeneration and acclimatization of transgenic plants. We hypothesized that shortening the process (and avoiding certain vulnerable stages such as plant regeneration) could facilitate production of transgenic plants constitutively expressing transgenes encoding toxic compounds. However, long term expression of the phytotoxic BP100-DsRed-tag54 in *Arabidopsis* plants proved to be incompatible with full development of fertile plants (this Thesis [Company et al., 2014a]). Alternatively, transient tobacco leaf agroinflitration (Vézina et al., 2009; Tremblay et al., 2011; Whaley et al., 2011) can allow production of phytotoxic peptides. Nevertheless, the downstream processing is expensive due to the high amounts of toxic compounds in the majority of tobacco cultivars.

Production of phytotoxic peptides in transgenic plants was approached using an alternative strategy based on strictly controlling the plant tissues and conditions in which the transgene is expressed (and thus, the phytotoxic peptide is produced and has a damaging effect on the host plant). Our main idea was to keep expression of toxic recombinant peptides to minimal levels especially along the transformation process, i.e. at developmental stages in which the GM plants are highly vulnerable (initial cell division steps, plant regeneration and acclimatization). Conversely, it was important that, in the absence of continuous synthesis of the recombinant peptide, transgene expression was as high as possible in the tissues and conditions in which the recombinant peptide had to be produced and accumulated. We tested this approach using, as an example, control of transgene expression by high temperature, an inexpensive stimulus easily applicable to plants growing under confined conditions (in a greenhouse). Inducible promoters have been used to drive transgene expression and confer plants resistance to pathogens or environmental conditions. Promoters induced in response to bacterial or fungal infection, treatment with elicitors or hormones, and mechanical wounding have been used to express e.g. Cry and AFP, which confer resistance to different plant pathogens (Breitler et al., 2004; Moreno et al., 2005; Gulbitti-Onarici et al., 2009; Kumar et al., 2009; Tiwari et al., 2011). Inducible promoters have been used as well to

produce recombinant proteins in plant biofactories; and a number of them have been described to drive transgene transcription to high levels in a wide range of plant tissues (Shin et al., 2010; Guzman et al., 2012; Wu et al., 2014). Although constitutive promoters could possibly result in higher yields of recombinant protein, here the main objective was to strictly control the expression of transgenes that were toxic to the host plant, thus allowing plant biofactories to survive.

We selected candidate promoters initially on the basis of published gene expression data, available through *in silico* databases. The large amount of data available in these databases (e.g. Gene Expression Omnibus [GEO] database [http://www.ncbi.nlm.nih.gov/geo/], ArrayExpress database [http://www.ebi.ac.uk/arrayexpress/] or Collection of Rice Expression Profiles [CREP] database [http://crep.ncpgr.cn/crep-cgi/home.pl]) allows this approach. Among the sequences analysed in the microarray experiment studying the response of rice to heat shock stress, gene selection was carried out considering a compromise between high expression after heat treatment and low expression in a range of tissues and developmental stages including callus at various stages (before and after agrotransformation), seeds, seedlings, tillering, flowering and mature plants of various rice genotypes. Two genes were selected: *Os.hsp18.0*, with strong expression upon heat shock and low expression in any other tissue; and *Os.hsp82*, with extremely low expression in all analysed tissues but moderate expression in response to high temperature. Use of the corresponding promoters to drive the expression of the phytotoxic peptides BP100.2 and BP100-DsRed-tag54 (i.e. BP100 fused to two reporter units, a fluorescent one and an antigenic one) showed that only the last one, pHsp82, allowed survival of transgenic plants for production of toxic compounds with the same transformation efficiency as those for production of the (nontoxic) reporter moiety of the same peptide, i.e. DsRed-tag54. Thus, use of inducible promoters allowed production of phytotoxic compounds in plant biofactories.

