Universitat Autònoma de Barcelona

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PhD thesis

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Dissertation presented by Cristina Peris Peris
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A la meua família

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Aquesta tesis és tant meua com teua.

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ABREVIATIONS

ADH	Alcohol Dehydrogenase
CLSM	Confocal Laser Scanning Microscopy
CSD	Cu/Zn Superoxide Dismutase
cv.	cultivar
DCL	Dicer like
DMT	Divalent Metal-ionTransporter
dpi	Days post-infection
DW	Dry Weight
EGTA	Ethylene Glycol Tetraacetic Acid
ENA	Efflux Nicotianamine transporter
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
FRO	Ferric Reductase Oxidase
GFP	Green fluorescence protein
HA	Hemaglutinin
hpi	Hours post-infection
HRZ	Haemerythrin motif-containing RING- and Zn-finger
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IRNLPI	Iron transcription factor
IRO	bHLH transcription factor
IROP	Iron oligopeptide transporter
IRT	Iron-Regulated transporter
LB	Left Border
LTI	Low-Temperature-Induced
NAS	Nicotianamine Synthase
NB-LRR	Nucleotide-binding/leucine-rich repeat
NRAMP	Natura Ressistance-Associated Macrophage Protein
PAMP	Pathogen-Associated Molecular Pattern
PR	pathogenesis-related protein
PRRs	Pattern Recognition Receptors
PTI	PAMP-triggered immunity
pv.	patovar
qRT-PCR	quantitative Reverse Transcription Polymerase Chain Rreaction.
RFP	Green fluorescence protein
RISC	RNA-induced silencing complex

RLK	receptor-like kinases
ROS	Reactive oxygen species
SLC	Solute Carrier
SMF	Suppressor of Mitochondria import Function
TM	transmembrane
Wak	Wall-associated kinases

SUMMARY

MicroRNAs (miRNAs) are key regulators of gene expression in a wide range of plant physiological processes, including developmental processes, hormone signaling, and responses to environmental stress, biotic and abiotic stress. That regulation is carried out by triggering sequence-specific cleavage or translational repression of target transcripts. This thesis addresses the functional characterization of miR7695, a novel miRNA from rice that targets an alternatively spliced transcript of the *OsNramp6* (*Natural-resistance-associated macrophage protein 6*) gene, and its contribution to disease resistance in rice. Members of the NRAMP family of proteins are known to function as metal transporters in a wide range of organisms, from bacteria to humans. The involvement of miR7695 through modulation of *OsNramp6* transcript accumulation in resistance of rice plants to infection by the rice blast fungus *Magnaporthe oryzae* has been demonstrated. This fungus is the causal agent of the rice blast disease, one of the most important diseases of cultivated rice. This thesis is organized into two chapters.

The **first chapter** focuses on the analyses of *MIR7695* expression in rice plants, including mutants impaired in miRNA biogenesis. This analysis allowed us to conclude that miR7695 is a novel, recently evolved miRNA from rice. The biological role of miR7695 was demonstrated in transgenic rice overexpressing the *MIR7695* precursor as well as in activation-tagged rice mutants of *MIR7695.* Accumulation of miR7695, with the concominant down-regulation of *OsNramp6* expression, confers enhanced resistance to *M. oryzae* infection in rice plants. Moreover, global transcript profiling of rice mutants accumulating miR7695 revealed alterations in an important number of genes involved in iron homeostasis.

The **second chapter** describes the subcellular localization and metal transport properties of NRAMP6 proteins, namely the full-length and the short NRAMP6 protein isoforms (only transcripts encoding the short-*Nramp6* isoform contain the target site for miR7695). Both NRAMP6 proteins localize to the plasma membrane and function as iron and manganese transporters. A role of *OsNramp6* in growth and resistance to infection by *M. oryzae* is reported.

Collectivelly, results here presented support that miR7695/*Nramp6* functioning is implicated in rice immunity, and broaden our knowledge about the molecular processes that sustain defense responses in plants. These studies are important to understand how the plant nutritional status affects disease resistance which might be useful in designing novel strategies for disease control in rice, a cereal of agronomic importance worldwide.

RESUMEN

Los microARNs (mirARNs) son un clase de pequeños ARNs no codificantes que actúan como reguladores de la expresión génica, y juegan un papel determinante en procesos fisiológicos de las plantas, en los que se incluyen procesos del desarrollo, señalización hormonal, y procesos tanto de estrés abiótico como biótico. Esta regulación es llevada a cabo por el reconocimiento específico de RNA mensajeros por complementaridad de secuencias, lo que determina bien la degradación o la represión traduccional de dichos tránscritos diana. Esta tesis aborda la caracterización funcional del miR7695, un nuevo mirARN de arroz cuya diana es el transcrito más corto generado por procesamiento alternativo del tránscrito primario del gen *OsNramp6* (*Natural-resistance-associated macrophage protein 6*), así como su contribución en resistencia a la infección por patógenos en plantas de arroz. Las proteínas pertenecientes a la familia de las NRAMP poseen actividad como transportadores de metales en un amplio rango de organismos, desde bacterias hasta humanos. En este trabajo se describe que miR7695 es un regulador post-transcripcional de *OsNramp6* que regula positivamente de la resistencia en plantas de arroz frente a la infección causada por el hongo *Magnaporthe oryzae*. Este hongo es el causante de la Piriculariosis, una de las enfermedades más importantes para el cultivo del arroz a nivel mundial. Esta tesis esta dividida en dos capítulos.

El **primer capítulo** se centra en el análisis de la expresión de *MIR7695* en las plantas de arroz, incluyendo mutantes afectados en la biogénesis de mirARNs. Estos análisis nos permitieron concluir que el mir7695 es un mirARN no descrito anteriormente en plantas que se ha generado recientemente desde el punto de vista evolutivo. La función biológica del mir7695 ha sido demostrado tanto en plantas transgénicas que sobreexpresan su precursor como en un mutante de T-DNA de activación del *MIR7695*. La acumulación del miR7695, y consiguiente disminución de la expresión de *OsNramp6,* confiere una mayor resistencia a la infección por el hongo *M. oryzae* en plantas de arroz. Además, los perfiles de transcripción obtenidos en el mutante de activación del miR7695 muestran cambios transcripcionales en un importante número de genes implicados en la homeostasis del hierro.

El **segundo capítulo** describe la localización subcelular y las propiedades de transporte de la proteínas NRAMP6, designadas como “full-lenght” and “short” en referencia a las dos isoformas de la proteína NRAMP6 (completa y truncada). De ellas, únicamente el transcrito que codifica para la isoforma corta de *Nramp6* contiene el sitio diana para el miR7695. Ambas proteínas se localizan en la membrana plasmática y actúan como transportadores de hierro y manganeso. En esta tesis también se describe el papel que juega *OsNramp6* en el crecimiento así como en la resistencia frente a la infección por *M. oryzae* en plantas de arroz.

En conclusión, los resultados aquí presentados apoyan la implicación de miR7695/Nramp6 en la inmunidad de arroz, y amplían nuestros conocimientos sobre los procesos moleculares que sustentan las respuestas de defensa de las plantas. Estos estudios son importantes ya que apoyan la idea de que el estado nutricional de las plantas afecta a la resistencia frente a patógenos. Ello, podría ser útil en el diseño de nuevas estrategias para el control de enfermedades en el arroz, un cereal de importancia agronómica en todo el mundo.

GENERAL INTRODUCTION

1. Rice

1.1. Ecosystems.

Rice belongs to the genus *Oryza* of the *Poaceae* (or *Gramineae*) family. As many cereals, rice evolved from grasses which were adapted to live in humid and warm regions. At present, rice grows in a wide range of environments and is productive in many situations where other crops would fail. Rice can grow in extremely diverse ecosystems, from tropical to temperate climates, and from sea level to altitudes up to 2.000 m. Based on soil water conditions, rice production ecosystems include flood-prone, rainfed (lowland and upland), and irrigated (lowland and upland) systems (Figure I.1). Irrigated systems and rainfed lowland rice systems provides the largest yields and account for about 80% of the worldwide harvested rice area and 92% of total rice production.

In Europe, rice is grown mainly in the Mediterranean basin. Italy and Spain are by far the biggest producers, but France, Greece, Portugal, Bulgary, Hungary and Romania all have a share in the paddy (flooded) rice grown annually in the European Union (http://ec.europa.eu/agriculture/markets/rice/index_en.htm).

Figure I.1. Rice ecosystems.
(A) Flood-prone rice system (http://www.elperiodico.cat/ca/noticias/societat/pla-proteccio-del-delta-lebre-comenca-fase-decisiva-380182).
(B) Upland Rainfed rice system (irri.org/our-impact/increase-food-security/philippines-gets-more-peso-per-hectare-from-rice-breeding).
(C) Irrigated rice system (http://www.rkmp.co.in/content/irrigated-rice-eco-system).

1.2. The rice plant: Morphological description

The rice cycle consists of three distinct phases: vegetative, reproductive and ripening (Figure I. 2A). The vegetative phase includes germination, emergence, seedling growth and active tillering, ending at the panicle primordial initiation. This is the first step in the reproductive phase, which continues with booting and heading or anthesis. The third and last phase in rice growth is ripening and can be subdivided into milky, dough, yellow-ripe and maturity stages.

The rice seed is a caryopsis type fruit (Figure I. 2B). The palea, lemmas, and rachilla constitute the hull which completely surrounds the outer side of the embryo and the endosperm. The embryo is divided in radicle, plumule, and epiblast, which are separated from the starchy endosperm by the scutellum. More in the pheripherical region are the aleurone layer, tegementum and pericarp.

The rice root system consists of crown roots and nodal roots (Figure I. 2C). The rice shoot is divided in different parts: the stem, which is cylindrical and erect and divided in nodes and internodes, and the leaf blade, which is lanceolate with parallel nervation and attached at the node by the leaf sheath, which encircles the stem..

Every tiller produces a panicle inflorescence with a main axis extending from the panicle base to the apex (Figure I. 2D). Secondary branches develop from the primary branches and pedicels develop from both. The spikelets are positioned above them. The flower is enclosed in the lemma and palea, which may be either awned or awnless. The flower consists of the pistil and stamens, and the components of the pistil are the stigmas, styles, and ovary (Rice Almanac; http://ricepedia.org/rice-as-a-plant).

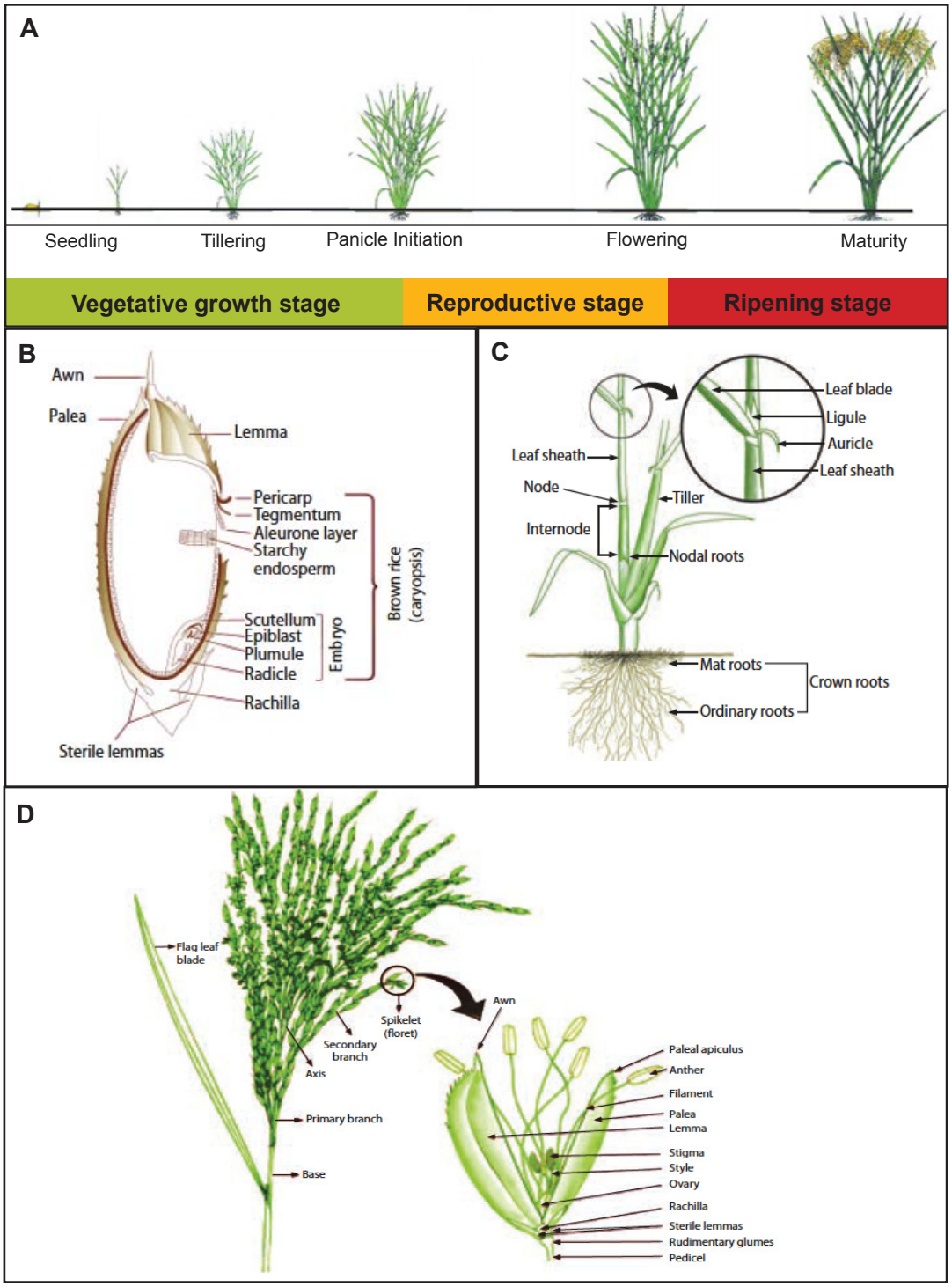


Figure I.2. Stages and rice morphology.
(A) Rice developmental stages. **(B)** Cross-section of the rice grain. **(C)** Parts of the rice stem and tillers. **(D)** Rice panicle and spikelets. Images obtained and modified from Rice Almanac (Maclean *et al.*, 2013).

1.3. Evolution and domestication of rice

The genus *Oryza* comprises 2 cultivated (2n=24) and 22 wild species (2n=24,48). The two cultivated species are *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice), with a worldwide and west Africa distribution, respectively. The wild species of *Oryza* includes *O. rufipogon, O. nivara, O. punctata, O. minuta, O. officinalis, O. alta, O. australiensis, O. granulata, O. ridleyi, O. brachyantha, O. barthii and O. coarctata,* among others. The wild species show diversity in its morphological traits like height, tillering, leaves, flowering, panicle, seeds characteristics and growth habits, comparing with the cultivated species (Figure I.3) (Zhang and Wing, 2013).

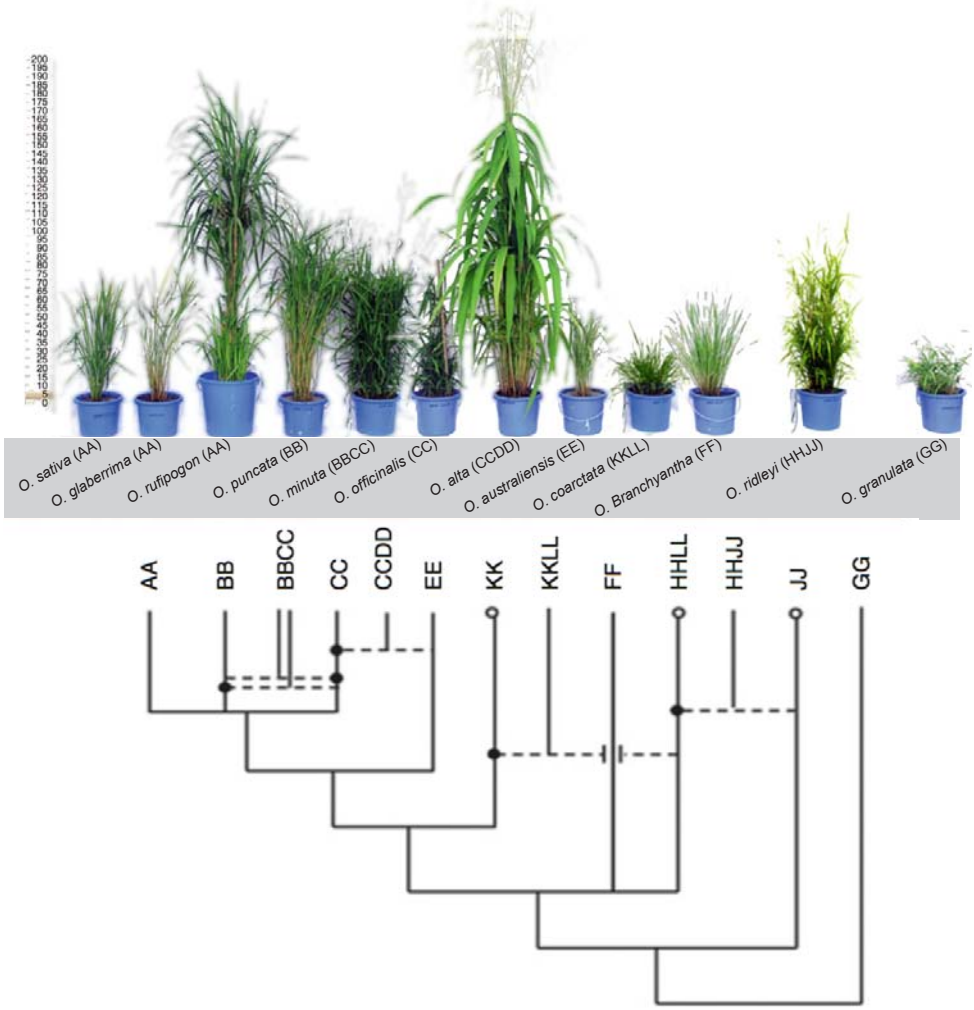


Figure I.3. The genus *Oryza*.
(A) Image of 12 representative species of *Oryza*.
(B) *Oryza* phylogenetic tree. Images modified from Genetics and Genomics of Rice (Zhang and Wing, 2013).

The Asian cultivated rice (*Oryza sativa*) contains two major cultivar groups, *japonica* and *indica*, which are the most cultivated and have a long history of natural selection and domestication. The *japonica* rice was first domesticated around the middle region of the Pearl River in Southern China whereas the *indica* rice was subsequently developed from crosses between *japonica* rice and local wild rice as the initial cultivars spread into southern Asia (Huang *et al.*, 2012). The *Oryza rufipogon* (perennial) and *Oryza nivara* (annual) species have been proposed to be the direct ancestors of *O. sativa* (Asian rice) (Sweeney and McCouch, 2007; Dogara and Jumare, 2014). On the other hand, the African cultivated rice (*Oryza glaberrima*) originated in the delta of the Niger river in West Africa and was independently domesticated from the wild progenitor *Oryza barthii* after the domestication of Asian rice (Wang *et al.*, 2014).

1.4. Agronomical importance

Rice is one of the most important crops around the world and it is a staple for more than half of the world's population. It is the second most produced cereal in the world, after maize (Figure I.4A). More than 90% of the world's rice is produced in Asia (Figure I.4B), being China and India the countries with most production (FAOSAT, 2016). In Asia, rice is a staple for a majority of the population and they consume more than 90% of its own production. (http://irri.org/rice-today/trends-in-global-rice-consumption). During the last ten years the rice harvested area and production has increased by 8% and 22%, respectively, dedicating 160 hectare millions to rice production, which corresponds to more than 700 millions tons of rice (FAOSAT, 2016).

During the Green Revolution (1960s), the combination of new high-yielding varieties and increased use of water, fertilizer, and pesticides, led to a rapid increase in rice production. At present, there is a continuing need to increase rice production to meet the ever growing demand of world food supply, with less arable land (due to urbanization and environmental pollution), less water supply and reduced input of pesticides and fertilizers. It is generally agreed that, to meet the food demands of the growing population, rice production will need to at least double by the year 2050.

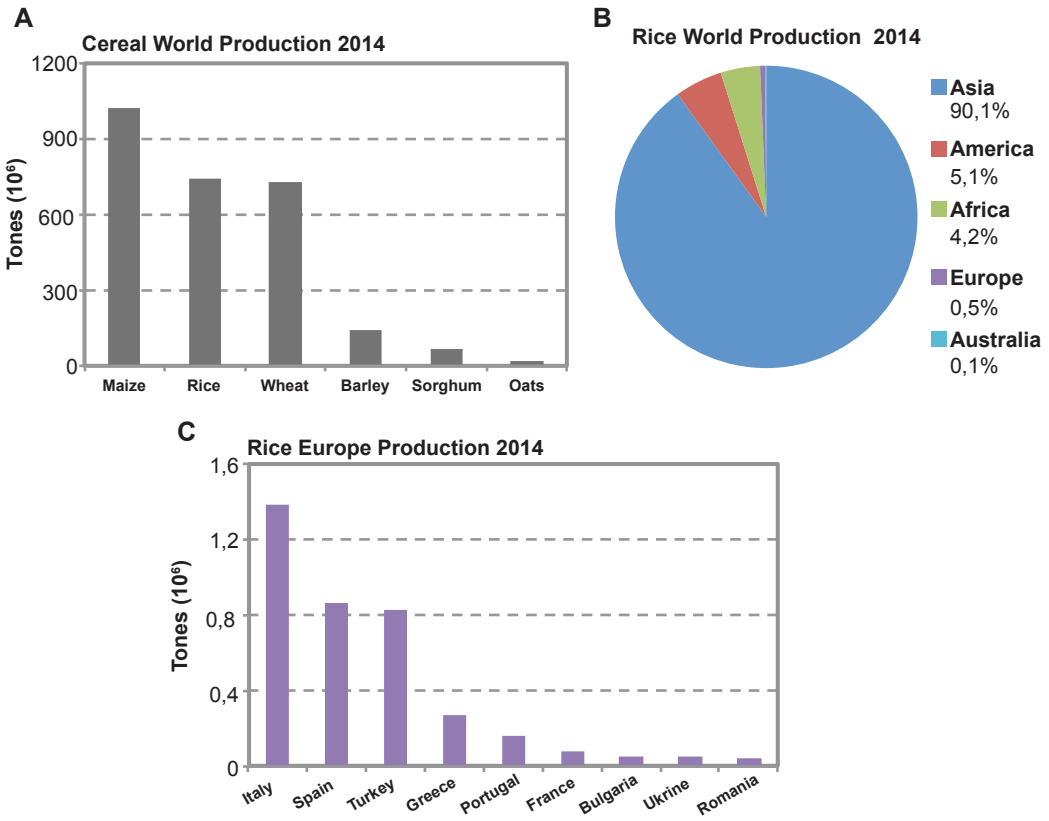


Figure I.4. Graphics on rice cultivation 2014.
(A) The most cereal produced in the word in 2014.
(B) Rice Word production represented in percentage.
(C) Rice production in Europe in 2014. Data obtained from FAOSSAT (faosat3.fao.org).

Rice has an important sociocultural significance and ecological importance in several Mediterranean countries of Europe. Annual consumption per capita ranges from 3.5 to 5.5 kg of milled rice in non rice-growing countries of northern Europe to 6–18 kg in southern Europe. The total rice-growing area within the 27 European Union (EU) member countries is about 450.000 ha, with an average annual production of about 3.1 million tons of paddy rice. However, Europe needs to import about 1.1 million tons of rice, which makes the EU's self-sufficiency in rice of about 70%. Most of the EU rice production takes place in Italy and Spain (80%) (Figure I.4C) (http://ricepedia.org/rice-around-the-world/europe).

1.5. Rice as a model system for monocotyledonous plants.

Rice is not only an important crop in the world but also the model species for studies in monocotyledonous plants. It has a relatively small genome of about 430 Mb, which is the smallest among all the cereal crops. Species within the *Oryza* genus represent different genome types, both diploid (AA, BB, CC, EE, FF, GG) and tetraploid, (BBCC, CCDD, KKLL and HHJJ) genomes (Figure I.3.). The AA genome includes the two cultivated species, *Oryza sativa* (Asian) and *Oryza glaberrima* (African). The combined international efforts facilitated the elucidation of the Asian rice genome (*japonica* and *indica* subspecies) (Goff, 2002; Yu *et al.*, 2002). Recently, the African rice genome sequence became available (Wang *et al.*, 2014). Also, physic and high-density genetics maps have shown extensive synteny between rice and other cereals (Izawa and Shimamoto, 1996).

Since 2003, the *Oryza* Map Alignment (OMAP, http://www.omap.org/resources.html) and *Oryza* Genome Evolution (OGEP, http://www.nsf.gov/awardsearch/showAward?AWD_ID=1026200) projects have led to the establishment of a comparative genomics platform to fully interrogate the genus *Oryza*. This work has led to the creation of a large array of publicly available genomic resources, including wild rice species, than can be used advantageously in genomics and breeding research.

Moreover, the availability of efficient protocols for rice transformation, germplasm collections (IRRI; International Rice Research Institute, NIAS; National Institute of Agrobiological Sciences), and cDNA and mutant resources provides essential resources in rice functional genomics. Available rice mutant resources include: GENOPLANTE- (CIRAD-INRA-IRD-CNRS), TRIM-Taiwan Rice Insertional Mutant (Academia Sinica), RMD-Rice Mutant Database, POSTECH-Rice Insertion Database (RISD), (Wang *et al.*, 2013). These advantages led the international scientific community to choose rice as the model cereal for functional genomics.

2. Problems in rice production

Despite rice plants are adapted to grow in diverse geographical areas and environmental conditions, biotic and abiotic stresses can impose limitations on rice productivity. Low and unstable rice productivity is often associated with abiotic stresses such as drought, salinity, nutrient starvation and submergence conditions (see section 2.1. Abiotic stress in rice). It also depends on the farming system used for rice production. For instance, rainfed rice fields may be affected by drought, whereas low soil fertility status is the major production constraint in upland rice fields.

Rice yield is also severely compromised by diseases caused by pathogens, including fungi, bacteria and viruses, as well as by insect pests and weeds. The fungus *Magnaporthe oryzae*, causing rice blast disease, is one of the most important fungal diseases of cultivated rice (Talbot, 2003) (see section 2.2. Biotic stress in rice).

2.1. Abiotic stress: nutrient deficiencies and toxicities

Abiotic stresses such as drought (water deficit), excessive watering (water-logging/flooding), extreme temperatures (cold, frost and heat), salinity, nutrient deficiency, and metal toxicity negatively impact growth, development, yield and seed quality of plants.

Rice is one of the most salt sensitive crops (Zeng and Shannon, 1998). Salinity affects both its early growth and reproductive stages, delays panicle emergence and flowering and decreases rice yield through reduced pollen viability. Yield is affected even under mild salinity as other nutritional and industrial qualitative traits are threatened by salinity. Salinity mostly affects coastal areas where periodic invasions of the sea water occurs. Here, semi-arid or arid lands with ineffective drainage will accumulate salts when irrigation water evaporates. The use of inappropriate irrigation practices has increased salinity levels in rice fields, which in many cases are located in the proximity of wetland habitats. Due to scarcer water availability, there is a clear tendency toward salinization in the river deltas where rice is grown in Europe (e.g. Ebro delta river in Tarragona, “Albufera” in Valencia, Camargue in France). High salinity imposes ionic stress, osmotic stress and other secondary stresses

such as nutritional disorders and oxidative stress, causing cell injury and photosynthesis reduction. Although salt and drought stresses are clearly different from each other in their physical nature, they share intrinsic attributes both leading to osmotic stress. The ionic stress generated by Na^+ would be specific to salt stress.

Rice requires larger amount of water throughout its life cycle as compared to other crops. Hence, water-related stress causes severe threat to rice production. With diminishing water supplies for agriculture worldwide, the needs to improve drought adaptation of rice and to screen resistant varieties are becoming increasingly important.

Among the various types of abiotic stress affecting rice, here we will focus on nutrient stress. For more information regarding abiotic stress affecting rice production, we refer to (Zhu 2011; Lafitte *et al*. 2004; Gao *et al*. 2007; Singh *et al*. 2008; Wani *et al*. 2014).

Nutrients (macronutrients and micronutrients) are essential for plant growth and development, and may also be important factors in plant-pathogen interactions as the nutritional status of the host plant might determine the outcome of the interaction with potential pathogens. Some of those nutrients, though, are required in relatively large amounts (macronutrients) and are frequently added as fertilizers in modern agricultural schemes. Among them are nitrogen (N), phosphorus (P), and potassium (K).

Trace elements, or micronutrients, are also essential for plant growth, including copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), molybdenum (Mo), and boron (B) (He *et al.*, 2005). They are required only in small amounts (5 to 200 ppm, or less than 0.02% dry weight). Plant growth and development could be delayed if any of these elements is lacking in the soil, not available or in excess (Figure I.5). Micronutrients takes part in metabolic activities, enzymatic process/catalysts (cofactors of metalloproteins), therefore they play an important role in balanced plant nutrition (Das, 2014). In this work, we focus on manganese and iron elements.

2.1.1. Iron

Fe is an essential element for plants as it is a co-factor for a variety of proteins mediating redox reactions. Plants require Fe for photosynthesis, mitochondrial respiration, nitrogen assimilation, and hormone biosynthesis, among others. Thus, Fe deficiency results in chlorosis, poor growth and reduced yields (Hänsch and Mendel, 2009) (Figure I.5A-B). Among grass species, rice is one of the most susceptible crops to Fe deficiency, especially during the early stages of plant development.

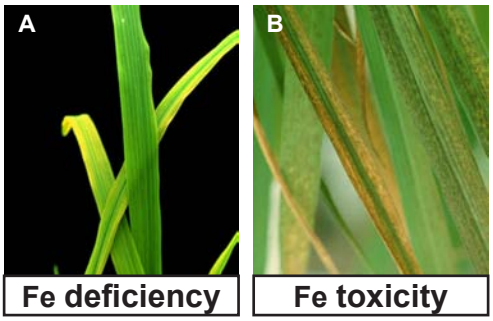


Figure I.5. Rice phenotype caused by iron disorders.

(A-B) Images of rice leaves affected by Iron deficiency **(A)**, Iron toxicity **(B)**. Images obtained from Rice Knowledge bank (www. knowledgebank. irri.org).

Despite the abundance of Fe in soils, its availability to plants is usually very low, and therefore iron deficiency is a common problem. Iron availability depends on soil conditions (e.g. soil pH). Most of the iron on the earth crust is in the ferric form (Fe^{3+}), but the ferrous form (Fe^{2+}) is physiologically more significant for plants. Fe^{3+} is insoluble in neutral and high pH, making iron unavailable to plants in alkaline and in calcareous soils (Guerinot and Yi, 1994). Furthermore, in these types of soil, iron readily combines with phosphates, carbonates, calcium, magnesium and hydroxide ions. Thus, high soil pH reduces Fe availability while acid soils increase Fe availability. Iron chlorosis caused by iron deficiency is a common problem in alkaline soils.

To overcome the generally low supply of Fe, higher plants have evolved two distinct strategies, Strategy I and Strategy II, to take up Fe from the soil (Marschner and Römheld, 1994; Hindt and Guerinot, 2012; Kobayashi and Nishizawa, 2012) (Figure I.6). The strategy I, also called the reduction strategy, is used by all dicotyledoneous and non-graminaceous monocotyledoneous

species, except rice, which is capable of utilizing both strategies (Ishimaru *et al.*, 2006; Kobayashi and Nishizawa, 2012). The strategy I, which is well described in *Arabidopsis thaliana*, involves the release of protons into the rhizosphere to acidify the soil to increase ferric ion (Fe^{3+}) solubility (Römheld and Marschner, 1986; Fox and Guerinot, 1998). Iron is subsequently reduced to ferrous form (Fe^{2+}) by a ferric reductase-oxidase (FRO) and it is then transported across the plasma membrane into the root cells by Iron-Regulated Transporter (IRT1, IRT2) (Eide *et al.*, 1996; Robinson *et al.*, 1997; Vert *et al.*, 2001). The Strategy II, also called the chelation strategy, is used by graminaceous monocotyledonous species (grasses) and consists in the biosynthesis and secretion of phytosiderophores belonging to the mugineic acid (MA) family from roots to the rhizosphere to solubilize Fe^{3+} (Kobayashi and Nishizawa, 2012). MAs present mineral chelating properties and are capable to form Fe^{3+}-phytosiderophore complexes which are taken up into root cells. Secretion of MAs (Strategy II) from rice roots to the rhizosphere is mediated by Os*TOM1* (Nozoye *et al.*, 2011), and the resulting Fe^{3+}-phytosiderophores are adsorbed into root cells by yellow-stripe like (YSL) transporters in the plasma membrane (Conte and Walker, 2011). In rice, OsYSL15 is the primary transporter responsible for uptake of Fe^{3+}-phytosiderophores from the rhizosphere (Inoue *et al.*, 2009).

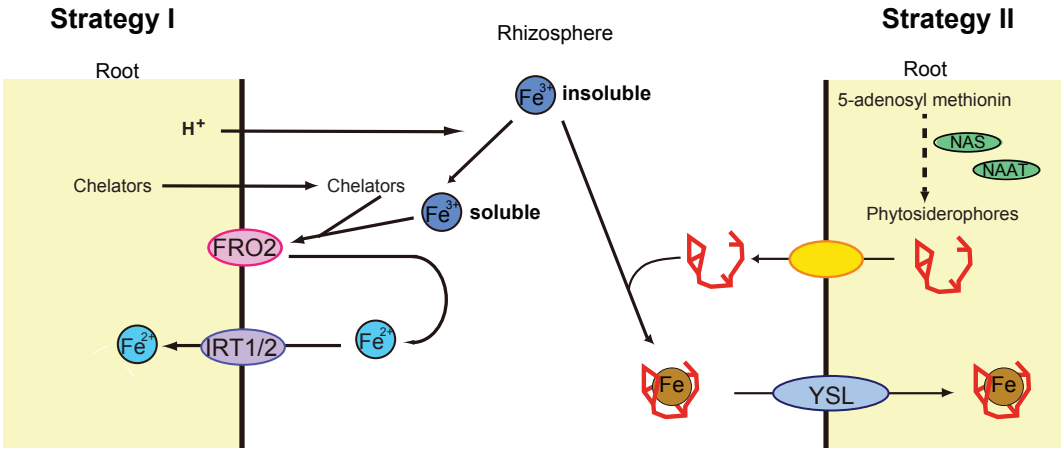


Figure I.6. Iron acquisition system in rice root.
Schematic representation of iron acquisition in roots upon iron deficiency. Ovals represent transporters and enzymes that play central roles in Fe uptake from the rhizosphere.. FRO; Ferric Reductase oxidase, IRT; Iron-Regulated transporter, NAS; Nicotianamine Synthase, NAAT; Nicotianamine aminotransferase. Broken lines indicate pathways with several steps. Modified from (Kobayashi *et al.*, 2014).

Rice was originally described as a Strategy II plant. However, evidence support that rice uses mechanisms from both strategies, Strategy I and Strategy II. Ishimaru and co-workers, (Ishimaru *et al.*, 2006) showed that rice plants are able to take up both Fe^{3+}-phytosiderophores and Fe^{2+}. Thus, in addition to absorbing an Fe^{3+}-phytosiderophore, rice possesses an Fe-uptake system that directly absorbs the Fe^{2+}. These authors demonstrated that the Iron-Regulated Transporter OsIRT1 (Strategy I system) is involved in Fe^{2+} uptake in rice plants, supporting that rice plants also possess the Fe^{2+}-transporter system. Two genes encoding Fe^{2+} transporters, Os*IRT1* and Os*IRT2*, are identified in rice (Bughio *et al.*, 2002; Ishimaru *et al.*, 2006).

Although Fe is an essential element for plants, when in excess, iron becomes toxic to the plant cells due to the formation of reactive oxygen species (ROS) via the Fenton/Haber-Weiss reactions through reduction-oxidation reaction between ferric (Fe^{3+}) and ferrous (Fe^{2+}). ROS leads to the oxidation of biomolecules (lipids, proteins, DNA) which might cause multiple damage to cellular structures (e.g. membranes), and eventually cell death. Therefore, plants must balance iron concentration in a homeostatic way to avoid toxicity. Along with this, iron homeostasis must be tightly controlled in plants through very dynamic processes to take up the metal from the soil, to traffic it throughout the plant, to compartmentalize it intracellularly, and ultimately to buffer and to store it in case of excess.

Iron in plant defense responses

Iron (Fe) is also a mediator in the plant defense response to pathogen infection (Liu *et al.*, 2006; Kieu *et al.*, 2012; Ye *et al.*, 2014). The role of Fe homeostasis in the control of redox-dependent defense responses has been demonstrated in wheat leaves during infection by *Blumeria graminis* f. sp. *tritici* (Liu *et al.*, 2006). These authors demonstrated that pathogen attack elicits secretion of Fe^{3+} to the apoplast, which leads to Fe depletion in the cytosol. The reactive Fe^{3+} accumulates at the cell wall appositions where it mediates the oxidative burst, which further stimulates Fe efflux and intracellular Fe deficiency. H_2O_2 and Fe deficiency induce expression of defense-related genes while suppressing the expression of Fe storage-related genes (Liu *et al.*, 2006). Moreover, iron starved Arabidopsis plants

display an enhanced resistance against the necrotrophic pathogens *Dickeya dadantii* and *Botritis cinerea* (Kieu *et al.*, 2012). An adequate Fe nutritional status has been shown to delay and partially supress infection and biotrophic growth of *Collecotrichum graminicola* in maize (Ye *et al.*, 2014). Collectively, these pieces of evidence suggest that alterations in iron homeostasis underlie plant defense responses. As both partners, host and pathogen, compete for this micronutrient, control of iron homeostasis is of central importance in host-pathogen interactions.

During pathogen infection, there is a competition between the host and the pathogen for iron. As the plant tissue provides the only source of iron for leaf pathogens, plants might develop Fe-withholding strategies to exert control over the attacking pathogen. This intricate relationship between the plant's nutritional status and the pathogen must be highly dynamic: the pathogen must acquire these vital elements from host tissues, and reciprocally, the host plant might defend itself by depriving the invader of nutritional resources. This means that under infection conditions, the nutrient content must be carefully regulated in the plant cell to allow normal growth and to arrest pathogen growth. The term "nutritional immunity" is used in human health and disease to define the dynamic interaction between pathogens and hosts as a non-specific host immune response against invading pathogens.

2.1.2. Manganese

Manganese (Mn) is essential for many cell processes, like photosynthesis and protection against oxidative stress. Mn is part of the oxygen-involving complex in photosystem II (PSII), which catalyses the water-splitting reaction, and acts as a cofactor of many enzymes involved in cellular redox reactions, like superoxide dismutases (MnSOD), the principal antioxidant enzymes in the mitochondria. Mn is also needed for carbohydrate, lipid, and lignin biosynthesis (Hänsch and Mendel, 2009; Socha and Guerinot, 2014).

At the phenotypical level, Mn deficiency and/or toxicity result in biomass reduction, interveinal chlorosis and tissue necrosis (Figure I.7 A-B). Plants subjected to Mn deficiency also suffer growth inhibition and have

decreased lignin content in their roots. On the other hand, the exposure to high Mn concentration originates brown spots on mature leaves and affects translocation of other essential elements (Ca, Mg, Fe or P) (Das, 2014; Socha and Guerinot, 2014).

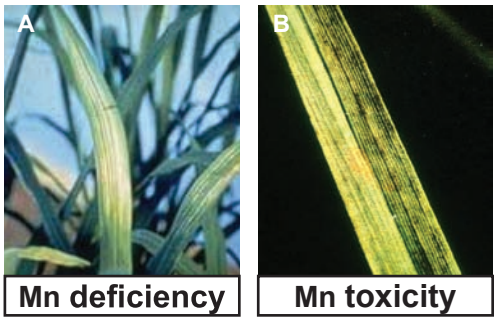


Figure I.7. Rice phenotype caused by Manganese disorders.

(A-B) Images of rice leaves affected by Manganese deficiency **(A)**, and Manganese toxicity **(B)**. Images obtained from Rice Knowledge bank (www.knowledgebank.irri.org).

2.1.3. Copper

Copper (Cu) is considered as an essential element for plant growth, it is involved in many physiological processes like photosynthesis and mitochondrial respiration, being an essential component of various proteins involved in these processes (plastiocyanins or Cytochrom oxydase). Both Cu deficiency and/or excess can cause inhibition in plant growth and developmental, as well as chlorosis. Also the exposure of rice plant to high concentration of Cu can generate Oxidative stress and ROS species (Burkhead *et al.*, 2009; Nagajyoti *et al.*, 2010).

2.1.4. Cadmium and arsenic toxicity

Cadmium (Cd) and arsenic (As) are non-essential trace elements for rice growth widely distribute in aquatic and terrestrial ecosystems. Both elements can be accumulated in grain rice, being a danger for human health (Panaullah *et al.*, 2009; Sebastian and Prasad, 2014).

Cd cause reduction of growth and biomass by affecting root and shoot length or root and leaf area. It also induces chlorosis and necrosis through damage of the photosynthetic apparatus and the indirect involvement in the production of ROS species. Cd also affects the uptake and translocation of other essential nutrients (Rizwan *et al.*, 2016).

Arsenic, as a redox active metalloid, directly stimulates ROS production, causing oxidative stress. It also inhibits several important cell functions, because it has high affinity with sulfurhydril (-SH) enzymes and can bind to reduced cysteines in peptides and proteins (Meharg, 2004; Shri *et al.*, 2009).

2.1.5. Metal transport proteins in rice.

In plants, metal transporters located at the plasma membrane are mainly involved in metal ion uptake and release, whereas those localized in subcellular compartments are mainly responsible for sequestration and remobilization of metal ions (the vacuole is the main storage compartment site for metals present in excess). A common characteristic in some of these proteins is their capability of transporting more than one divalent cation.