Conversely, the pHsp18.0 was considered not workable to produce transgenic plants encoding highly phytotoxic peptides. Experimental determination of the *Os.hsp18.0* and *Os.hsp82* mRNA levels in rice along the process of agrotransformation confirmed higher expression of *Os.hsp18.0* in these tissues. We concluded that the pHsp18.0 promoter was too active at some stages along the transformation process (particularly in response to hygromycin selective agent) for use to drive the expression of phytotoxic compounds in plants. This allowed defining an expression threshold in these stages (i.e. that of *Os.hsp18.0*), above which survival of plants for production of toxic compounds was not possible. Transformation of constructs with the promoter of *Os.hsp12*, with expression patterns similar to *Os.hsp18.0*, further confirmed the established threshold. In a subsequent *in silico* exercise we used the same approach (i.e. search for genes strongly induced upon a given condition, analysis of expression in the selected tissues using gene expression

databases and application of the threshold) to identify other candidate promoters combining equal or lower mRNA levels than *Os.hsp82* along the transformation process and a high expression level under other types of induction. A single gene was identified which expression pattern was better than that of *Os.hsp82*, strongly suggesting that its promoter would be suitable to drive the expression of phytotoxic BP100ders in transgenic rice, although with better yields.

Active recombinant BP100ders are produced in transgenic plants

The cationic, amphypatic α-helical peptides related to BP100 are difficult to detect, quantify and isolate in plant tissues due to the extreme physicochemical properties of BP100. It has strong cationic character (pI = 11), low extinction coefficient and lack of immunogenicity as determined *in silico* and experimentally. It is known that peptides are often poorly immunogenic; and many ongoing efforts aim at increasing peptide immunogenicity using very different strategies such as attachment of lipids to the peptide (Moyle et al., 2006), use of different chitosan formulations (Sáenz et al., 2009), use of single-wall carbon nanotubes (SWNTs) (Villa et al., 2011), or use of recombinant nanoparticles derived from *Turnip mosaic virus* (Sánchez et al., 2013). For that reason, we initially demonstrated the correct functionality of transgenes encoding BP100ders in plants by quantification of transgene mRNA; and production of active recombinant BP100ders through their toxic effects to host plants. These included, for constitutive *bp100der* expression, pathogen resistant and oxidative stress tolerant phenotypes, ultrastructural alterations at the ER level (i.e. where recombinant BP100ders were to accumulate) and, especially for peptides with moderate and high phytotoxicity, low transformation efficiencies. Synthesis of BP100.2 in transgenic plants in which the transgene was regulated by the pHsp82 heat inducible promoter was indirectly demonstrated by a significant delay in recovery from heat shock (that is, in the presence of the toxic recombinant peptide).

In an alternative approach, we directly detected recombinant BP100ders by fusion to an epitope of the *Tobacco Mosaic Virus* 54K replicase (tag54) (Rasche et al., 2011) and/or the fluorescent reporter DsRed, a tetrameric fluorescent protein of 25.4 kDa that emits a bright red fluorescent light (Matz et al., 1999). We used tag54 alone to monitor the accumulation of a BP100ders with high antimicrobial activity and low haemolytic activity: BP100.gtag, which was constitutively produced in transgenic rice. The tag54 sequence had no antimicrobial or haemolytic activity. Other recent reviews have also used an epitope-tagging system to detect the recombinant peptide in *Arabidopsis* (Wu et al., 2013). In that case, the epitope tag used was FLAG tag. Similar to our results, this tag did not significantly influence on the peptide activity (this Thesis [Company et al., 2014a]; [Badosa et al.,

2013]). The tag54 allowed measurement of the accumulation of recombinant BP100ders in transgenic rice plants by western blot using an anti-tag54 antibody (Rasche et al., 2011). As it could be expected, independent events carrying the *bp100.gtag* coding sequence under the control of the constitutive maize Ubiquitin promoter had different levels of recombinant BP100.gtag in their leafs; which correlated well with the recombinant *bp100.gtag* mRNA levels quantified by RT-qPCR. This is a common phenomenon and can be explained by different transgene integration sites (Bhat and Srinivasan, 2002; Gelvin and Kim, 2007). Recombinant BP100.gtag was produced in transgenic plants and accumulated up to 0.5% total soluble protein (TSP). These yields are comparable to those obtained in other plants producing e.g. human serum albumin and anti-HIV-1 antibody 2G12 using constitutive promoters in *Triticum aestivum* and *N. tabacum* (Khan et al., 2012).