One of these families is Zn-regulated transporters (ZRT) and Fe-regulated transporter-like Protein (IRT) (ZIP) family protein, which are capable of uptaking and transporting divalent metal ions and are suggested to play critical roles in balancing metal uptake and homeostasis (Guerinot, 2000). In rice, the ZIP family includes the iron transporters OsIRT1 and OsIRT2, both are localized in plasma membrane of root cells and during iron deficiency both genes are induced expression (Ishimaru *et al.*, 2006). The most characterized zinc transporter members of this family in rice are OsZIP1, OsZIP3, OsZIP4, OsZIP5, and OsZIP8 (Bashir *et al.*, 2012). OsZIP1 and OsZIP3 are involved in Zn uptake from soil, OsZIP4, OsZIP5 and OsZIP8 in root to shoot translocation, while OsZIP4 and OsZIP8 seems particularly important for Zn transport to seed (Ramesh, 2003; Ishimaru *et al.*, 2005; Yang *et al.*, 2009; Lee *et al.*, 2010*a*,*b*; Suzuki *et al.*, 2012).

Members of the Yellow stripe-like (YSL) mediate metal-phytosiderophore uptake and/or metal-nicotianamine translocation, playing important roles in metal homeostasis (Hall and Williams, 2003; Palmer and Guerinot, 2009). In rice, eighteen proteins have been identified in this family involved in different metal transport capabilities. For instance, OsYSL6 is a rice Mn-nicotianamine transporter required for Mn detoxification when in excess (Sasaki *et al.*, 2011). Moreover, OsYSL2 contributes to long-distance Mn and Iron translocation to the shoots and the seed (Ishimaru *et al.*, 2010). In addition, OsYSL18

involved in iron-phytosiderophore transport in reproductive organs and phloem of lamina joints (Aoyama *et al.*, 2009). YSL-metal transporting abilities are dependent of the phytosiderophores production, such as Nicotianamine (NA), which is synthesized by the enzyme Nicotianamine Synthase (NAS). Three NAS enzymes exists in rice: OsNAS1, OsNAS2 and OsNAS3. Both *OsNAS1* and *OsNAS2* expression are induced in roots and leaves, whereas *OsNAS3* was induced in roots but suppressed in leaves in response to Fe-deficiency (Higuchi *et al.*, 2001; Inoue *et al.*, 2003). The increased expression of *OsNAS3* in a rice mutant activation resulted in higher metal content in leaves and mature seeds, and the increased amounts of Fe in grains was bioavailable to rice (Lee *et al.*, 2009).

Vacuole sequestration is an important mechanism in metal homeostasis regulation. Little is know in rice, but recent studies involve some rice families in this process: the Vacuole Iron Transporter (VIT) and the Cation Diffusion Facilitator (CDF). OsVIT1 and OsVIT2 are localized to the vacuole and are able to transport Mn, Fe and Zn in yeast, however physiological studies of plant mutants suggest only their activity to transport Fe and Zn *in planta* (Zhang *et al.*, 2012). Plant CDF members are usually called Metal Tolerance Protein (MTP). In rice, OsMTP8.1 plays an important role in Mn homeostasis, presumably by sequestering Mn specifically into vacuoles in the cells of rice shoots (Chen *et al.*, 2013).

Less is known about Mn export, but some studies in yeast point to a potential involvement of OsCAX1a, OsCAX3 (Ca^{2+}/cation antiporters) in Mn^{2+}/H^{+} exchange (Kamiya *et al.* 2005).

Natural Resistance-associated macrophage proteins

The Natural Resistance Associated Macrophage Protein (NRAMP) was originally identified as a Fe transporter in macrophages of rat (Vidal *et al.*, 1993). Nowadays, NRAMP proteins are recognized as a ubiquitous family of metal transporters with homologues in fungi, animals, plants and bacteria (Cellier et al. 1995; Cellier et al., 1996; Nelson 1999). Plant NRAMP proteins complement yeast mutants deficient in metal uptake, demonstrating their

conserved function as metal transporters among all kingdoms (Curie et al., 2000, Gross et al., 2003, Thomine et al., 2000). In plants, NRAMP proteins are usually encoded by large gene families indicating possible functional specialization within the NRAMP family.

NRAMP proteins are integral, membrane spanning proteins, containing 10-12 transmembrane (TM) domains and a conserved motif (CTM, consensus transport motif) (Gross *et al.*, 2003). Different subcellular localizations have been described for plant NRAMPs, including plasma membrane and vacuolar membranes, vesicles or the plastid envelope (Thomine *et al.,* 2003; Lanquar *et al.,* 2005; Xiao *et al.,* 2008; Cailliatte *et al.,* 2009,2010; Takahashi *et al.,* 2011; Sasaki *et al.,* 2012; Yang *et al.,* 2013). With such diverse subcellular localization, plant NRAMPs are expected to play a multifaceted role in intracellular metal homeostasis.

Members of the NRAMP family differ in their selectivity and are capable of transporting divalent metal cations into the cytoplasm. Thus, plant NRAMPs are known to transport a broad range of metals, such as Fe, Mn, Zn, Cd, As or Al (Nebo and Nelson 2006). Whereas Mn and Fe are essential for metabolism, Cd and As are toxic for virtually all living organisms (Nath *et al.*, 2014; Rizwan *et al.*, 2016). The metal transport capabilities of distinct NRAMP proteins have been described, and a biological function has been assigned to some of them. Most of these studies were carried out in the model plant *Arabidopsis thaliana*. The AtNRAMP3 and AtNRAMP4 are vacuolar metal transporters responsible for iron mobilization in early embryo development (Curie *et al.* 2000; Thomine *et al* 2000, 2003; Lanquar *et al.* 2005). They are also involved in the export of vacuolar Mn in photosynthetic tissues of adult plants (Lanquar *et al.* 2010) and contribute to Cd sensitivity (Thomine *et al.*, 2000). AtNRAMP6 functions as an intracellular Cd transporter, the *AtNramp6* gene being predominantly expressed in seeds (Cailliatte *et al.*, 2009), whereas AtNRAMP1 acts as a Mn transporter in Arabidopsis roots (Cailliatte *et al.*, 2010). In addition to their important roles during plant growth, certain NRAMP proteins might also have a role during plant-microbe interactions. For instance, the Arabidopsis AtNRAMP3 and, to a lesser extent, AtNRAMP4 have been reported to be involved in resistance against the bacterial pathogen *Erwinia chrysanthemi* (Segond *et al.*, 2009).

In spite of the relative wealth of information about NRAMPs in Arabidopsis, much less is known about the exact metal transport capabilities and physiological role of NRAMP proteins in rice, the staple food of over half the world's population. The rice genome contains eight genes encoding NRAMP proteins (*OsNramp1* to *OsNramp8*) (Belouchi *et al.*, 1997; Gross *et al.*, 2003). So far, the metal transport activity of only four rice NRAMP proteins has been characterized. OsNRAMP1 was reported to be a Fe and Cd transporter also involved in arsenic transport but not in Mn transport (Curie *et al.*, 2000; Takahashi *et al.*, 2011; Tiwari *et al.*, 2014). OsNRAMP3 functions in Mn transport (Yang *et al.*, 2013), whereas OsNRAMP5 is a Mn, Fe and Cd transporter (Ishimaru *et al.*, 2012; Sasaki *et al.*, 2012). The rice OsNRAMP4 (also known as Nrat1, Nramp aluminium transporter 1) protein shares relatively low similarity with the other NRAMP members and was identified as a transporter for aluminium that does not show transport activity for divalent cations (Xia *et al.*, 2010).

2.2. Biotic stress

Organisms that cause disease in rice include fungi, bacteria, viruses, insects, nematodes and parasitic plants.

Rice blast (*Magnaporthe oryzae*) and sheath blight (*Rhizoctonia solani*) are the two most devastating fungal diseases of rice because of its worldwide distribution and destructiveness. Information about the rice blast disease is presented below (section 2.1.1). **Sheath blight**, caused by the fungus *Rhizoctonia solani*, is the second most devastating fungal disease of rice (Banniza and Holderness, 2001). The fungus *lives in the soil and produces leaf and young tiller senescence.* Although variations in the level of resistance to sheath blight are observed among rice genotypes, no resistance genes for *R. solani* have been identified. Also, *R. solani* is difficult to control chemically since it spreads by water using floating sclerotia. **Bakanae** ("foolish seedling" in Japanese), is a seedborne fungal disease caused by the fungus *Fussarium* spp. (*Gibberella fujikuroi* species complex) (Wulff *et al.*, 2010). Among the *Fusarium* species causing this disease are *F. fujikuroi*, *F. proliferatum* and *F. verticillioides*. Infected seedlings exhibit abnormal elongation which is attributed to gibberellins (a plant growth hormone) produced by the fungus.

Furthermore, rice yields can be severely compromised by the bacterial pathogens *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv *oryzicola* (**bacterial blight** and **bacterial leaf streak**, respectively). The disease is most likely to develop in areas that have weeds and stubbles of infected plants. It can occur in both tropical and temperate environments. Moreover, **bacterial foot rot** (*Dickeya zeae*, previously known as *Erwinia chrysanthemi* pv. *zeae*) and **bacterial panicle blight** of rice (*Burkholderia glumae*) have emerged as important pathogens affecting global rice production.

Rice virus diseases are generally restricted to specific rice growing areas, e.g., **Rice Hoja Blanca Virus** (RHBH) in South-America, and **Rice Yellow Mottle Virus** (RYMV) in Africa. Rice Stripe Virus, Rice Dwarf Virus, and Rice Tungro Viruses (RSV, RDV, RTBV, RTSV) are found in Asia (Hibino, 2006; Wei and Li, 2016).

Recently, the **apple snail** (*Pomacea insularum*) entered accidentally the Ebro river delta (Spain). The Apple snail species (genus *Pomacea*) are native of the South America wetlands and are considered important exotic invasive species. They have been widely introduced in Asia and Central and North America to be used as aquarium pets or human food. The apple snail destroys rice paddy fields, eating the sown seed and the rice plantlets, although they also feed on other plants (Lach *et al.*, 2000). In Europe, the apple snail was detected for the first time on the left bank of the Ebro delta in early August 2009. Nowadays, it's also present on the right bank. In August 2015, the apple snails was detected in the rice fields located in the region of Baix Empordà (Pals, Girona). The range expansion of these snails is considered as explosive, this invasion representing a serious threat to the European rice fields.

2.2.1 The rice blast disease

The ascomycete fungus *Magnaporthe oryzae* is the causal agent of the rice blast disease (or Piriculariosis), which is easily recognizable by their symptoms in rice leaves. Blast lesions have a diamond shape with whitish to gray centers and red to brownish or necrotic border. With time, lesions enlarge and coalesce (Figure I.8A). When attacking young plants, the fungus can completely destroy the plants (leaf blast disease). Although *M. oryzae*

has been traditionally regarded as a foliar pathogen, this fungus also infects stem, node and panicles (Wilson and Talbot, 2009). The most important injury during the reproductive stage is associated to neck blast (Bonman *et al.*, 1989). *M. oryzae* was also shown to infect some cereal roots, in particular those of rice and barley (Sesma and Osbourn, 2004; Campos-Soriano and San Segundo, 2009). Based on its scientific and economic relevance, rice blast was considered the most important disease caused by fungi in plants (Dean *et al.*, 2012).

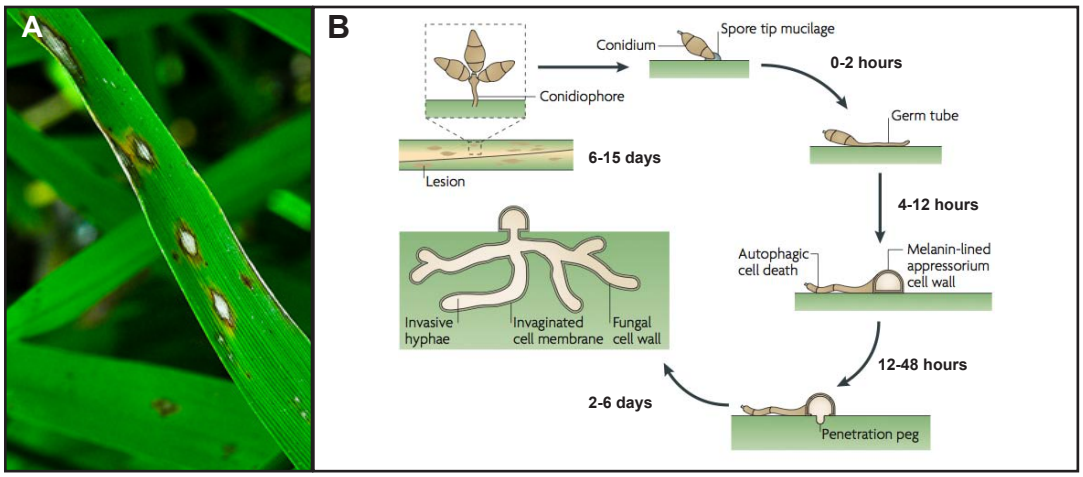


Figure I.8. Rice blast disease.
(A) Blast rice leaf lesion image from Rice Knowledge bank (www. knowledgebank.irri.org).
(B) Life cycle of the blast fungus *Magnaporthe oryzae*. The arrow shows the timing of different phases. Modified from Wilson and Talbot, 2009.

M. oryzae is a hemibiotrophic pathogen which maintains an initial biotrophic relationship with its host, followed by a necrotrophic lifestyle (Wilson and Talbot, 2009). The infection cycle is initiated when a three-celled conidium lands on the rice leaf surface (Figure I.7B). The spore attaches to the hydrophobic cuticle and germinates, producing a narrow germ tube, which subsequently flattens and hooks at its tip before differentiating into an appressorium. The appressorium matures and the conidium collapses and dies in a programmed process that requires autophagy. The appressorium becomes melanized and develops substantial turgor which translates into a physical force. Then, a narrow penetration peg forms at the base of the appressorium that penetrates the cuticle and allows the fungus to enter into the rice epidermis.

Tissue invasion occurs by means of bulbous, invasive hyphae that invade epidermal cells (biotrophic stage). Cell-to-cell movement initially occurs via plasmodesmata. The fungus then switches to the necrotrophic phase, a more aggressive phase that involves lysis of the host cells. Under favorable conditions (e.g. high humidity), the fungus sporulates from disease lesions between 72 and 96 hours after infection. Spores are dispersed by wind or rain splash to re-initiate the infection cycle in new neighbor plants (Wilson and Talbot, 2009).

The *M. oryzae* genome has been sequenced and efficient transformation methods have been developed (Dean *et al*. 2005). *M. oryzae* has emerged as a model organism in phytopathology, and a model for studying fungal plant interactions.

3. Plant innate immunity

Plants are constantly exposed to pathogen attack. To survive under such conditions, plants have evolved an innate immune system that efficiently detects potential microbial pathogens for activation of defense responses. The plant immune system is composed of two layers of defense responses, originally described as the zig-zag model (Jones and Dangl, 2006). The first layer is initiated by the recognition of Pathogen-Associated Molecular Pattern (PAMP) molecules by host Pattern Recognition Receptors (PRRs) at the cell surface, a phenomenon referred to as PAMP-triggered immunity (PTI) or basal defense (Jones and Dangl, 2006; Boller and Yang HE, 2009; Zipfel, 2014). PAMPs such as flg22 (22-amino-acid peptide) are well characterized pathogen-derived molecules that trigger PTI responses in the host cell (Gómez-Gómez and Boller, 2000). In addition, many pathogens produce degrading enzymes during plant infection that damage plant cells and generate degradation products (i.e. cell wall fragments) that serve as elicitors of the plant defense (Damage-Associated Molecular Patterns, or DAMPs).

The PTI pathway includes a variety of plant immune responses, both physical and chemical responses, which are orchestrated through a complex network of signaling pathways. Among others, PTI components include

deposition of callose, production of ROS species, activation of protein phosphorylation/dephosphorylation processes, production of antimicrobial compounds (e.g phytoalexins) and accumulation of Pathogenesis-related proteins (PRs) (Jones and Dangl, 2006; Van Loon *et al.*, 2006; Boller and Felix, 2009). To counteract this innate defense, pathogens deploy effectors that suppress PTI triggering susceptibility in the host plant (Effector-triggered susceptibility, ETS).

Plants have evolved a second layer of defense through recognition of pathogen, the so called Effector-triggered Immunity (ETI, also known a gene-for-gene resistance). Here, pathogen secreted effectors (or the biochemical consequences of their activity) are recognized by host resistance (R) proteins, usually mediated by a family of nucleotide-binding/leucine-rich repeat (NB-LRR or NLR) proteins. The ETI response could be considered as a faster and stronger version of PTI that often culminates in hypersensitive cell death response (Greenberg and Yao, 2004; Jones and Dangl, 2006). ETI and PTI are both associated with qualitatively similar, but quantitatively and kinetically different transcriptional reprogramming in the host (Tao *et al.*, 2003).

However, although the zig-zag model has been a good model for the evolutionary history of the plant immune system, it has some limitations (Pritchard and Birch, 2014) It is not a quantitative or predictive framework for the direct study of plant microbe interactions. For instance, the zig-zag model, as defined by Jones and Dangl (2006), is based only on interactions between the host immune system and biotrophic microbes that impair plant growth and reproduction, and does not take into account endogenous elicitors (DAMPs), symbiosis or necrotrophy. In order to understand host-microbe interactions, more dynamic models need to be developed, including the nature of interactions and the enviromental context.

In rice, resistance to bacterial and fungal pathogens is conferred by both basal resistance (PTI) and resistance genes (ETI) (Liu *et al.*, 2014). A broad array of blast resistance genes have been described and are being effectively used in breeding programs to increase resistance to the rice blast fungus. However, rice improvement for durable resistance to blast based on *R* genes is difficult as most of them break down in a few years because of

blast race specificity and rapid change in blast pathogenicity.

PTI and ETI responses to bacterial and fungal pathogens have been historically considered as protein-based defense mechanisms that are regulated at the transcriptional level, largely independent from the RNA silencing mechanisms that typically operate during antiviral defense in plants. Increasing evidence supports that plants also use posttranscriptional regulation of immune responses triggered by fungal and bacterial pathogens (Navarro *et al.*, 2006; Katiyar-Agarwal and Jin, 2010; Pumplin and Voinnet, 2013; Seo *et al.*, 2013; Staiger *et al.*, 2013).

4. Micro RNAs and other endogenous small RNAs in plants

Plants carry two major classes of small RNAs, namely microRNAs (miRNAs) and small interfering RNAs (siRNAs) that regulate gene expression in a sequence-specific manner (Baulcombe, 2004; Xie *et al.*, 2005*b*; Jones-Rhoades *et al.*, 2006; Voinnet, 2009).

MiRNAs and siRNAs are distinguished by their precursor molecules and different modes of biogenesis (Voinnet, 2009; Axtell, 2013; Rogers and Chen, 2013). MiRNAs are transcribed by RNA polymerase II (Pol II) as long single-stranded RNA precursors forming unique stem-loop double-stranded RNAs (dsRNAs) structures that are processed by RNAse III DICER-LIKE proteins (DCL) generating miRNA duplexes (see below, section 4.1). The second major class of small RNAs, the siRNAs, are generated from long dsRNAs resulting from the activity of RNA-dependent RNA polymerases (RDRs) that are sliced by DCL activities into siRNA duplexes (Xie *et al.*, 2004; Axtell, 2013; Baldrich *et al.*, 2014). Thus, the most distinguishing feature of siRNA biogenesis is the requirement of RDR activity for generation of dsRNA substrates.

4.1. MicroRNAs in plants

Plant miRNAs are generally transcribed from *MIRNA* genes by RNA polymerase II (Pol II) into a primary miRNA transcript (pri-miRNA), which forms unique stem–loop structures which are sequentially processed in the nucleus, typically by the RNaseIII DICER-like 1 (DCL1), to miRNA precursors

(pre-miRNA) and an miRNA–miRNA* duplex (also named miRNA-5p/miRNA-3p duplexes). Alternative pathways for miRNA biogenesis involving DCL4 or DCL3 have also been described (Rajagopalan *et al.*, 2006; Vazquez *et al.*, 2008). The two strands of the miRNA–miRNA* duplex are methylated at their 3' end and transported from the nucleus into the cytoplasm, where the functional miRNA is loaded into the RNA-induced silencing complex (RISC) that contains the ARGONAUTE1 (AGO1) protein as the core component (Kurihara and Watanabe, 2004; Baumberger and Baulcombe, 2005; Xie *et al.*, 2005*a*; Jones-Rhoades *et al.*, 2006; Vaucheret, 2008). MiRNAs function by guiding the effector AGO proteins to its target transcripts by sequence complementarity, directing post-transcriptional gene silencing by cleavage or translational repression (Llave *et al.*, 2002; Brodersen *et al.*, 2008).

Numerous studies in different plant species have demonstrated the crucial role of miRNAs in a wide range of plant developmental processes, such as organ polarity and morphogenesis, flowering, shoot and root development, and hormone signalling, among others (Palatnik, 2003; Mallory *et al.*, 2004; Chen, 2009; Rubio-Somoza and Weigel, 2011). There are also reports indicating that miRNAs respond to different types of abiotic and biotic stress (Sunkar and Zhu, 2004; Chiou *et al.*, 2006; Navarro *et al.*, 2006; Jeong and Green, 2013; Staiger *et al.*, 2013; Yang and Huang, 2014). Most research on plant miRNAs has been conducted in the model dicotyledonous plant *A. thaliana*.

The majority of miRNAs that were initially discovered are highly conserved throughout the plant kingdom and have conserved targets functioning in the regulation of developmental processes (Jones-Rhoades *et al.*, 2006). The target genes of most conserved miRNAs are transcription factors controlling diverse developmental processes (Legrand *et al.*, 2010; Lakhotia *et al.*, 2014). Plants also have species-specific miRNAs that are believed to play more specific roles. Indeed, the discovery of new miRNAs in plants, including rice, appears to have reached a plateau, and now the challenge is to identify novel miRNAs showing species- or tissue-specific expression, and miRNAs that are conditionally expressed (i.e. pathogen-regulated miRNAs).

During the last years, the adoption of deep sequencing technologies

has significantly contributed to plant miRNA discovery. The microRNA database (miRBase) is the official miRNA repository that keeps the updated annotations on newly discovered miRNAs (http://www.mirbase.org) (Kozomara and Griffiths-Jones, 2014). Rice is actually the second plant species in number of annotated miRNAs in miRBase, only after *Medicago truncatula*. Based on their nucleotide sequence, miRNAs are grouped into distinct families, each family comprising one or more members.

In rice, a great effort has been made for the identification of miRNAs accumulating in different tissues and/or developmental stages (e.g. seedlings, shoot, root, panicles), or in response to biotic and abiotic stresses (i.e. drought, salt, temperature and nutrient stress) (Jeong *et al.*, 2011; Campo *et al.*, 2013; Li *et al.*, 2014; Baldrich and San Segundo, 2016). Along with this, the relevance of distinct rice miRNAs in controlling traits of agronomic importance, such as tiller growth, early flowering, panicle and grain production, is well documented (Miura *et al.*, 2010; Wang *et al.*, 2012; Zhang *et al.*, 2013). Very recently, it was reported that blocking miR396 greatly increases grain yield in rice by modulating development of auxiliary branches and spikelets through direct induction of the miR396-targeted *OsGRF6* (*Growth Regulating Factor 6*) gene (Gao *et al.*, 2015). The current knowledge on the involvement of rice miRNAs in disease resistance is discussed below.

4.2. MicroRNAs in plant disease resistance.

An important number of plant miRNAs are regulated by pathogen infection in several plant species (Staiger *et al.*, 2013; Gupta *et al.*, 2014; Weiberg *et al.*, 2014; Yang and Huang, 2014). However, our understanding of miRNA function in plant disease resistance is far less than that in plant development. Moreover, most of the studies were carried out in the model plant *Arabidopsis thaliana.* Evidence in the literature support that miRNAs might function during PTI and/or ETI responses in Arabidopsis and other plant species.

Distinct miRNAs have been functionally characterized and are known to be components of the PTI response. The first evidence for miRNA contributing to PTI was discovered in Arabidopsis plants where perception of the bacterial elicitor flagelin (flg22) induces accumulation

of miR393, which negatively regulates the expression of a F-box auxin receptor and confers resistance to the bacterial pathogen *Pseudomonas syringae* (Navarro *et al.*, 2006). Furthermore, the activity of miR393* has been shown to be involved in modulation of PR1 exocytosis (Zhang *et al.*, 2011). MiR398 is involved in PTI responses through the control of ROS production, this miRNA targeting two Cu/Zn superoxide dismutase genes (CSD1 and CSD2) and a copper chaperone for superoxide dismutase. Cu/Zn CSDs are a group of metalloenzymes which act as scavengers of ROS, thus, protecting plants against oxidative stress associated to pathogen infection (Sunkar and Zhu, 2004; Jagadeeswaran *et al.*, 2009). Transgenic Arabidopsis lines overexpressing miR398 are compromised in resistance to the bacterial pathogen *P. syringae* (Li *et al.*, 2010). Here, there are two examples of plant miRNA controlling positive and negative regulators of plant defense responses: the auxin pathway (controlled by miR393) is generally considered as a repressor of plant immunity (Naseem *et al.*, 2015), whereas ROS production (controlled by miR398) is linked to plant resistance (Torres *et al.,* 2006) Thus, pathogen-induced and pathogen-repressed miRNAs might target negative and positive regulators of the plant defense responses, respectively. Recently, it has been described in Arabidopsis the miR863-3p which sequentially targets both negative and positive regulators of immunity through two modes of action, mRNA degradation and translational inhibition, to fine-tune the timing and amplitude of defense responses (Niu *et al.*, 2016). In other studies, Arabidopsis plants overexpressing either miR400 or miR844 showed much severe disease symptoms compared to wild-type plants when challenged with pathogenic bacteria (*P. syringae* pv *tomato DC3000*) or fungi (*Botrytis cinerea*) (Park *et al.*, 2014*a*; Lee *et al.*, 2015). MiR400 guides the cleavage of transcripts encoding pentatricopeptide repeat (PPR) proteins, whereas miR844 targets *cytidinephosphate diacylglycerol synthase3* (*CDS3*) transcripts. It is also known that miR160a functions as a positive regulator of PAMP-induced callose deposition, whereas miR398 and miR773 negatively regulate PAMP induced callose deposition and hence disease resistance to *P. syringae* (Li *et al.*, 2010; Baldrich and San Segundo, 2016).

Several miRNAs have been shown to be also involved in ETI responses by controlling *R* gene expression in *Solanaceae* (Li *et al.*, 2011; Shivaprasad

et al., 2012; Vries *et al.*, 2015) and *Leguminosae* species (Zhai *et al.*, 2011), as well as in Arabidopsis (Lu *et al.*, 2006; Boccara *et al.*, 2014), apple (Ma *et al.*, 2014) and cotton (Zhu *et al.*, 2013). This is the case of miR482/miR2118 and miR6019/miR6020, among others. Currently, functional characterization of miRNA involved in ETI has been done only in *Solanaceae* (Li *et al.*, 2011) and apple (Ma *et al.*, 2014).

4.3. Role of miRNAs in rice innate immunity

A substantial fraction of the rice miRNA transcriptome has been reported to be pathogen-responsive (Li *et al.*, 2010; Campo *et al.*, 2013; Baldrich *et al.*, 2015). However, the exact role of most of these pathogen-regulated miRNAs in rice immunity remains elusive. Only certain miRNAs have been functionally characterized in the interaction of rice plants with the fungus *M. oryzae* and viral pathogens (Baldrich and San Segundo, 2016). The contribution of rice miRNAs to antibacterial resistance is less understood, thus reflecting the important gap that occurs in our knowledge of the biological function of rice miRNAs.

Deep sequencing of small RNA transcriptomes allowed the identification of previously unknown rice miRNAs which expression is regulated by *M. oryzae* elicitors (Campo *et al.*, 2013; Baldrich *et al.*, 2015). Furthermore, combined small RNA and degradome analysis revealed the existence of regulatory networks enriched in elicitor-responsive rice miRNAs supported by the identification of their corresponding target genes, such as those associated with hormone signaling and crosstalk between defense-related hormones (ET, SA, JA, auxin) and polyamine biosynthesis. An important number of miRNAs that are regulated by *M. oryzae* elicitors are known to be involved in small RNA pathways, including the miRNA and heterochromatic pathways. Among them are miR162 and miR168 (targeting *DCL1* and *AGO1* transcripts, respectively), this observation points to a pathogen regulation of the miRNA machinery itself (Baldrich *et al.*, 2015).

Rice miRNAs for which a function in blast disease resistance has been demonstrated are miR160a, miR398b and miR7695 (Campo *et al.*, 2013; Li *et al.*, 2014). MiR160 targets Auxin Response Factors (ARFs) involved in auxin signaling. Auxins have a crucial role in development and control plant

immune responses in both Arabidopsis and rice plants (Navarro *et al.*, 2006; Domingo *et al.*, 2009) Overexpression of miR160a or miR398b in transgenic rice results in increased H_2O_2 accumulation at the infection site, up-regulation of defense gene expression (i.e. *PR1* and *PR10*) and enhanced resistance to *M. oryzae* (Li *et al.*, 2014).

4.4. Role of miRNAs in nutrient stress

A number of studies demonstrated the involvement of miRNAs in responses to nutrient and metal stress (Gielen *et al.*, 2012; Zeng *et al.*, 2014; Paul *et al.*, 2015).

Sulfate is an essential element for many compounds, such a aminoacids, sulfolipids and vitamins, which play a critical role in several physiological processes (Leustek *et al.*, 2000; Zeng *et al.*, 2014). It is well known the involvement of miR395 in plant **Sulfur (S) homeostasis** during Sulfur starvation through posttranscriptional regulation of its *APS1/3/4* and *SULTR2;1* targets (Jones-Rhoades *et al.*, 2006; Kawashima *et al.*, 2009; Liang *et al.*, 2010).

It has also been described the participation of several mRNAs in plant **Phosphate (P) homeostasis** such as miR827 and miR399, which are transcriptionally up-regulated under Pi deficiency. Accumulation of miR827 results in down-regulatation *NLA* (NITROGEN LIMITATION ADAPTATION, an E3 ligase), whereas miR399 accumulation down-regulates *PHO2* (PHOPHATE2, an ubiquitin conjugating enzyme in *Arabidopsis*; and also known as *LTN,* or LEAF TIP NECROSIS 1 in Rice) (Bari *et al.*, 2006; Hu *et al.*, 2011, 2015). PHO2 and NLA function cooperatively as negative regulators of Pi acquisition by degradation of PHT1 phosphate transporters (Huang *et al.*, 2013; Lin *et al.*, 2013; Park *et al.*, 2014*b*).

Plant **Copper (Cu) homeostasi**s is also regulated by several miRNAs, such as miR408, miR857 and miR398, all of them down-regulated under Cu starvation conditions. Up-regulation of these miRNA was negatively correlated to the accumulation of their targets (Zeng *et al.*, 2014). As mentioned before, the implication of the miR398 in Cu homeostasis through CDS1/2 not only involved this miRNA in plant immunity, but also in the Cu availability for other essential Cu proteins such as plastocyanin, when Cu is limited for plants (Sunkar *et al.*, 2006; Yamasaki *et al.*, 2007).

OBJECTIVES

The general aim of this PhD Thesis was the study of a small RNA sequence previously identified in our laboratory by deep sequencing of small RNA populations from rice tissues. This particular small RNA sequence mapped into a genomic region with the ability to fold into the stem-loop hairpin structure typical of miRNA precursors, thus, representing a miRNA candidate. The work focused in the following specific objectives:

1. To investigate whether the small RNA sequence identified in small RNA libraries represents a novel miRNA from rice. In order to accomplish this specific objective, the criteria for the annotation of novel plant miRNAs were followed, which include the excision from a stem-loop precursor structure and *dcl* dependency for miRNA accumulation. Once this small RNA sequence was validated as a miRNA, it was registered in the miRBase database as miR7695.

2. To investigate the possible contribution of miR7695 in rice immunity. For the analysis of miRNA functioning in disease resistance, we generated transgenic rice lines overexpressing the *MIR7695* precursor (*MIR7695* plants) which were molecularly and phenotypically characterized (resistance to infection by the rice blast fungus *M. oryzae*). These studies were complemented with the analysis of a rice mutant for *MIR7695*.

3. To identify the target gene of miR7695. Expression analysis of predicted targets for miR7695 in *MIR7695* plants was used to identify the target gene for this novel miRNA which was found to be an alternatively spliced transcript isoform of the *Nramp6* gene (e.g. the shortest transcript isoform).

4. To explore miR7695-regulated processes potentially involved in the rice response to pathogen infection. To achieve this goal, transcription profiling using RNA-Seq was carried out.

3. To determine the subcellular localization of the NRAMP6 protein. This study was approached by transient expression assays of *OsNramp6-GFP* fusion genes in *Nicotiana benthamiana* leaves.

4. To characterize the metal transport properties of the NRAMP6 protein. The metal transport function of NRAMP6 proteins (long- and short protein isoforms) has been demonstrated by functional complementation of yeast mutants impaired in metal transport.

5. Analysis of rice mutants for miR7695 target gene(s) focusing on their properties of disease resistance or susceptibility to infection by the rice blast fungus *M. oryzae*.

CHAPTER I

Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the *Nramp6* (*Natural resistance-associated macrophage protein 6*) gene involved in pathogen resistance.

ABSTRACT

Plants have evolved efficient defence mechanisms to defend themselves from pathogen attack. Although many studies have focused on the transcriptional regulation of defence responses, less is known about the involvement of microRNAs (miRNAs) as post-transcriptional regulators of gene expression in plant immunity. Here, we report the functional characterization of a new rice miRNA, osa-miR7695, which negatively regulates an alternatively spliced transcript of the *OsNramp6* (*Natural resistance-associated macrophage protein 6*) metal transporter gene. Accumulation of osa-miR7695 in overexpressor (Nipponbare) and activation-tagged mutant (Tainung 67) rice lines confers resistance against *M. oryzae* infection. Transcriptomic analysis of osa-miR7695 activation mutant rice plants reveals an important number of genes whose expression is altered in control and *M. oryzae* infected conditions. Among them, key genes related to iron homeostasis were specifically down-regulated in infected conditions. This study highlights a miRNA-mediated regulation of *OsNramp6* in disease resistance, whilst illustrating the existence of a novel regulatory network that integrates miRNA function and mRNA processing in plant immunity, most probably, through the regulation of plant iron status.

INTRODUCTION

Plants are naturally exposed to a variety of pathogenic microbes, such as fungi, bacteria, viruses and viroids. To protect themselves from pathogen attack, plants have developed a multilayered immune system that helps them to counteract pathogen infection. The first line of defense consists in the recognition of Pathogen-Associated Molecular Pattern (PAMP) molecules, the so called PAMP-triggered immunity (PTI), by host Pattern Recognition Receptors (PRRs) (Jones and Dangl, 2006; Boller and Yang HE, 2009; Zipfel, 2014). Successful pathogens can suppress PTI by delivering effectors into plants. Some plants in turn possess nucleotide-binding/leucine-rich repeat (NB-LRR or NLR) proteins (R proteins) that recognize pathogen effectors often culminating in hypersensitive plant cell death response. This second layer of plant immunity is called effector-triggered immunity (ETI) (Greenberg and Yao, 2004; Jones and Dangl, 2006). PTI and ETI trigger qualitatively similar, but quantitatively and kinetically different transcriptional reprogramming in the host (Tao *et al.*, 2003). Although much has been learned about the components of the plant immune system, at the molecular level, most studies have been performed on protein-coding genes. Some studies have shown the implication of microRNAs (miRNAs) in this reprogramming process through post-transcriptional regulation of gene expression (Navarro *et al.*, 2006; Katiyar-Agarwal and Jin, 2010; Pumplin and Voinnet, 2013; Seo *et al.*, 2013; Staiger *et al.*, 2013).

MiRNAs are small non-coding RNAs that regulate gene expression in a sequence specific manner (Voinnet, 2009). Plant miRNAs are mostly transcribed by RNA polymerase II as long single-stranded primary miRNA (pri-miRNA) transcripts forming stem-loop structures. The nuclear RNase III DICER_LIKE (DCL) protein sequentially processes pri-miRNAs into shorter stem-loop precursor (pre-miRNA) molecules to give rise to miRNA-miRNA* duplexes (also named miRNA-5p and miRNA-3p species). The miRNA/miRNA* duplexes are transported into the cytoplasm where the functional strand (miRNA guide) is incorporated into the ARGONAUTE1 protein, which is the effector component of the RNA-induced silencing complex (RISC), and guides the RISC complex to its target transcript by sequence complementarity

(Baumberger and Baulcombe, 2005; Xie *et al.*, 2005*b*; Jones-Rhoades *et al.*, 2006; Vaucheret, 2008). MiRNAs control post-transcriptional gene silencing by cleavage or translational repression of their target transcripts (Llave *et al.*, 2002; Brodersen *et al.*, 2008).

Regarding *DCL* genes, genome-wide identification of *DCL* genes in different plant species reveals redundancy of this gene family in plants. Members of the *DCL* gene family can be grouped into four subgroups, namely *DCL1* to *DCL4*, which have overlapping and diversified functions in small RNA biogenesis (Margis *et al.*, 2006). Production and function of each class of small RNAs has very consistent requirements for specific members of the *DCL* gene family. For instance, DCL1 is mainly involved in the generation of miRNAs, whereas DCL2, DCL3 and DCL4 contribute to the generation of the different classes of siRNAs (Chan *et al.*, 2004; Xie *et al.*, 2004, 2005*a*; Borsani *et al.*, 2005; Rajagopalan *et al.*, 2006; Kasschau *et al.*, 2007). However, miRNAs have experienced a progressive shift in DCL usage due to the accumulation of mutations in miRNA precursors. In this way, ancient *MIR* genes are processed by DCL1 and give rise predominantly to canonical miRNAs (21-nt) while recently evolved *MIR* genes are processed by DCL3 or DCL4 to produce different sizes of miRNAs (Vazquez *et al.*, 2008; Cuperus *et al.*, 2011) .

In recent years, a significant progress has been made in the identification of miRNAs from rice. The relevance of distinct rice miRNAs controlling traits of agronomic importance, such as tiller growth, early flowering, panicle and grain production, is well demonstrated (Miura *et al.*, 2010; Wang *et al.*, 2012; Zhang *et al.*, 2013). Numerous studies have shown also the participation of miRNA in a wide range of plant processes such as development, hormone signaling, oxidative stress, nutrient stress (mainly P starvation), abiotic and biotic stress (Chen, 2009; Rubio-Somoza and Weigel, 2011; Staiger *et al.*, 2013; Zhang *et al.*, 2013; Yang and Huang, 2014; Paul *et al.*, 2015; Baldrich and San Segundo, 2016). The first example of a miRNA involved in plant immunity was described in Arabidopsis plants were perception of flagellin22 (flg22) induces the accumulation of miR393, which contributes to PTI by repressing the auxin pathway (Navarro *et al.*, 2006). The Arabidopsis miR393b* also contributes to immunity, by promoting the

secretion and accumulation of the pathogenesis-related protein PR1 (Zhang *et al.*, 2011). In rice and Arabidopsis, MiR398 is down-regulated in response to bacterial and fungal infection (Jagadeeswaran *et al.*, 2009; Lu *et al.*, 2010). miRNA398 targets transcripts encoding ROS-scavenging enzymes, namely Cu/Zn superoxide dismutase (CSD1 and CSD2) transcripts, thus, protecting plants against oxidative stress associated to pathogen infection (Sunkar *et al.*, 2006; Yamasaki *et al.*, 2007; Li *et al.*, 2010). Distinct miRNAs have been shown to contribute to ETI by guiding cleavage of resistance (*R*) genes in *Solanaceae*, *Leguminosae* species as well as in *Arabidopsis* (Shivaprasad *et al.*, 2012; Boccara *et al.*, 2014). Although many other plant miRNAs have been shown to respond to pathogen infection in different plant species (Gupta *et al.*, 2014), little is known about the molecular mechanisms underlying their role in plant defense. Even less is known about plant miRNAs in defense against fungal pathogens as most studies so far were focused on Arabidopsis immunity against the bacterial pathogen *Pseudomonas syringae* (Navarro *et al.*, 2006; Li *et al.*, 2010; Boccara *et al.*, 2014; Lee *et al.*, 2015; Niu *et al.*, 2016).

Historically, miRNAs functioning in plant immunity and nutrient homeostasis have been studied separately from each other, although information independently obtained by different researchers points to a link between them. For example, nitrogen fertilization is required to maintain yield in rice but also increases susceptibility to the rice blast fungus *M. oryzae*. This observation supports that nutrient availability affects plant immune responses, although the mechanisms underlying regulatory functions of miRNAs in plant immunity remain elusive. MiR393, which is involved in resistance to bacterial infection, is also up-regulated by Cd, Hg and Al (Mendoza-Soto *et al.*, 2012). As an example, miR398 involved in the plant response against pathogen infection is regulated by heavy metals such as Fe^{3+} and Cu^{2+}. (Mendoza-Soto *et al.*, 2012; Waters *et al.*, 2012). There is then the possibility that miRNAs might integrate multiple signal transduction pathways and that nutrient homeostasis-related miRNAs might act as regulatory elements in plant immunity. A better understanding of the relationship between nutrient homeostasis and immunity in plants at a molecular level will provide a basis for the development of more efficient strategies in crop protection.

Great efforts have been made on the last years for the identification of miRNA populations accumulating in rice tissues in response to biotic stress (Baldrich and San Segundo, 2016). A substantial fraction of the rice miRNA transcriptome has been also shown to be regulated during *M. oryzae* infection, or treatment with elicitors obtained from this fungus in rice (Campo *et al.*, 2013; Li *et al.*, 2014; Baldrich *et al.*, 2015). However, the biological role of most of these pathogen-responsive miRNAs remains unknown. MiRNAs for which a function in blast resistance has been demonstrated are: miR160a, miR398b (Li *et al.*, 2014) and miR7695 (present work).