On the other hand, highly phytotoxic BP100ders were as well produced in transgenic plants under the control of the heat-shock induced promoter pHsp82. Other authors have tried to produce, under inducible promoters, other recombinant proteins such as Cry1B under the control of the maize proteinase inhibitor (Mpi) wound inducible promoter achieving 0.2% TSP (Breitler et al., 2004). Higher yields of recombinant *Acidothermus cellulolyticus* endoglucanase E1 were achieved in transgenic tobacco using the tomato Rubisco small subunit (RbcS-3C) light-inducible promoter, i.e. 1.3% TSP (Dai et al., 2000). However, this type of strong inducible promoters would not allow production of phytotoxic compounds. In our case, recombinant BP100-DsRed-tag54 reached only about 0.07% TSP in leaves eight hours after heat induction, about tenfold lower yield than using constitutive promoters. Curiously, the same heat shock promoter drove the accumulation of about 0.15% TSP of the reporter DsRed-tag54 as demonstrated using the same antibody in western blot. This difference was also observed in transient transformation experiments in which transgenes encoding BP100-DsRed-tag54 and DsRed-tag54 under the control of the constitutive CaMV35S promoter were agroinfiltrated into *N. benthamiana* leaves. Both in transiently transformed tobacco and stably transformed rice there were statistically similar levels of recombinant BP100-DsRed-tag54 and DsRed-tag54, as assessed by DsRed fluorescence quantification in confocal microscopy observations. This indicated that western blot based quantifications tended to underestimate the levels of recombinant BP100ders compared to recombinant proteins harbouring the same antigen but in the absence of BP100. These discrepancies could be explained by the mentioned unusual physicochemical properties of BP100, which result in nonspecific interactions with different components of the plant cells and unspecific adherence to surfaces and materials used during protein extraction, thus reducing the efficiency of isolation of BP100ders from complex matrices. Intense research is currently being carried out at the UdG to optimize protocols for efficient purification of BP100ders from this type of complex matrices (Montesinos, 2014).

Importantly, recombinant BP100.gtag proved to have antimicrobial activity. Due to the difficulty of purifying recombinant BP100ders from plant tissues, crude TSP extracted from leafs of transgenic plants constitutively expressing BP100.ders were used to inhibit bacterial growth in an *in vitro* assay. In conclusion, our approach allowed production of antibacterial peptides with high antibacterial and low haemolytic activities in plants at workable yields. BP100ders with high haemolytic activity could also be produced in plants in a temperature induction strategy, but with moderate yields.

Recombinant BP100ders accumulate in newly formed ER-derived vesicles

As previously mentioned, our approach was systematically based on targeting recombinant BP100ders to the ER to achieve higher yields as it has been described for other proteins (Nuttall et al., 2002; Benchabane et al., 2008), partly due to protection of the recombinant protein from the activity of plant proteases (López-García et al., 2002; Monroc et al., 2006b; Badosa et al., 2007) and also to minimize the putative toxic effect of high levels of antimicrobial peptides to the host plant (as we have proved in this work). Thus, high expression of our *bp100der* transgenes should entail high activity at the ER level and could potentially have some unexpected effects at the ER level (Vitale and Ceriotti, 2004).