In our group, deep sequencing of small RNA populations from rice tissues (leaves and roots) that have been treated, or not, with elicitors prepared from the fungus *M. oryzae* revealed dynamic alterations in the accumulation of an important number of rice miRNAs (Campo *et al.*, 2013; Baldrich *et al.*, 2015). These studies allowed us to identify previously uncharacterized miRNAs from rice (novel miRNAs), some of them already registered in miRBase (release 21, June 2014). They are: *osa-miR7692, osa-miR7693, osa-miR7694* and *osa-miR7695* (the latest one being the object of the present work).

Analysis of miR7695 accumulation in monocotyledonous (rice, sorghum and maize) and dicotyledonous (soybean, tobacco or Arabidopsis) species, revealed that this novel miRNA might by specific to rice (*O. sativa*) (Campo *et al.*, 2013). Furthermore, when examining miR7695 accumulation in cultivated rice varieties belonging to the *O. sativa* group (including *japonica* and *indica* subspecies), only *japonica* subspecies, both temperate and tropical, were found to accumulate miR7695. As for cultivated rice species belonging to *O. glaberrima*, miR7695-derived small RNA species were detected in these varieties, but differences in miR7695 precursor processing appear to occur between *O. sativa* and *O. glaberrima* cultivars. Moreover, miR7695 was detected in some wild rice species were miR7695 precursor processing resembled that of cultivated *O. glaberrima* subspecies (rather than cultivated *O. sativa* japonica subspecies) (Campo *et al.*, 2013).

In this work, we report the functional characterization of a novel miRNA from rice, osa-miR7695, which negatively regulates an alternatively spliced transcript of the *OsNramp6* (*Natural resistance-associated macrophage*

protein 6) metal transporter gene. Accumulation of osa-miR7695 in overexpressor and activation-tagged mutants rice plants results in enhanced resistance to *M. oryzae* infection. Transcriptomic analysis in osa-miR7695 activated mutant rice lines supports a link between rice immunity and iron homeostasis.

RESULTS

Mir7695 is a novel DCL4-processed miRNA from rice.

Deep sequencing of small RNA populations from rice tissues allowed us to identify miR7695, a novel miRNA from rice (Campo *et al.*, 2013). This miRNA has been annotated in the microRNA database (http://www.mirbase.org) as osa-miR7695 (hereafter called miR7695). The predicted *MIR7695* precursor structure is shown in Figure CI.1A. Five small RNA sequences identified in small RNA sequence datasets mapped to the *MIR7695* precursor. In this respect, the production of two or more miRNAs from a *MIRNA* precursor has been previously described in rice and Arabidopsis (Zhu *et al.*, 2008; Zhang *et al.*, 2010). Although at different levels, all 5 miR7695-related species accumulated in rice leaves (Campo *et al.*, 2013). RT-PCR confirmed that the entire *MIR7695* precursor is transcribed as a single transcriptional unit that comprises five small RNAs mapping to this precursor (Figure CI.1A).

We examined the accumulation of *MIR7695*-related species in loss-of-function *dcl1* and *dcl4* mutants (Liu *et al.*, 2005, 2007). Although rice *dcl2, dcl3a*, *dcl3b* and *rdr* knockdown rice mutants have been reported (Urayama *et al.*, 2010), this material was unavailable for our study. Of interest, the accumulation of miR7695 was severely compromised in the *dcl4* mutant, but remained unaffected in the *dcl1* mutant (Figure CI.1B). This finding, together with the observation that *MIR7695* shows a high degree of complementarity in the stem-loop region, a feature typical of young miRNAs, supports the notion that *MIR7695* represents a novel, recently evolved miRNA-generating locus that is processed by DCL4 to produce multiple unique small RNAs (possible miRNAs and/or miRNA-like RNAs). Similar results have been reported previously for other young miRNAs in several plant species (Rajagopalan et *al*., 2006; BenAmor et *al*., 2009).

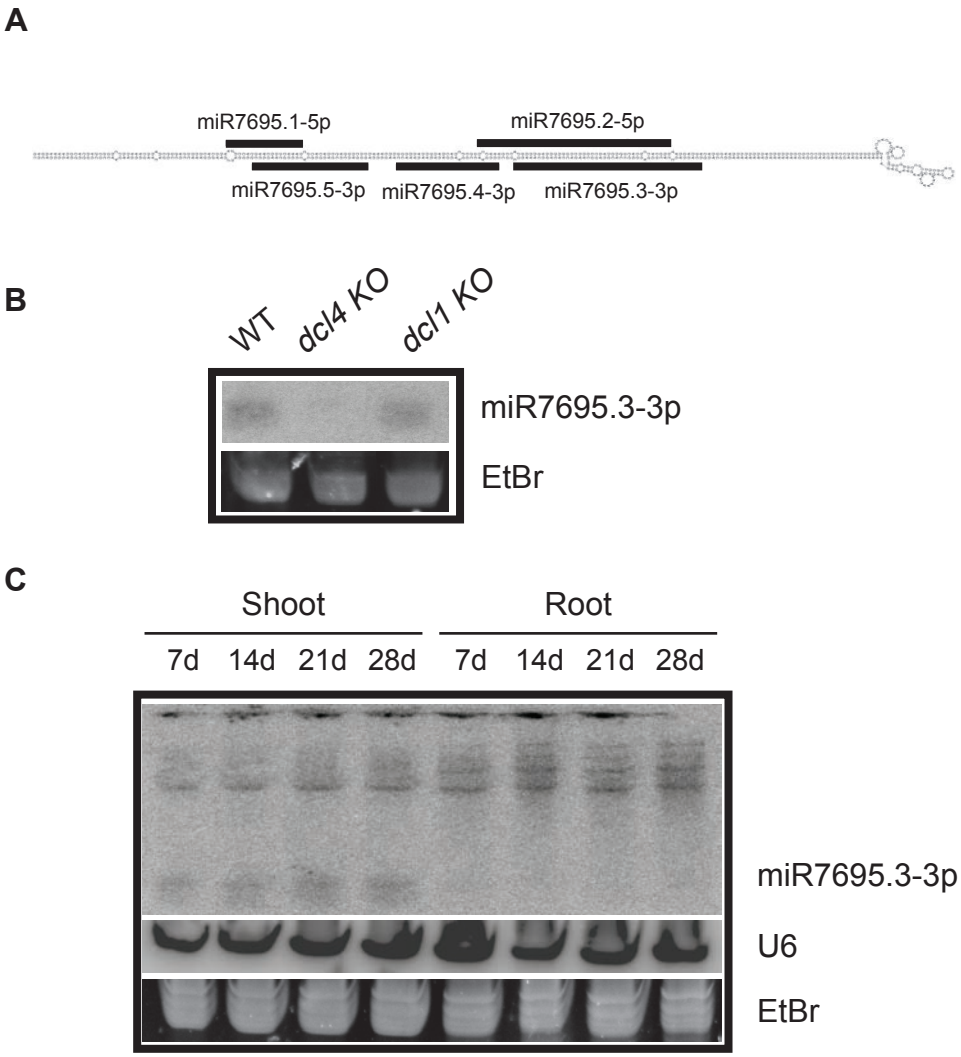


Figure CI.1. Accumulation of miR7695 in mutants defective in miRNA biogenesis and wild-type rice plants.

(A) *MIR7695* precursor structure. Black bars indicate the small RNA sequences identified in small RNA sequencing datasets (Campo et al., 2013)

(B) Northern blot analysis of miR7695 in *dcl1* and *dcl4* knock-out (KO) rice mutants. WT, wild-type (*O. sativa* cv. Nipponbare).

(C) Accumulation of miR7695 in shoots and roots of wild-type rice plants at different developmental stages (7, 14, 21 and 28 days). A synthetic oligonucleotide sequence complementary to the miR7695.3-3p small RNA was used as the hybridization probe. Hybridization with the small nuclear RNA U6 probe and Ethidium bromide (EtBr) were used as control (bottom panels).

miR7695 accumulates in rice shoots during development.

We examined miR7695 accumulation in tissues (e.g. shoots, roots) of rice plants at different developmental stages (7, 14, 21 and 28 day-old plants). Accumulation of miR7695 was consistently detected in rice shoots, but not in roots, at the various developmental stages here analysed (Figure CI.1C). We also noticed the presence of higher molecular weight RNA in both shoot and root tissues, most probably corresponding to *MIR7695* precursor processing intermediates. These results suggest that *MIR7695* is expressed in both shoot and roots, but *MIR7695* precursor processing and accumulation of mature miR7695 occurs only in shoot tissues. Further analyses are needed to verify whether improper processing of *MIR7695* precursor occurs in the rice root.

miR7695 down-regulates the expression of an alternatively spliced transcript of the *Nramp6* gene.

For the identification of putative target genes of miR7695, it was used the psRNATarget tool (http://plantgrn.noble.org/psRNATarget/). However, when considering all 5 small RNA species mapping into the pre-miR7695 structure, multiple target genes were predicted (Table CI.1).

To further investigate into the biological function of miR7695, we generated transgenic rice overexpressing the *MIR7695* precursor. For this, the DNA fragment containing the *MIR7695* fold-back structure was PCR amplified from genomic DNA and cloned into the pCAMBIA1300 plant expression vector (Figure CI.2A) under the control of the constitutive maize *Ubiquitin* (*Ubi*) promoter. Prior to rice transformation, we analysed the functionality of the cloned precursor by detecting *MIR7695*-derived small RNAs in *rdr6 N. benthamiana* leaves transiently expressing our construct. This analysis confirmed the processing of the *MIR7695* precursor and the production of all five expected small RNAs (Figure CI. 2B).

Transgenic rice (*O. sativa* japonica cv *Nipponbare*) were generated by *Agrobacterium*-mediated transformation of mature embryos-derived embryogenic calli. Transgenic lines harbouring either the *MIR7695* precursor or the empty vector (control plants) were produced. Accumulation of high levels of *MIR7695*-related small RNAs in transgenic rice was confirmed by

Northern blot analysis (Figure CI. 2C).

Table CI.1. Predicted target genes for miR7695.

Precursor	small RNA	Sequence	Target Description	
			Gene name (Accesion)	Score
miR-7695	miR7695.1-5p	UAAGUCCACGAACUUGCAAAGC	EMB1270 (EMBRYO DEFECTIVE 1270) (Os06g09880	3
			A/G-specific adenine DNA glycosylase (Os12g10910	3
			protein kinase (Os05g44290)	3
	miR7695.2-5p	UGCCUAUGUGGCACGCCACGUGAA	ribulose-phosphate 3-epimerase (Os09g32810)	2.5
		AUUGUGGUCUUGCCUAUGUGGC	AMP deaminase 2 (Os05g28180)	2.5
		UUGUGGUCUUGCCUAUGUGGC	CRS2-associated factor 1 (Os08g07790)	2.5
		AUUGUGGUCUUGCCUAUGUGG	A/G-specific adenine DNA glycosylase (Os12g10910)	2.5
		GUUUGAUUGUGGUCUUGCCUA	ankyrin repeat family protein (Os11g24840)	2.5
		CGUUUGAUUGUGGUCUUGCCU	myosin head family protein(Os05g46030)	3
		CACGUUUGAUUGUGGUCUUGCC	RINT-1 / TIP-1 family protein (Os02g51330)	3
		ACGUUUGAUUGUGGUCUUGCC	monoglyceride lipase (Os12g01030)	3
		GGUCCAAAGCCACGUUUGAUUGUG	monoglyceride lipase (Os11g01040)	3
		CCAAAGCCACGUUUGAUUGUG	mitotic-chromosome-associated protein mix-1 (Os01g67740)	3
		UCCAAAGCCACGUUUGAUUGUG	ubiquitin carboxyl-terminal hydrolase, UBP15 (Os02g14730)	3
		UCCAAAGCCACGUUUGAUUGU	NBS-LRR resistance-like protein, RGH2A (Os12g18360)	3
	miR7695.3-3p	GUAGGCAAGACCACAGUCAAAUGC	**metal transporter Nramp6** (Os01g31870)	0
		ACGUGAUGUGCCACGUAGGCAAGA	**lectin-like receptor kinase 1** (Os08g03002)	2.5
		CGUGAUGUGCCACGUAGGCAAG	cytochrome P450 76C4 (Os10g05490)	2.5
		CACGUGAUGUGCCACGUAGGCAAG	reticulon (Os04g57420)	3
		ACGUGAUGUGCCACGUAGGCA	Leucine Rich Repeat family protein (Os06g43670)	3
		UCCACGUGAUGUGCCACGUAGGC	F-box/LRR-repeat protein 14 (Os04g30320)	3
		CACGUGAUGUGCCACGUAGGC	speckle-type POZ protein (Os11g45560)	3
		CCACGUGAUGUGCCACGUAGG	cytokinin-O-glucosyltransferase 2 (Os02g36810)	3
		UCCACGUGAUGUGCCACGUAG	bile acid sodium symporter (Os02g27490)	3
		UCAUUAUCCACGUGAUGUGCCAC	RERJ1 Transcription Factor JA-responsive) (Os04g23550)	3
		CAUUAUCCACGUGAUGUGCCA		
		UCAUUAUCCACGUGAUGUGCCA		
		UCAUUAUCCACGUGAUGUGCC		
	miR7695.4-3p	UUUGAACCGGAAUGAUAUAAU		
	miR7695.5-3p	AUCUCGAUGUGCACUUUGCAAG	**lectin-like receptor kinase 1** (Os08g03002)	0.5
		GGAUCUCGAUGUGCACUUUGCAAG	glutathione synthetase, chloroplast precursor (Os11g42350)	1
		UUAGGGAUCUCGAUGUGCACUUU	**metal transporter Nramp6** (Os01g31870)	2
		UAGGGAUCUCGAUGUGCACUUU	ubiquinone biosynthesis protein ubiB (Os07g27480)	2.5
		UAGGGAUCUCGAUGUGCACUU	terpene synthase 7 (Os01g42610)	2.5
		UUAGGGAUCUCGAUGUGCACU	proteasome subunit alpha type 7 (Os09g36710)	3
		GUUUAGGGAUCUCGAUGUGCAC	glucan endo-1,3-beta-glucosidase 7 precursor (Os01g64170)	3
		UUUAGGGAUCUCGAUGUGCAC	IAP100 (Os10g35030)	3
		GUUUAGGGAUCUCGAUGUGCA	IN2-2 protein (Os04g26890)	3
		AGUUUAGGGAUCUCGAUGUGCA	GTP-binding protein lepA (Os06g05250)	3

The indicated predicted targets had a score ≤ 3.0 and UPE ≤ 25 (allowed maximum energy to unpair the target site, target accessibility).

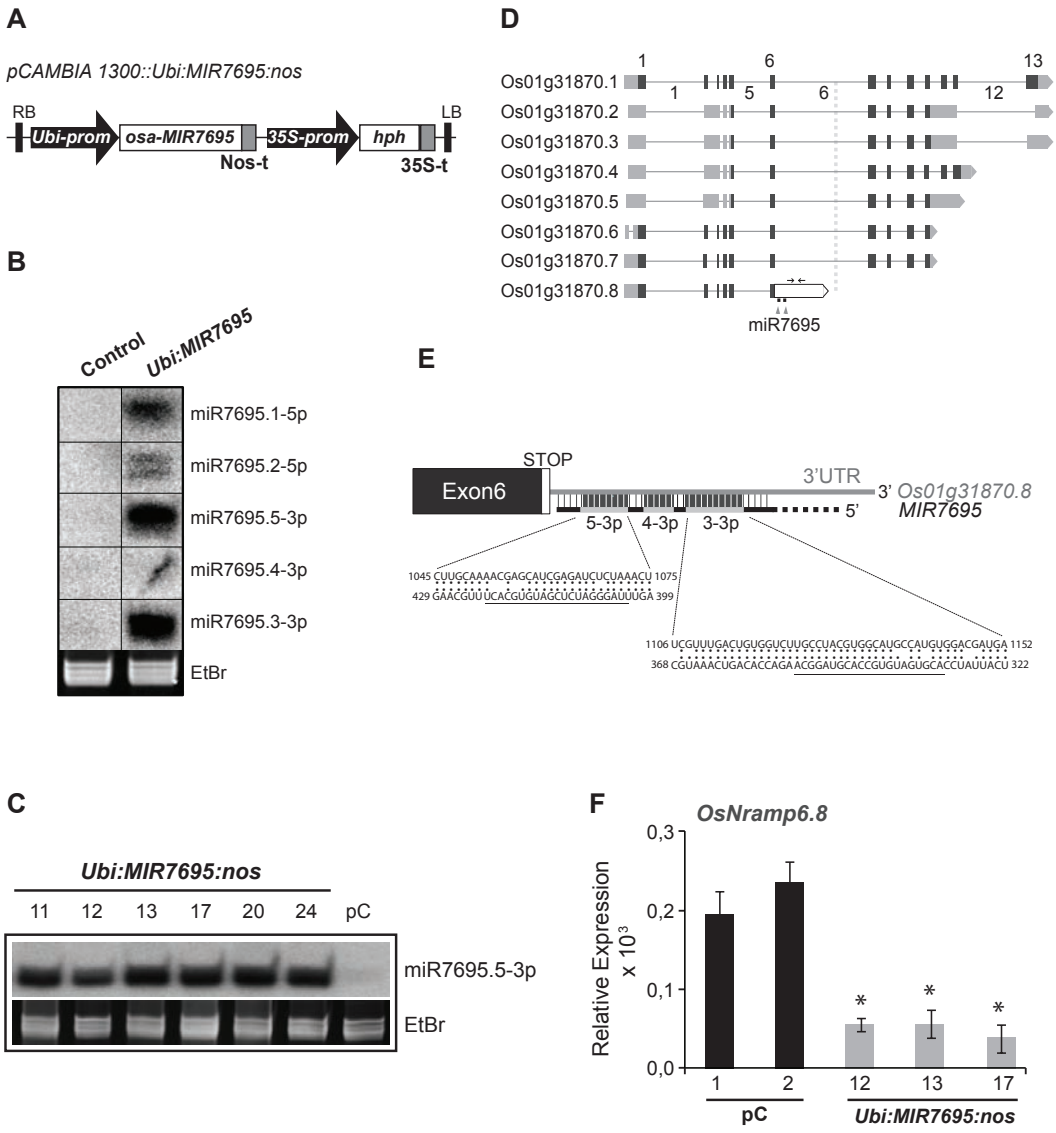


Figure CI.2 miR7695 targets an alternatively spliced transcript of the *OsNramp6* gene.
(A) Schematic representation of the cassette for constitutive expression of *MIR7695* in rice (*ubi::MIR7695::nos*). *Ubi*, Maize *Ubiquitin*; *nos*, *nopaline synthase*; *35S*, *Cauliflower mosaic virus 25S*; *hph*, *hygromycin*; prom, promoter; t, terminator.
(B) Functional analysis of the *MIR7695* precursor in *Nicotiana benthamiana* leaves (*rdr6IR* line). *MIR7695*-derived small RNAs were detected by agroinfiltration of *N. benthamiana* leaves, followed by Northern blot analysis using oligonucleotides complementary to the five sequenced small RNAs produced by the *MIR7695* precursor. No signals were detected in control leaves transformed with the empty pCAMBIA vector (control). Results from Campo *et al.*, 2013.
(C) Accumulation of miR7695 (miR7695.5-3p) in leaves of independently generated transgenic rice lines overexpressing the *MIR7695* precursor (*ubi::MIR7695::nos*), pC, plants transformed with the empty vector. Representative transgenic lines are presented. The same RNAs stained with EtBr are shown in the lower panel.
(D) Alternative splicing transcript variants of *OsNramp6.* The Os01g31870.1 transcript variant encodes the full-length protein and was taken as reference for intron/exon numbering. Only the shortest variant

(Os01g31870.8) contains miR7695 target sites (black bars) at the 3′ untranslated region (UTR). Arrows in the 3′UTR denote the primers used for Os01g31870.8 expression analyses. Image from Campo *et al.*, 2013.
(E) Complementarity of miR7695.3p-related small RNAs with the 3'UTR region of Os01g31870.8 transcripts. Image from Campo *et al.*, 2013.
(F) Accumulation of short *OsNramp6* transcripts (Os01g31870.8) in leaves of rice plants overexpressing the *MIR7695* precursor (*Ubi::MIR7695::nos*) and vector control (pC) lines, as determined by qRT-PCR (primers used indicated in Table CI.2). Samples were normalized to the rice *Ubiquitin* (*OsUbi1*, Os06g46770). Error bars represents ±SD. Asterisks indicate significant differences (t-Student test, *p<0,05).

Because miRNAs negatively regulate their target genes, we tested the expression of miR7695-predicted targets in transgenic rice. The best predicted miR7695 target was the metal transporter *Nramp6* (*Natural resistance-associated macrophage protein 6*) gene (Os01g31870) (Table CI.1). The *OsNramp6* gene produces eight transcript variants by alternative splicing (Figure CI.2D), but only the shortest transcript (Os01g31870.8) contained complementary sites for *MIR7695*-derived small RNAs (miR7695.5-3p and miR7695.3-3p), which are located at the 3' untranslated region (Figure CI.2E). Interestingly, accumulation of *OsNramp6* short transcripts (Os1g31870.8) was reduced in *MIR7695* overexpressor rice lines, indicating a miR7695-mediated regulation of the *OsNramp6* shortest splice variant (Figure CI. 2F). As for other predicted targets of miR7695, namely the lectin-like receptor kinase, NBS-LRR and lipase genes, their expression of was not significantly altered in transgenic plants compared to control plants (Campo *et al.*, 2013).

Transgenic rice plants overexpressing *MIR7695* show enhanced resistance to the rice blast fungus *M. oryzae.*

To test if miR7695 contributes to rice immunity, we locally inoculated *MIR7695* overexpressor rice lines with spores of the rice blast fungus *M. oryzae*. Three independent T2 homozygous lines were assayed. We found by visual inspection that all transgenic lines displayed enhanced resistance to *M. oryzae* relative to control plants (non-transformed, empty-vector) (Figure CI.3A). While *MIR7695* overexpressor rice exhibited only 1.04–5.53% of the leaf area affected by blast lesions, control plants showed a highest percentage (15.21% and 14,9% for wild-type and empty vector plants, respectively) of infection (Figure CI.3B). The best performing *MIR7695* overexpressor rice

line was line 13. From this, it is concluded that miR7695 positively regulates resistance to the rice blast fungus *M. oryzae*, most probably, by modulating the accumulation of short *Nramp6* transcripts (Os1g31870.8).

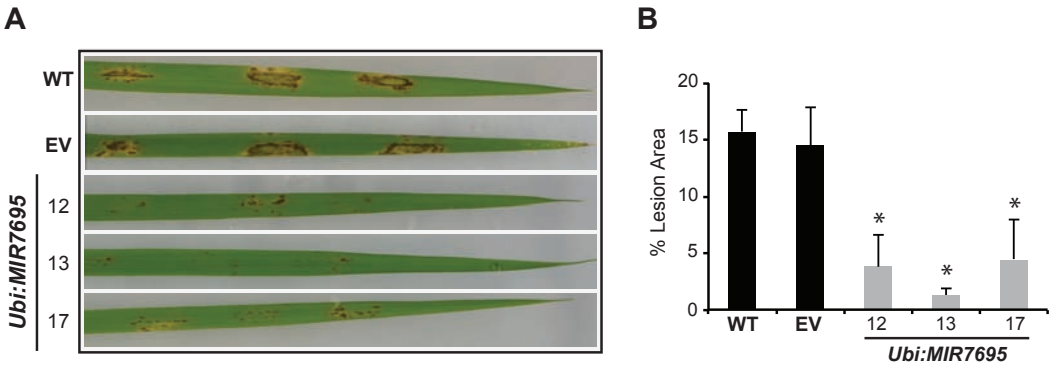


Figure CI.3. Resistance of *MIR7695* overexpressor rice plants to *Magnaporthe oryzae*.
(A) Leaves were locally inoculated with a *M. oryzae* spore suspension (10^5 spores/ml). Disease symptoms of leaves at 4 days post-infection (dpi) are shown. Three independent osa-miR7695 transgenic lines, empty vector (EV) plants and wild-type (WT, Nipponbare) plants were assayed. Representative results from one of three independent experiments that produced similar results are presented.
(B) Percentage of leaf area affected by blast lesions at 4 dpi as determined by image analysis (Assess 2.0 software, (Lamari, 2008). Error bars represents ±SD. Asterisks indicate significant differences (t-Student test, *p<0,05).

Increased resistance to *M. oryzae* infection in a *MIR7695* activation-tagged rice mutant.

To further establish a relationship between miR7695 function and disease resistance, we searched for rice mutants affected in *MIR7695* expression. Here, it is worth mentioning that finding rice miRNAs knock-outs in T-DNA insertional lines is difficult due to the small size of *MIRNA* genes. We identified a rice mutant line (M0107013) in the Taiwan Rice Insertion Mutant (TRIM) database, a T-DNA-tagged rice mutant collection designed for gene knockout and activation tagging in Tainung67 (TNG67) *japonica* varieties (Hsing *et al.*, 2007; http://trim.sinica.edu.tw). Activation tagging in these lines is achieved by positioning an octamer of transcriptional enhancer derived from the cauliflower mosaic virus 35S (CaMV35) promoter next to the left border (LB) of the T-DNA. In TRIM mutants, gene activation can be found at a distance of 15 kb from the CaMV35 enhancers (Hsing *et al.*, 2007).

The rice mutant M0107013 line carries the T-DNA insertion about 5,5 Kb upstream of the *MIR7695* gene (Figure CI.4A). Accordingly, we hypothesised that *MIR7695* might be activated in this mutant line.

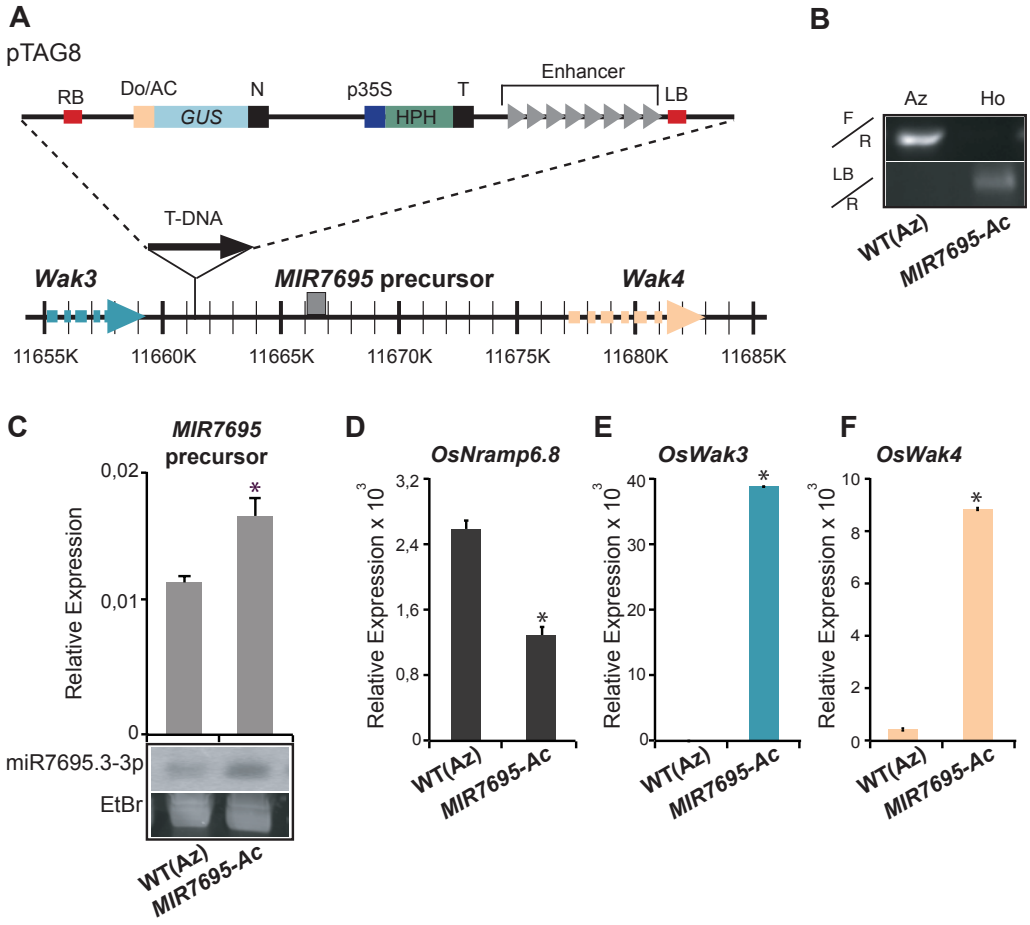


Figure CI.4. Analysis of the *MIR7695* T-DNA activation-tagged mutant rice line.
(A) Schematic representation of the T-DNA insertion mutant line from the TRIM collection (line M0107013). The location of the T-DNA and nearby genes *MIR7695*, *Wak3* (*Wall-associated kinase3*) and *Wak4 (Wall-associated kinase4*), and T-DNA components, is indicated. Key: LB, left border; RB, right border; *GUS*, β-glucuronidase; N, nopaline synthase terminator; P35S, cauliflower mosaic virus (CaMV) 35S promoter; HPH, hygromycin phosphotransferase; T, CaMV 35S terminator; Enhancer, CaMV 35S enhancer.
(B) Identification of homozygous mutant plants. PCR of genomic DNA was carried out using the primers indicated in Table CI.2. The nucleotide sequence of the amplification fragment was confirmed. Ho, homozygous for the T-DNA insertion; Az, segregated azygous; WT, wild-type (TN67).
(C) Accumulation of *MIR7695* precursor (upper panel) and miR7695 (lower panel) in homozygous mutant (*MIR7695-Ac*) and wild-type (WT(Az), segregated azygous) plants was determined by RT-qPCR and Northern blot analysis, respectively. Expression values (upper panel) represent the mean ±SE (n=3) after normalization to the *Ubiquitin1* gene (Os06g46770). Northern blot was done using the miR7695.3-3p sequence as the hybridization probe (lower panel; as a loading control, RNA blot was stained with EtBr prior to transferring).

(D-F) Accumulation of **(D)** short *Nramp6* (Os01g31870.8), **(E)** *OsWak3* (Os01g20880) and **(F)** *OsWak4* (Os01g20900) transcripts in the *MIR7695-Ac* rice mutant line, as determined by RT-qPCR. The short *Nramp6* transcript contains the miR7695 target site(s) at the 3' UTR (see Figure CI.2.D). Values represent the mean ±SD (n=2) after normalization to the *OsUbiquitin1* gene (Os06g46770). Asterisks indicate significant differences (t-Student test, *p<0,05)

Confirmation of the T-DNA insertion site was confirmed by PCR analysis of genomic DNA followed by DNA sequencing of the PCR products. Homozygous and azygous plants were identified and further characterized (Figure CI.4B). RT-qPCR and Northern blot analysis revealed an increase in *MIR7695* precursor and mature miR7695 accumulation, respectively, in homozygous mutant plants compared to azygous/control plants (Figure CI.4C), thus, confirming that M0107013 is an activation mutant for *MIR7695*. Accordingly, we designated this activation mutant as *MIR7695-Ac*. As we previously observed in the *MIR7695* overexpressor lines, *OsNramp6.8* transcripts were down-regulated in leaves of *MIR7695-Ac* plants (Figure CI.4D).

Since the CaMV35 enhancers can activate gene expression at a distance of 15kb, we searched if other genes located nearby the T-DNA insertion site (from now on flanking genes) might be activated. Two genes encoding wall-associated kinases (*Wak*), namely the *OsWak3* (Os01g20880) and *OsWak4* (Os01g20900) genes were located at 6 Kb upstream and 15 Kb downstream, respectively, of the T-DNA. Both *OsWak3* and *OsWak4* were up-regulated in the *MIR7695-Ac* mutant (Figure CI.4 E, F).

Next, we tested *MIR7695-Ac* plants for resistance to *M. oryzae* infection. Disease resistance assays using two methods, local inoculation on detached leaves (Figure CI. 5A) and spray inoculation of whole plants (Figure CI. 5B). Control azygous plants showed an 11.25% and a 5,57% of their leaf area infected with *M. oryzae* using the detached leaf and whole plant assay, respectively. However, *MIR7695-Ac* rice exhibited blast symptoms at a much lower extent (1,15% and 0,28%). Thus, *MIR7695*-Ac plants showed enhanced resistance against *M. oryzae.*

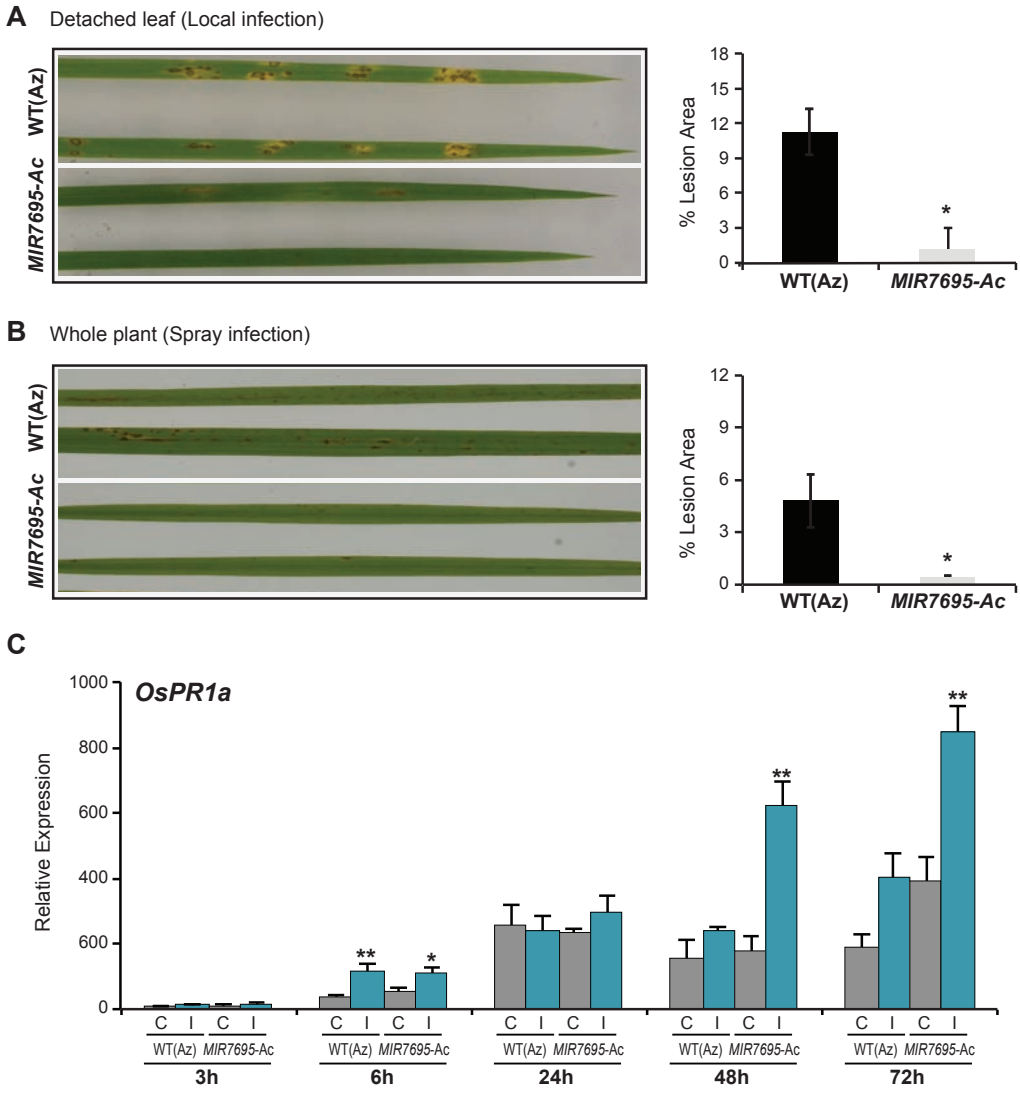


Figure CI.5. Resistance of *MIR7695-Ac* mutant lines to *Magnaporthe oryzae.*

(A) Detached leaf infection assay. The second leaf of 3-week old wild type-azygous (WT(Az)) and *MIR7695* activation-tagged mutant (*miR7695-Ac*) plants was placed in solid agar medium, locally inoculated with a *M. oryzae* spore suspension (10^5 spores/ml) and photographed at 4 days post-inoculation. Error bars represent ±SE (n=3)

(B) Whole plant infection assay. Leaves of 3 week-old WT(Az) and *MIR7695-Ac* rice plants were sprayed with a *M. oryzae* spore suspension (10^5 spores/ml) and the second leaf was photographed at 7 days post-inoculation. (**A-B**) Right panels indicate the percentage of leaf area affected by blast lesions, as determined by image analysis, (APS Assess 2.0 software (Lamari, 2008). Error bars represent ±SE (n=4). Two independent infection assays were done with similar results.

(C) Accumulation of *OsPR1a* transcripts in WT(Az) and *MIR7695-Ac* plants at different times after inoculation with *M. oryzae* spores, as determined by RT-qPCR. Blast infection was done as in B. Three leaves from each plants were taken at the indicated times after inoculation and used for RNA extraction (each sample consisting a pool of three plants). Values are means ±SE (n=3) after normalization to the *Ubiquitin1* gene (Os06g46770). Asterisks indicate significant differences (infected vs non-infected for each genotype; t-Student test, *p<0,05 **p<0,01). C, Mock-inoculated (control) plants; I, *M. oryzae* infected plants.

Resistant phenotypes are usually accompanied by the up-regulation of defense-related genes. The *OsPR1a* gene has been identified as a defense marker of the rice response to *M. oryzae* infection (Agrawal *et al.*, 2001). Thus, we analysed the expression of the *OsPR1a* gene in wild-type (azygous) and *MIR7695-Ac* rice plants inoculated with *M. oryzae* by spray inoculation of whole plants. Results obtained showed an initial response to fungal infection in both azygous and *MIR7695-Ac* plants, with up-regulation of *OsPR1a* expression at 6 hours after inoculation (Figure CI.5C). Interestingly, *MIR7695-Ac* plants accumulated higher amounts of *OsPR1a* transcripts than WT (azygous plants) at later time points of infection (48h and 72h) (Figure CI.5C). These findings suggest that the resistance phenotype that is observed in *MIR7695-Ac* plants might be, at least in part, due to a stronger activation of defense responses during pathogen infection, a situation that resembles the so called defense priming phenomenon (Balmer *et al.*, 2015; Conrath *et al.*, 2015).

Altogether our results show that both *MIR7695-overexpressor* (Nipponbare) and *MIR7695-Ac* mutant (Tainung67) plants show enhanced resistance to infection by the rice blast fungus *M. oryzae,* pointing to an important function of the miR7695/Nramp6 (*OsNramp6.8* transcripts) pair in rice immunity against *M. oryzae*.

Finally, knowing that *OsWAK* genes are up-regulated in *MIR7695-Ac* plants, there is the possibility that up-regulation of *OsWAK* genes might also contribute to the phenotype of resistance that is observed in these plants. Thus, the WAK3 and WAK4 proteins belong to a large family of receptor-like kinases (RLK) (Dong *et al.*, 2004; Zhang *et al.*, 2005). Even though the function of OsWAK3 and OsWAK4 has not been characterized yet, increasing evidence support that members of this family play a role in rice immunity against *M. oryzae*. In particular, OsWAK1, OsWAK14, OsWAK91 and OsWAK92 have been reported to be positive regulators of blast resistance, whereas OsWAK112d is a negative regulator (Li *et al.*, 2009; Delteil *et al.*, 2016). In this work, we examined the fungal-responsiveness of *OsWak3* and *OsWak4* in wild type plants. Expression analysis revealed a transient down-regulation of *OsWak3* at 3h post-inoculation with *M. oryzae* spores, whereas *OsWak4* was transiently up-regulated after 48h of *M. oryzae* inoculation

(Figure CI.6). Further studies are however needed to determine whether alterations in *OsWak3* and/or *OsWak4* expression contribute to resistance to *M. oryzae* infection in the *MIR7695-Ac* rice mutant.

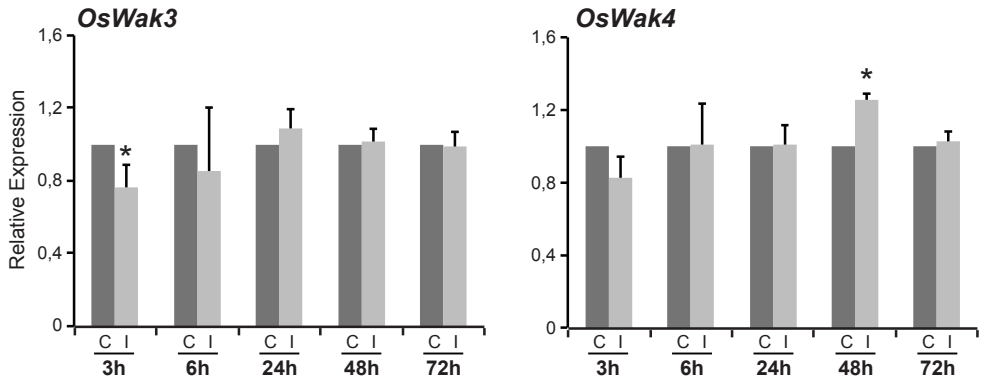


Figure CI.6. Expression of Os*WAK3* and Os*WAK4* in leaves of *M. oryzae*-infected wild-type rice. Accumulation of *OsWak3* (Os01g20880) and *OsWak4* (Os01g20900) transcripts in wild-type (*O. sativa* cv. Nipponbare) plants was determined at different times points of *M. oryzae* infection by RT-qPCR. Leaves of 3 week-old rice plants were sprayed with a suspension of *M. oryzae* spores (10^5 spores/ml). Three leaves from individual plants were taken at the indicated times after inoculation and used for RNA extraction (each sample consisting in a pool of three plants). Values were normalized to of the *OsUbiquitin1* (Os06g46770) and the mean expression for infected (I) samples for each time point is represented as a relative value compared to the corresponding control (C) mock inoculated plants. Values represent means ±SE (n=3). Asterisks indicate significant differences (t-Student test, *p<0,05).

The expression of iron-related homeostasis genes is down-regulated in *MIR7695-Ac* plants in response to *M. oryzae* infection.