In agreement, microarray hybridization studies carried out by our group (Montero, 2012) showed that transgenic rice constitutively producing small amounts of the moderately phytotoxic BP100.2mi had a transcriptomic pattern compatible with the response to ER stress. This involved alteration of the expression levels of four genes encoding class III peroxidase precursors, and as well of other genes such as those encoding aquaporin, certain isomerases, etc., similar to reported for rice overexpressing Cecropin A targeted to the ER (Campo et al., 2008). The expression patterns of six of these genes were studied in the GM rice line producing the highest amounts of BP100.gtag using RT-qPCR. Their expression patterns were altered in BP100.gtag as well (Figure 5), which is compatible with a situation of stress at the ER level, associated with the high production of this BP100der and accumulation in the ER (this Thesis, [Company et al., 2014a]). This is in contrast with rice GM plants expressing a different recombinant peptide, the antifungal protein AFP.

Probe Set ID	Accession number	Annotation	Recombinant peptide BP100.gtag	BP100.2mi*	AFP*
Os.2957.1.S1_at	AK073360.1	Class III peroxidase 45 precursor			
Os.264.1.S1_at	AK103189.1	Class III peroxidase 64 precursor			
Os.27793.1.S1_x_at	AK064825.1	Class III peroxidase 30 precursor			
Os.9301.1.S1_x_at	AK120411.1	Bet v I allergen defense protein			
Os.10310.1.S1_at	AK108037.1	Peptidyl-prolyl cis-trans isomerase, FKBP-type			
Os.5045.1.S1_at	AK102138.1	BBTI8 - Bowman-Birk type bran trypsin inhibitor precursor			

Figure 5. Changes in the expression patterns of 6 genes related to ER stress, in leafs of GM plants expressing BP100.gtag, BP100.2mi and AFP under the control of the constitutive pUbi promoter. The mRNA levels were assessed in three biological replicates per event using RT-qPCR and the *ef1a* constitutive gene for normalization. Dark to light colour scale represents high to low fold expression levels in GM plants expressing BP100.gtag, BP100.2mi or AFP, vs. untransformed rice var. Senia plants. The darkest colour corresponds to 60-fold change. Asterisk: extracted from Montero, 2012.

In parallel, TEM observations of rice plants constitutively producing various BP100ders showed alterations in the ER morphology as compared to untransformed plants and transgenic plants uniquely producing the reporter protein. As previously mentioned, this included a variable dilation of intra-cisternal spaces, abundant dictyosome vesicles and larger ER derived vesicles, and accumulation of electrondense granules (this Thesis, [Nadal et al., 2012; Company et al., 2014a]). This is similar to the ultrastructural effects of short exposure to metal ions such as copper, cadmium or lead (Liu et al., 2009; Jiang and Liu, 2010; Fan et al., 2011). The increased amount of vesicles with diverse sizes, derived from dictysomes and ER, tend to fuse to originate larger vacuoles that store heavy metals. Retention of heavy metals in specific vacuoles has been considered a detoxification mechanism to prevent cell damage (Einicker-Lamas et al., 2002). Dictyosomes and ER vesicles would carry proteins or polysaccharide components that participate in membrane and cell wall repairing processes following damage; and newly synthesized proteins that bind to the toxic substances to form stable complexes. In this way, the concentration of free metal ions or toxic compounds decreases (Jiang and Liu, 2010). We could hypothesize that the cationic recombinant BP100ders trigger a similar cellular response as the one described for short exposure to metal cations; and accumulation of recombinant BP100ders in the ER derived vesicles would protect the host cell from the toxic effects of these recombinant peptides. Furthermore, it has been demonstrated that high levels of toxic substances such as heavy metals entail serious cell and organelle injury and loss of functionality of detoxification pathways; and this leads to cell death (Liu et al., 2009; Jiang and Liu, 2010; Fan et al., 2011). This is similar to the severe ultrastructural alterations detected in rice cells upon exogenous application of high doses of chemically synthesized BP100.gtag: swollen and irregular mitochondria, with alteration of the internal membranes and disruption of cristae structure; and extension of the vacuolar system into the plasma membrane.

One of the important results of the present work was the observation that recombinant BP100ders did accumulate in newly formed vesicles that derived from the ER. This was clearly observed in confocal microscopy images of *N.benthamiana* leaves transiently transformed with a construct encoding BP100-DsRed-tag54, using filters to detect the DsRed fluorescence. Recombinant BP100-DsRed-tag54 but not recombinant DsRed-tag54, both designed for accumulation in the ER, was rapidly accumulated in vesicles up to 15 µm in diameter, residual fluorescence only being detected in the overall ER at very initial stages of transgene expression. As expected, recombinant DsRed-tag54 accumulated in the typical ER pattern, demonstrating that the special localization of BP100-DsRed-tag54 was triggered by the BP100 sequence. Co-transformation with a transgene encoding a modified cyan fluorescent protein targeted to the ER (eCFP) showed accumulation of recombinant eCFP in a typical ER pattern and co-localization of recombinant eCFP and BP100-DsRed-tag54 in the newly formed vesicles. Thus, BP100 induced the formation of ER derived vesicles, where the recombinant BP100der accumulated, together with other luminal ER proteins. The same vesicular pattern was observed in stably transformed plants from different species, particularly *A. thaliana* constitutively expressing BP100.DsRed.tag54 (this Thesis [Company et al., 2014a]), and rice producing the same BP100der in response to high temperature (this Thesis [Company et al., 2014b] submitted). Thus, it seems to be a transversal behaviour of BP100. Similar to BP100, a few protein motifs have been described to induce the formation of stable novel endoplasmic reticulum (ER)-derived protein bodies (PBs) (Conley et al., 2011). This includes the elastin-like polypeptides (ELP), synthetic biopolymers composed of a series of tandemly repeated VPGXG sequence (Conley et al., 2009), the Zera© domain, a Pro-rich domain derived from the maize (*Zea mays*) seed storage protein γ -zein (Torrent et al., 2009; Joseph et al., 2012), and hydrophobin HFBI, a surface-active protein derived from filamentous fungi (Joensuu et al., 2010a). They all share with BP100 an amphypatic structure that facilitates self-assembly (Llop-Tous et al., 2010) and membrane interactions ([Alves et al., 2010]; this Thesis [Company et al., 2014a]) and this has been related to their ability to induce the formation of novel vesicles. Accumulation of recombinant fusion proteins in cell vesicles has been used as a tool to increase accumulation levels (yields) and to facilitate purification of the recombinant proteins. This could be particularly useful for production of BP100ders in plants, considering the above mentioned difficulties to isolate this type of peptides from complex matrices because of their unspecific interactions with many cellular components. In preliminary experiments we successfully obtained cell fractions enriched in membranous components and vesicles from transgenic rice leafs producing BP100.gtag; and proved (in a western blot assay) that they contained high amounts of the recombinant protein.

In conclusion, α-helical cationic AMPs such as BP100ders are highly promising molecules for phytosanitary and other applications, but its production in transgenic plants is a difficult challenge. We proved that, although their toxicity to eukaryotic and non-target cells can be fully compatible with its commercial use (as determined in rat toxicity tests), high accumulation in the transgenic plant often causes phytotoxicity and impairs use of plants as biofactories.

We demonstrated that the haemolytic activity of chemically synthesized BP100ders can be used as a tool to predict phytotoxicity of the corresponding recombinant peptide against the host plant. This way, design of new BP100ders, especially by adding short sequences derived from some natural AMPs such as Magainin and Cecropin A, resulted in peptides with high antimicrobial activity against the target bacterial strains of interest, and at the same time low phytotoxic activity. These BP100ders could be produced in transgenic plants using a constitutive expression and accumulation in the ER approach, yielding up to 0.5% TSP.