To investigate the mechanism(s) by which miR7695 (or WAK) confers resistance to *M. oryzae*, we collected samples for RNA sequencing (RNA-seq) analysis from mock-treated and *M. oryzae*-infected leaves of rice *MIR7695-Ac* mutant and azygous control plants (48 dpi). We focused our analysis in genes that were differentially expressed in *MIR7695-Ac* plants in comparison to wild-type (Azygous) plants in control and infection conditions (log2FC >1, false discovery rate [FDR] <0.05). Under control conditions, a total of 291 were differentially expressed in *MIR7695-Ac* plants relative to wild-type (azygous) plants, of which 163 and 128 were up- and down-regulated, respectively (Figure CI.7A)(see Annex II). Under infection conditions (fungal-infected *MIR7695-Ac vs* fungal-infected wild-type azygous) 188 genes were found to be differentially expressed (92 up-regulated, 96 down-regulated) (Figure CI.7B).

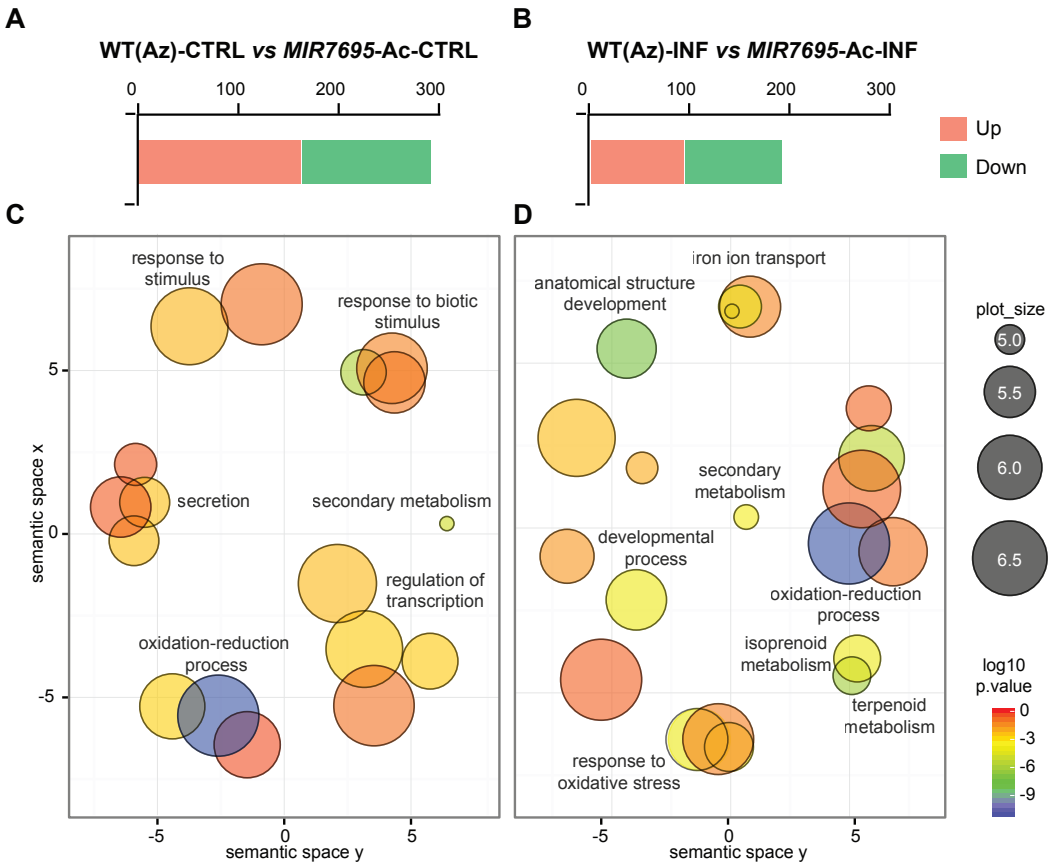
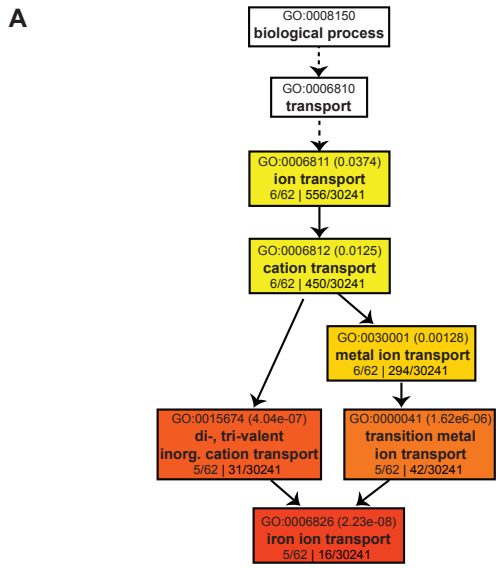


Figure CI.7. RNA-Seq data analysis of *M. oryzae*-infected *MIR7695-Ac* mutant leaves. Leaves of 3 week-old rice plants (wild-type azygous and *MIR7695-Ac*) were sprayed with a suspension of *M. oryzae* spores (10^5 spores/ml), and collected at 48 after inoculation. **(A-B)** Number of genes differentially expressed in *MIR7695-Ac* plants compared to wild-type azygous plants in **(A)** control conditions (CTR) and under **(B)** *M. oryzae* infection (INF) with a FC >2 (log2FC > 1, FDR < 0.5). **(C-D)** Gene ontology (GO) analysis of differential genes function in *MIR7695-Ac* plants in **(C)** control and **(D)** infected conditions. Significant enrichment was determined by AgriGO analysis ($p < 0.05$, Fisher's test) for all GO categories. GO terms are represented by discs. Disc colors (blue to red) represent de degree of GO enrichment (p-val) while disc size is proportional to the frequency of the GO term in the GO Database (larger and smaller discs represent more general and more specific terms, respectively). The scatter plots represent terms after reducing redundancy by REVIGO (http://revigo.irb.hr/) (Supek *et al.*, 2011) Discs representing similar GO terms are clustered closer to each other.

A gene ontology (GO) search for biological processes among the differentially expressed genes was carried out using the website software programs AgriGO (Du *et al.*, 2010) and ReviGO (Supek *et al.*, 2011). This analysis revealed several enriched terms in *MIR7695*-Ac plants. In control conditions, the most significant terms were related to response to biotic stimulus, secretion, transcriptional regulation, oxidation-reduction and

secondary metabolism (Figure CI.7C). During infection, the most significant terms enriched in *MIR7695-Ac* plants were involved in oxidative stress, oxidation-reduction, anatomical structure development, iron transport and secondary metabolism (isoprenoids and terpenoids) (Figure CI.7D). Of interest, the only category represented among the 96 down-regulated genes in *M. oryzae*-infected *MIR7695-Ac* plants (relative to *M. oryzae*-infected wild-type azygous plants) was related to "iron ion transport" (Figure CI.8A). This category included genes belonging to the iron deficiency responses involved in the reduction Strategy I: the Fe^{3+} reductase (*OsFRO2) and* Fe^{2+} transporter (*OsIRT2*) genes, and the chelation Strategy II: two nicotianamine synthase (*OsNAS1* and *OsNAS2)* and a transcription factor (*OsIRO2*) genes (Figure CI. 8B). A closer search revealed that six additional genes related to iron homeostasis not identified by AgriGO analysis were down-regulated in *MIR7695-Ac* plants: a Fe^{2+} transporter (*OsNramp1*, another member of the rice *Nramp* gene family), a transcription factor (*OsIRNLPl*), two ubiquitin ligases (*OsHRZ1* and *OsHRZ2*), an oligopeptide transporter (*OsIROPT)* and a nicotianamine efflux transporter *(OsENA1)* genes (Figure CI. 8B).

RT-qPCR analysis was carried out to validate RNA-seq data for selected genes in the down-regulated category of iron ion transport (*OsFRO2, OsIRT2, OsNramp1, OsIRO2, OsNAS1* and *OsIRNLP1*) (Figure CI.8C). Results obtained revealed that the iron homeostasis-related genes were strongly repressed in *MIR7695-Ac* plants after 48 hours of *M. oryzae* infection (Figure CI.8C). No transcriptional changes were observed in control azygous plants in response to infection at the time point here analyzed (Figure CI.8C). Of interest, the Fe^{2+} transporter (*OsIRT2*) and the *Nicotianamine synthase* (*OsNAS1)* from the Strategy I and Strategy II uptake system, respectively, were down-regulated in *MIR7695*-Ac plants in control conditions (no infection), suggesting alterations in iron-homeostasis in *MIR7695*-Ac previous to *M. oryzae* infection (Figure CI.8C).



B

	WT(Az) *vs* *MIR7695*-Ac	
	CTRL	INF
"Iron Ion transport" AGRIGO		
OsFRO2		-1.50
OsIRT2		-2.17
OsNAS1	-1.50	-4.07
OsNAS2	-2.39	-3.12
OsIRO2		-1.39
OsNRAMP1		-1.21
OsIRNLPl		-2.81
OsHRZ1		-1.05
OsHRZ2		-1.78
OsIROPT1		-1.67
OsENA1		-3.64

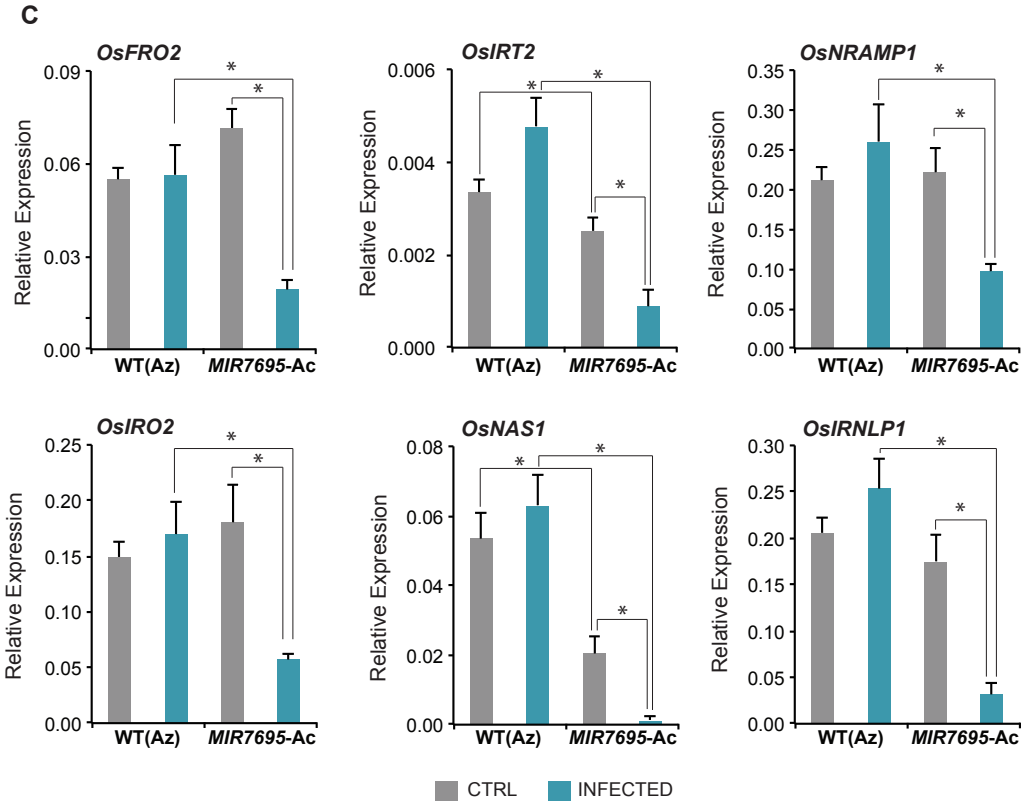


Figure CI.8. *M. oryzae* induced down-regulation of genes related to iron homeostasis in *MIR7695-Ac* plants.

(A) Hierarchical tree graph of down-regulated genes under *M. oryzae* infection in *MIR7695-Ac* mutant. The significant term (adjusted P<0.05) are marked with colour, while non-significant terms are shown as white boxes. The diagram, the degree of colour saturation of a box is positively correlated to the enrichment level of the term. Solid and dashed, lines represent, one and zero enriched terms at both ends connected by the line, respectively.

(B) Rice iron homeostasis genes down-regulated in *MIR7695-Ac* compared to wild-type azygous (WT(Az)) plants. Values represent log2 fold changes (pvalues < 0.5, FDR < 0.5).
(C) Validation of RNAseq data by RT-qPCR. RNA samples used were the same as for RNA-seq analysis. Genes analysed: *OsFRO2* (Os04g48930), *OsIRT2* (Os03g46454), *OsNramp1* (Os07g15460), *OsNAS1* (Os03g19420), *OsIRO2* (Os01g72370), *OsIRNLP1* (Os12g18410). Values represent means ±SE (n=3) after normalization to the *Ubiquitin1* gene (Os06g46770). Asterisks indicate significant differences (t-Student test, *p<0,05).

All together, these results suggest that an increase in miR7695 accumulation results in enhanced resistance to *M. oryzae* infection through down-regulation of genes involved in iron homeostasis. The resistance of rice plants overexpressing *MIR7695* (transgenic and mutant plants) might be based on a more robust immune response during *M. oryzae* infection as it was observed for *OsPR1a* expression (a marker of the rice defense response). Potentiation of defense responses will increase the plant's ability to resist pathogen infection. Whether these responses are a direct consequence or an indirect effect of miR7695/Nramp6 (short isoform) functioning needs to be elucidated. Further experiments will be needed to decipher the genetic components governing miR7695-mediated defense responses.

DISCUSSION

Plant's ability to mount and effective response against pathogen attack is essential to guarantee its survival. Increasing evidence support that microRNAs are important regulators of plant innate immunity (Campo *et al.*, 2013; Yang and Huang, 2014; Baldrich and San Segundo, 2016). In this study, we characterize a novel rice miRNA, miRNA7695, which targets an alternatively spliced transcript of the metal transporter Os*Nramp6* gene (*OsNramp6.8*). Accumulation of miR7695 in both *MIR7695* overexpressor and *MIR7695-Ac* mutant plants confers enhanced resistance to *M. oryzae,* which is accompanied by the down-regulation of iron homeostasis genes.

A large proportion of *MIR* genes are generated by inverted gene duplication events that give rise to new 'young' *MIR* genes. Transcription of young *miR* genes produces fold-back structures with few bulges that are processed by DCL4/DCL3, giving rise to miRNAs of variable length. During evolution, *MIR* genes accumulate mutations in their sequence which leads to a switch to DCL1 processing. These old 'ancient' *MIR* genes produce canonical miRNAs of 21nt (Vazquez *et al.*, 2008; Cuperus *et al.*, 2011). In this work, we show that processing of *MIR7695* precursor is largely dependent on DCL4. This finding, together with the long extensive base pairing within the *MIR7695* precursor stem region, supports the hypothesis that *MIR7695* is probably an evolutionarily recent *MIR* gene.

In the last years, an increasing amount of non-conserved miRNAs has been identified in plants. Although the biological role has not been described for most of them, many are related to stress-responses or cell-specificity (Leung and Sharp, 2010). As plant immune systems tend to evolve rapidly to counteract the rapidly evolving pathogens (Chisholm *et al.*, 2006), one might thus predict the emergence of new non-conserved *MIR* genes to control the expression of rapidly evolving protein-coding genes involved in plant immunity.

Here, we report that miR7695 negatively regulates an alternatively spliced transcript of the metal transporter Os*Nramp6* (Os01g31870.8) gene. The first evidence came from the observation that accumulation of miR7695 in overexpressor (Nipponbare) and activation mutant (*MIR7695-Ac*, Tainung

67) rice plants results in a reduction of *OsNramp6.8* transcripts (present work). Both Nipponbare and Tainung 67 are japonica varieties. This result is in agreement with observations in our group (Campo *et al.*, 2013), were they observed lower levels of accumulation of short *Nramp6* transcripts (*OsNramp6.8*) in *japonica* varieties compared to *indica* varieties, the former ones not accumulating miR7695 species. Most probably, miRNA functioning and processing occurs only in the *japonica* group. Altogether these results support the notion that miR7695 targets the short splice variant *OsNramp6.8.*

We show that accumulation of miR7695 contributes to rice blast resistance in both *MIR7695* overexpressor and *MIR7695-Ac* mutant lines. The first evidence of a plant miRNA playing a role in plant immunity came from studies in Arabidopsis. Navarro and co-workers (2006) showed that miR393 acts in bacterial resistance through repression of the auxin pathway. Only two examples of rice miRNA have been functionally characterized in rice immunity against the rice blast fungus: miR160a and miR398b, which target auxin response factors (ARFs) and superoxide dismutases (SOD2) transcripts, respectively (Li *et al.*, 2014). Overexpression of both miR160a and miR398b in *indica* rice confers resistance against *M. oryzae*, which is accompanied by the up-regulation of defense marker genes (i.e *OsPR1a*). In this work, we show that *MIR7695-Ac* plants show a stronger accumulation of *OsPR1a* transcripts at 48h and 72h of infection, suggesting that miR7695 might be acting as a positive regulator of post-invasive defense responses against *M. oryzae.* Consistent with the accumulation of miR7695 in transgenic rice, its target gene *OsNramp6.8* was down-regulated, suggesting that the enhanced resistance observed might be in part due to decreased *OsNramp6.8* levels.

On the other hand, NRAMP proteins are divalent-metals transporters playing a role in maintenance of metal homeostasis in a wide range of organisms, including plants (Cellier *et al.*, 1995). A relationship between *Nramp* gene expression and plant immunity has been described in Arabidopsis. Here AtNRAMP3 and AtNRAMP4 participate in iron mobilization and are involved in resistance against the bacterial pathogen *Erwinia chrysanthemi* (Lanquar *et al.*, 2005; Segond *et al.*, 2009). In rice, *OsNramp1, OsNramp2* and *OsNramp3* transcripts are altered after infection with the bacterial and fungal pathogens, *Burkholderia glumae* and *M. oryzae* (Zhou and Yang, 2004). OsNRAMP1 and

OsNRAMP3 transport iron and manganese, respectively (Curie *et al.*, 2000; Takahashi *et al.*, 2011; Yamaji *et al.*, 2013; Yang *et al.*, 2013). Therefore, an intricate connection between metal homeostasis and plant immunity appears to exist in Arabidopsis and rice plants. This connection has long been documented in humans and other vertebrates (Hood and Skaar, 2012), were the term “Nutritional immunity” was coined to refer to host-mediated restriction of Fe availability (Weinberg, 1975).

In this work, transcriptomic analysis of *MIR7695-Ac* rice plants allowed us to identify a set of iron homeostasis-related genes, all of them being down-regulated in *MIR7695-Ac* plants in response to *M. oryzae* infection. They included genes related to the iron uptake Strategy I (*OsFRO2* and *OsIRT2*) (Connolly *et al.*, 2003; Ishimaru *et al.*, 2006) and Strategy II (*OsNAS1* and OsNAS2) (Inoue *et al.*, 2003; Nozoye *et al.*, 2014), Fe-regulated transcription factors (*OsIRO2* and *OsIRNLPl)* (Ogo *et al.*, 2006, 2007, 2011), Fe transport and mobilization (*OsNRAMP1*) (Curie *et al.*, 2000; Takahashi *et al.*, 2011) two ubiquitin ligase (*OsHRZ1* and *OsHRZ2*) (Kobayashi *et al.*, 2013; Kobayashi and Nishizawa, 2015), an oligopeptide transporter (*OsIROPT)* (*Zheng et al., 2009*) and a nicotianamine efflux transporter *(OsENA1)* (Nozoye *et al.*, 2011). These findings further support the existence of links between iron homeostasis and immunity, a feature that has been previously observed in other graminaceous and Arabidopsis plants (Liu *et al.*, 2006; Kieu *et al.*, 2012; Ye *et al.*, 2014).

An important number of iron homeostasis genes were found to be down-regulated in response to blast infection in *MIR7695-Ac* plants, these genes being induced under iron deficiency (Ogo *et al.*, 2006; Zheng *et al.*, 2009; Bashir *et al.*, 2014; Kobayashi *et al.*, 2014). The iron-deficiency response comprises a set of induced processes that facilitate iron uptake and mobilization to ensure a proper iron homeostasis (Kobayashi and Nishizawa, 2012). As an strategy to arrest pathogen infection, plants might activate mechanisms to withhold iron (and other essential metals) from invading pathogens. Alternatively, the plant may expose the pathogen to an excess of iron at the site of pathogen attack, acting as a toxin or as inducer of ROS production. All these strategies rely on transcriptional reprogramming of gene expression for iron mobilization.

According to Liu and co-workers (2007), fungal attack in wheat stimulates Fe^{3+} accumulation into apoplast, which mediates an oxidative burst causing Fe depletion in the cytosol and down-regulation of the wheat *TmNAS1* gene. Iron reduction together with H_2O_2 induce the expression of defense-related genes, including *PR1* (Liu *et al.*, 2006). This observation is in agreement with results obtained in the present work, were down-regulation of iron-homeostasis genes is accompanied by the up-regulation of the *OsPR1a* gene in infected *MIR7695-Ac* plants. It will be of interest to determine if Fe and ROS content is altered in *MIR7695*-Ac plants compared to wild-type plants. Equally, the expression of other rice defense genes (e.g. rice PRs) needs to be investigated in *M. oryzae*-infected *MIR7695*-Ac plants. Of interest, *OsIRT2* and *OsNAS1*, both related to iron uptake, were also down-regulated in *MIR7695-Ac* in control conditions (non-infected), suggesting that iron content might be altered in these plants previous to fungal infection. Then, it is reasonable to speculate that *MIR7695-Ac* plants are preconditioned for a fast immune response through iron homeostasis modulation.