We additionally demonstrated that BP100ders with high phytotoxic activity could be produced in transgenic plants through strict regulation of their expression patterns. Use of the high temperature induced promoter pHsp82 led to viable transgenic plants producing moderate levels of highly phytotoxic recombinant BP100ders. In identifying the pHsp82 promoter we developed an approach, based on publically available gene expression databases, to identify promoter sequences that could be used to drive the expression of this type of transgenes in plants.

CHAPTER VII

CONCLUSIONS

According to the studies enclosed in this Thesis, it can be **concluded:**

1. High doses of BP100ders (i.e. largely above their MIC) were toxic against plant cells upon infiltration to tobacco leaves and inclusion in rice germination media. Similarly, incubation of rice plantlets with BP100ders resulted in ultrastructural alterations comparable to those caused by cationic heavy metals. This demonstrates that accumulation of recombinant BP100ders in plant cells can lead to phytotoxicity.

2. Using up to nine BP100ders we could not find a clear correlation between their capacity to damage tobacco leaves or alter rice seedling development and viability of GM plants expressing it.

3. The haemolytic activity can be considered as a marker for the phytotoxicity of recombinant BP100ders. Peptides with haemolytic activity below the 15% at 150 µM threshold were suitable for production in plant biofactories, with transformation efficiencies close to half that of the selection marker using constitutive promoters. On the contrary, for those with haemolytic activities above 60% at 150µM the chances of obtaining viable transgenic plants expressing it were minimal.

4. The phytotoxicity against the host plant is not directly linked to the antimicrobial activity of BP100ders. BP100 modifications based on elongation of the basic undecapeptide with portions of natural AMPs such as Magainin and Cecropin A (BP100.g, BP100.gtag, BP100.g2 and BP100.c, for accumulation in the ER) had potent antibacterial activity and could be constitutively expressed as recombinant peptides in transgenic plants with yields up to about 0.5% TSP (for BP100.gtag). These recombinant peptides were active against the target microbial species.

5. Fusion of a BP100 derived peptide to a fluorescent moiety such as DsRed or the epitope tag54 permitted detection of the recombinant protein without decreasing its antimicrobial activity or affecting its phytotoxicity.

6. Both in transient and stably transformed plants of various species (tobacco, rice and *Arabidopsis*), recombinant BP100ders targeted to the ER accumulate in newly formed ER derived vesicles, together with other ER luminal proteins. Their formation was dependent on the BP100 sequence and should facilitate purification of recombinant proteins.

7. Besides the new ER derived vesicles, rice plants constitutively producing BP100.gtag, with low phytotoxicity, had changes in the ER ultrastructure similar to those observed upon short exposure to cationic heavy metals. They also showed altered expression of a number of genes such as peroxidases, etc. in a pattern that is compatible with the response to ER stress.

8. Highly phytotoxic BP100 derived peptides could be produced in plant biofactories using the heat shock inducible promoter pHsp82, although only reaching moderate yields (estimated around 0.3% TSP upon exposure to gradual temperature increase).

9. The deleterious effect of recombinant phytotoxic peptides on the host plant is determinant in the initial stages of production of transgenic plants, i.e. during the transformation process and regeneration of GM plantlets. Inducible promoters suitable to drive the expression of transgenes encoding highly phytotoxic peptides in transgenic plants should have extremely reduced activity in these specific plant tissues and developmental stages, as it is the case of *Os.hsp82*. Promoters with slightly higher activities in these specific stages, such as that of *Os.37773.1.s1_at* or *Os.165.1.s1_at* (both encoding Hsps), could not be used to that purpose since GM plants did not survive.

10. A procedure for selection of candidate promoters to drive the expression of transgenes encoding phytotoxic peptides in plants was developed using publically available transcriptomic data. A single, drought inducible promoter sequence was identified that could potentially allow higher yields of phytotoxic recombinant peptides than pHsp82.

CHAPTER VIII

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