Currently, miRNA398b (targeting SOD genes) provides a good example of post-transcriptional regulation linking both plant defense and nutrient homeostasis responses. The involvemenet of miR398b in pathogen immunity and copper homeostasis has been described in rice, Arabidopsis and common bean (Li *et al.*, 2010, 2014; Naya *et al.*, 2014) However, resistance phenotypes observed in miR398 overexpressor plants are more related to ROS accumulation rather than Cu homeostasis responses (Li *et al.*, 2014).

In addition to miR7695, *MIR7695-Ac* plants also accumulate *OsWak3* and *OsWak4* transcripts, which genes are located close to the T-DNA insertion site. We also show that infection with *M. oryzae* is accompanied by dynamic alterations of *OsWak3* and *OsWak4* expression in wild-type rice plants. In other studies, *OsWAK* genes were reported to be involved in immunity to the rice blast fungus *M. oryzae* (Li *et al.*, 2009; Delteil *et al.*, 2016). In particular, OsWAK1, OsWAK14, OsWAK91 and OsWAK92 act as positive regulators of blast resistance, whereas OsWAK112d is a negative regulator (Delteil *et al.*, 2016). Therefore, a possible contribution of both WAK genes in the resistance phenotype observed in *MIR7695*-Ac plants should not be

ruled out. Currently, no information is available on the involvement of *WAK* genes in iron homeostasis in rice. Only in Arabidopsis, a WAK-like protein, AtWAKL4, has been shown to participate in mineral nutrition responses (Hou, 2005). Future studies are needed to gain further insights into the molecular mechanisms in which the miR7695/Nramp6 (short isoform) pair function for maintenance of metal homeostasis in the context of rice immunity.

To conclude, results presented here support that miR7695, and its corresponding target gene, can be considered an integral part of the rice response to *M. oryzae* infection. Since *M. oryzae* is one of the primary causes of rice losses worldwide, unraveling the miR7695-mediated mechanisms underlying resistance to blast has the potential to aid in the development of novel strategies to improve resistance to blast. Also, taking into account that rice has been adopted as the model species in cereal genomics, efforts to identify gene regulation networks that integrate miRNA functioning and alternative splicing events of target genes will improve our understanding of the molecular mechanisms involved in adaptation to pathogen infection in cereal species of agricultural importance.

MATERIALS AND METHODS

Rice transformation

For rice transformation, the *pC1300::ubi1::MIR7695::nos* was transferred to the *A. tumefaciens* EAH105 strain. The parent pCAMBIA 1300 vector contains the *hptII* (*hygromycin phosphotransferase*) gene encoding hygromycin resistance in the T-DNA region as the selectable marker. Transgenic rice lines expressing the *osa-MIR7695* precursor were produced by *Agrobacterium*-mediated transformation of embryogenic calli derived from mature embryos as previous described (Sallaud *et al.*, 2003). Transgene expression was confirmed by Northern blot analysis of primary T0 transformants. The stability of transgene expression was monitored in the selected transgenic lines at the T1 and T2 generations.

DNA analyses

For rice mutant genotyping, genomic DNA (100ng/PCR reaction) was extracted according to the method of (Murray and Thompson, 1980) but using mixed alkyltri-methylammoniumbromide (MATAB) as the extraction buffer (0.1M Tris–HCl pH 8.0, 1.4M NaCl, 20 mMEDTA, 2% MATAB, 1% PEG 6000 and 0.5% sodium sulphite). PCR genotyping was done using a set of gene-specific primers (*PRE7695-For* and *PRE7695-Rev*) and T-DNA-specific primers (RB) (Table CI.2).

RNA analyses

Total RNA was extracted from plant tissues using the TRizol reagent (Invitrogen™). For northern blot analysis of rice miRNAs, RNAs were fractionated in a 17.5% denaturing polyacrylamide gel containing 8 M urea, transferred to nylon membranes and probed with γ-^{32}P-ATP end-labeled oligonucleotide (miR7695.3-3p: 5'-GGTCTTGCCTACGTGGCACATCACGTGGA-3'). Blots were pre-hybridized and hybridized in Perfect-Hyb Plus buffer (Sigma). Hybridization signals were detected using STORM Phosphorimager (GE Helthcare).

Gene expression anlaysis was done by quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis. The first complementary

DNA was synthesized from DNase-treated total RNA (1ug) with high Capacity cDNA Reverse Transcription (Life technology, Applied Biosystems) according to the manufacturer's instruction. Amplification was done with two microliters of cDNA (20ng/µl) in optical 96-well plates (Roche Light Cycler® 480; Roche Diagnostics, Mannheim, Germany) using SYBR Green I dye and gene-specific primers (Table CI. 2). Data were normalized with *OsUbi1* (Os06g46770) and *OsCyc* (Os02g02890) as internal controls.

Blast Resistance Assays

The fungus *M. oryzae* (strain Guy-11, courtesy of Ane Sema) was grown in CMA medium (9 cm plates, containing 30 mg/L chloramfenicol) for two weeks at 28ºC under a 16h/8h light/dark photoperiod condition. *M. oryzae* spores were prepared as previously described (Campo et al., 2013). Infections were carried out in soil-grown plants (3-4 leaf stage) by two different methods, i) drop inoculation on detached leaves (Coca *et al.*, 2004) and ii) whole-plant spray inoculation assays (Valent *et al.*, 1991; Sesma and Osbourn, 2004). Briefly, the drop inoculation method consists in placing the second detached leaves into plate dishes with 1% (w/v) water agar containing kinetine at 2 mg/l. Whatman filter paper discs saturated with a *M. oryzae* spore suspension (10^4 - 10^6 spores/ml) were placed onto the upper face of the leaf for 60 h and then removed. The spray inoculation method consists in spraying whole rice plants with a *M. oryzae* spore suspension (10^5 spores/ml; 0.2ml/plant) using an aerograph at 2 atmospheres of pressure. Plants were maintained overnight in the dark under high humidity conditions. The percentage of leaf area affected by blast lesions was determined at 4 (drop-inoculated leaves) or 7 days (spray-inoculated leaves) post-inoculation with *M. oryzae* spores using the APS Assess 2.0 programme.

RNA-Seq Library samples Preparation and Sequencing

Total RNA was extracted from rice leaves tissues that had been treated or not with a *M. oryzae* spores solution following the whole plant infection method (10^5 spores/ml, 0.2ml/plant) at 48h, using Maxwell® 16 LEV Plant RNA Kit (Promega), according manufacture's instructions. Raw reads were checked for quality with FastQC v0.11.3 (www.bioinformatics.babraham.ac.uk/

projects/fastqc/) and then trimming and removal of adapters were performed with Trimmomatic v0.33 (Bolger *et al.*, 2014) (minimum quality score 35, minimum length 25). The obtained reads were then mapped against the *Oryza sativa* reference genome (MSU 7.0) with STAR (v2.4.0j) (Dobin *et al.*, 2013) providing the reference gene annotation file with known transcripts (RGSP 7.0). Alignment files were filtered to remove reads with MAPQ <30. FeatureCounts (Liao *et al.*, 2014) (v1.4.5-p1) was used to perform read summarization at gene level, with the strand-specific option “reversely stranded”. Statistical analysis of the read counts was performed with R, using the HTSFilter (Rau *et al.*, 2013) package to remove lowly expressed genes and the edge R package (Robinson *et al.*, 2009) to perform differential expression analysis. To identify genes with significant expression differences, a cut-of of FDR < 0.05 and |log2FC| ≥ 1 was applied. Gene Ontology (GO) enrichment analysis of the differentially expressed genes was performed with the AgriGO webtool ($p<0.05$, Fisher’s test) (http://bioinfo.cau.edu.cn/agriGO/) (Du *et al.*, 2010). Enriched GO terms were grouped and summarized, as well as 2D-plotted by semantic clustering using the online analysis tool ReviGO (http://revigo.irb.hr/) (Supek *et al.*, 2011).

Table CI.2. Primer sequence used for genotyping and gene expression analysis.

Gene name	Gene Locus	Primer sequences	
PRE7695 (PCR)		For	5'-ATTCCCAAACAAGTTACCGTGAC-'3
		Rev	5'-GATAGTGACTACTGTACCAACAT-'3
RB		For	5'-ACTCATGGCGATCTCTTACC-'3
OsNramp6.8 (s-NRAMP6)	Os01g31870.8	For	5'-TCTCCGTCCGACATCCGTA-'3
		Rev	5'-TCTCCGTCCGACATCCGTA-'3
OsUbiquitin1	Os06g46770	For	5'-TTCCCCAATGGAGCTATGGTTT-'3
		Rev	5'-AAACGGGACACGACCAAGG-'3
OsCycclophilin2	Os02g02890	For	5'-GTGGTGTTAGTCTTTTTATGAGTTCGT-3'
		Rev	5'- ACCAAACCATGGGCGATCT-3'
pre-miR7695 (RT-qPCR)		For	5'-GCGCACATCGAGGTCTCTAAA-'3
		Rev	5'-CTTTGGACCGGGATGATACAA-'3
OsWaK3	Os01g20880	For	5'-CGCTCTTCCCCTTGTCTTCTT-'3
		Rev	5'-GCATAGGGTTGTCGGAGGC-'3
OsWaK4	Os01g20900	For	5'-TCCAGTGTTTAGAGGGCTTGGTT-'3
		Rev	5'-CGCGATTCAGGATTTT CAGC-'3
OsFRO2	Os04g48930	For	5'-CGTCCCGATCTCAGAAGTACG-'3
		Rev	5'-CAAATTTCGCATTTCACGCA-'3
OsIRT2	Os03g46454	For	5'-CATGTCCGTCATGGCCAAG-'3
		Rev	5'-TCAACACATACGGTGTCACCTTC-'3
OsNAS1	Os03g19420	For	5'-CTCGCTGTCCGTGACATCG-'3
		Rev	5'-CGCCTAGCATCATCCACACA-'3
OsNAS2	Os03g19427	For	5'-GCTGTCCGTCTAACAGCCG-'3
		Rev	5'-TTAGCTTCGCAAATGCAGCA-'3
OsIRO2	Os01g72370	For	5'-GGGCTTTCTGCGTGTTATGAG-'3
		Rev	5'-CATGCTATGATGCGGTCCAT-'3
OsIRNLP1	Os12g18410	For	5'-ATCGGATTTGGATTCTGGAGG-'3
		Rev	5'-ACGTTGGCACACTCCCAATT-'3
OsNramp1	Os07g15460	For	5'-GAGGTTGTGGTCGCGCTATT-'3
		Rev	5'-TGCTCATCTCCACGAAGAAGC-'3

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CHAPTER II

The *OsNramp6* gene, an iron and manganese transporter from rice that functions in disease resistance.

ABSTRACT

Metal ions are life elements for all living cells. However, metals can be toxic when present in excess. In plants, metal homeostasis is partly achieved through the function of metal transporters, including the diverse Natural Resistance-Associated Macrophage Proteins (NRAMPs). We investigated the metal transport activity and biological role of NRAMP6, a previously uncharacterized member of the rice NRAMP family. Alternative splicing of the *OsNramp6* gene produces transcript variants encoding different NRAMP6 proteins. In this work, we determined the function of the full-length and the shortest NRAMP6 proteins (l-NRAMP6 and s-NRAMP6, respectively). Transient expression of OsNramp6-GFP fusion genes in *Nicotiana benthamiana* leaves showed that both OsNRAMP6 proteins localize to the plasma membrane. Functional complementation of yeast mutants revealed that l-NRAMP6 and s-NRAMP6 are iron and manganese transporters, with no relevant cadmium or arsenic transport activity. When grown under sufficient metal supply, loss of OsNramp6 function results in biomass reduction, thus, supporting a role of OsNramp6 in plant growth. Interestingly, the nramp6 mutant displayed enhanced resistance to the fungal pathogen *Magnaporthe oryzae*, indicating that OsNramp6 participates in the rice immune response. A better understanding of OsNramp6-mediated mechanisms underlying disease resistance in rice will help in developing appropriate strategies for crop protection.

INTRODUCTION

Mineral nutrients are essential for normal plant growth and development. Some metals are required in trace amounts as essential micronutrients, such as iron (Fe), copper (Cu), manganese (Mn) and Zinc (Zn) that serve structural roles in proteins or function as enzyme cofactors. They are also components of cellular redox reactions (Hall and Williams, 2003). However, if present in excess in a bioavailable form, these essential micronutrients can be harmful to cells through the generation of toxic reactive oxygen species (ROS) or by replacement of other metal ions from metalloproteins rendering these proteins non-functional. On the other hand, if plants do not have sufficient supply of these metals, then deficiency symptoms develop (e.g. chlorosis, necrotic spots, loss of leaves, or stunted growth). Therefore, plants have developed finely tuned homeostatic mechanisms to ensure the appropriate concentrations of essential metals at the cellular and the whole-plant levels.

Diverse protein families are known to be components of the metal homeostatic network in plants, such as the zinc and iron ZIP (Zrt- and Irt-related protein) transporters, the NRAMP (Natural Resistance-Associated Macrophage Protein) family of divalent metal transporters, the COPT (COPper Transporter) copper transporters, the copper-transporting P-type ATPases, the ferric-chelate reductases, and the YSL (*Yellow Stripe-Like*) transporters (Thomine *et al*., 2000; Guerinot, 2000; Curie and Briat, 2003; Puig and Peñarrubia, 2009; Williams and Pittman 2010; Kobayashi and Nishizawa 2012; Victoria *et al.,* 2012; Thomine and Vert 2013). Transporters localized at the plant cell plasma membrane control metal ion uptake and release, whereas those localized in endogenous subcellular compartments are mainly responsible for sequestration and remobilization of metal ions in the cell. Metal transporters are also involved in detoxification of heavy metals such as cadmium (Cd) or arsenic (As) (Thomine *et al.*, 2000; Cailliatte *et al.*, 2009; Tiwari *et al.*, 2014). The NRAMP proteins were first identified in rat macrophages (Vidal *et al*., 1993) and nowadays they are recognized as a ubiquitous family of metal transporters present in fungi, animals, plants and bacteria (Cellier *et al.*, 1995; Cellier *et al.*, 1996; Nelson, 1999). NRAMPs are integral, membrane-spanning proteins usually encoded by large gene

families in plants, indicating a possible functional specialization. Moreover, plant NRAMP proteins complement yeast mutants deficient in metal uptake (e.g. Fe or Mn transport), demonstrating their conserved function as metal transporters across all kingdoms (Curie *et al.,* 2000; Thomine *et al.,* 2000; Gross et *al.,* 2003). Members of the NRAMP family are capable of transporting divalent metal cations (Fe^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, As^{2+}) into the cytoplasm (Nevo and Nelson, 2006), with the exception of the rice Nrat1 (OsNRAMP4) protein which tranports the trivalent Aluminium ion (Al^{3+}) (Li *et al.,* 2014), although they differ in their specificity. Whereas Fe, Mn and Zn are essential for metabolism, Cd, As and Al are toxic for virtually all living organisms. Moreover, NRAMP proteins localize at different subcellular compartments, including the plasma membrane, tonoplast, vesicles or the plastid envelope (Thomine *et al.,* 2003; Lanquar *et al.,* 2005; Xiao *et al.,* 2008; Cailliatte *et al.,* 2009,2010; Takahashi *et al.,* 2011; Sasaki *et al.,* 2012; Yang *et al.,* 2013).

The biological function of distinct NRAMPs in controlling plant growth and development has been elucidated, mostly in the model plant *Arabidopsis thaliana.* For instance, AtNRAMP3 and AtNRAMP4 contribute to iron nutrition in germinating seeds by remobilizing vacuolar iron stores (Curie *et al.,* 2000; Thomine *et al.,* 2000, 2003; Lanquar *et al.,* 2005), to the export of vacuolar Mn in photosynthetic tissues of adult plants (Lanquar *et al.,* 2010) and to Cd sensitivity in Arabidopsis plants (Thomine *et al.,* 2000). AtNRAMP1 appears to be essential for Arabidopsis growth in low Mn conditions (Cailliatte *et al.,* 2010) whereas AtNRAMP6 functions as an intracellular Cd transporter that increases Cd sensitivity when overexpressed in Arabidopsis (Cailliatte *et al.,* 2009). In addition to their important roles during plant growth, certain NRAMP proteins appear to mediate plant-microbe interactions. For instance, the Arabidopsis AtNRAMP3 and, to a lesser extent, AtNRAMP4 have been reported to be involved in resistance against the bacterial pathogen *Erwinia chrysanthemi* (Segond *et al.,* 2009). At present, the exact mechanisms by which NRAMP proteins and metal homeostasis control plant immunity remain unclear.

Rice is one of the most important cereal crops in the world and the main staple food crop for more than 50% of the world's population. In spite of the relative wealth of information about NRAMPs in Arabidopsis, much less

is known about the exact metal transport capabilities and biological role of NRAMP proteins in rice (*O. sativa* L). The rice genome contains eight genes encoding NRAMP proteins (*OsNramp1* to *OsNramp8*) (Belouchi *et al.,* 1997; Gross *et al.,* 2003). Among them, OsNRAMP1 was reported to transport Fe, Cd and As but not Mn (Curie *et al.,* 2000; Takahashi *et al.,* 2011; Tiwari *et al.,* 2014), OsNRAMP3 functions in Mn transport (Yang *et al.*, 2013) and OsNRAMP5 is a Mn, Fe and Cd transporter (Ishimaru *et al.,* 2012; Sasaki et *al.,* 2012). The rice OsNRAMP4 (also known as Nrat1, Nramp aluminium transporter 1) was identified as a transporter for aluminium that does not show transport activity for divalent cations in yeast (Xia *et al.,* 2010)

In this study, we investigated the metal transport activity and biological function of a previously uncharacterized member of the rice NRAMP family, OsNRAMP6, in the context of disease resistance. Rice yields are severely compromised by the fungal pathogen *Magnaporthe oryzae*, the causal agent of the rice blast disease (Wilson and Talbot, 2009), considered the most important plant fungal disease (Dean et *al.,* 2012). Owing to the scientific and economic relevance of the rice/*M. oryzae* pathosystem, we approached the functional characterization of OsNRAMP6 within the context of the rice/*M. oryzae* interaction.

The *OsNramp6* gene (Os01g31870) has a complex transcriptional pattern producing up to eight transcript variants by alternative splicing, generating OsNRAMP6 proteins of different size (Campo *et al*., 2013). We show that both the full-length and the shortest OsNRAMP6 protein localize at the plasma membrane. Functional complementation of yeast mutants revealed that the two NRAMP6 protein isoforms function as Fe and Mn transporters. We also provide evidence that loss-of-function of *OsNramp6* confers enhanced resistance to infection by the rice blast fungus *M. oryzae*.

RESULTS

The primary *OsNRAMP6* transcript (Os01g31870.1) is known to produce up to 8 transcript isoforms, of which the shortest transcript variant (Os01g31870.8) is generated by miss-splicing of intron 6 (Figure CII.1A). Quantitative RT-PCR using transcript-specific primers revealed co-existence of the full-length and short *NRAMP6* transcripts (hereinafter referred to as *l-Nramp6* for *long-Nramp6*, and *s-Nramp6* for *short-Nramp6*) in shoots of rice (*O. sativa* cv Nipponbare) plants at different developmental stages (Figure CII.1B). The *l-Nramp6* transcripts accumulated at much higher levels than *s-Nramp6* transcripts.

The protein encoded by the full *OsNramp6* transcript is 550 amino acids long while that encoded by the short *OsNramp6* transcript is 210 amino acids in length (corresponding to amino acids 1–203 of the full-length protein plus a C-terminal extension of 7 amino acids) (Figure CII. 1A and C). SOSUI transmembrane (TM) domain searches (Hirokawa *et al.,* 1998) predicted nine TM domains in l-NRAMP6 (Figure CII.1C). In the case of s-NRAMP6, only 3 TM domains are predicted (Figure CII.1C), as it is also the case for the protein deduced from the *OsNramp8* cDNA sequence. By using the same prediction system for other rice NRAMPs, 10 (NRAMP2, NRAMP5) and 11 (NRAMP1, NRAMP3, NRAMP4, NRAMP7) TM domains are predicted (data not shown).

The amino acid sequence of NRAMP6 shares the highest sequence identity with OsNRAMP5 and OsNRAMP1 (51.3% and 48.7%, respectively), OsNRAMP4 (44.8%), OsNRAMP3 (42.0%), with lesser identity with OsNRAMP2 (28.5%) and OsNRAMP7 (26.6%) (Figure CII.2). The consensus transport motif (CTM; GQSSTITGTYAGQFIMGGFLN) which is common among plant NRAMP proteins is only partially conserved in l-NRAMP6, and it is absent in s-NRAMP6 (Figure CII.2).

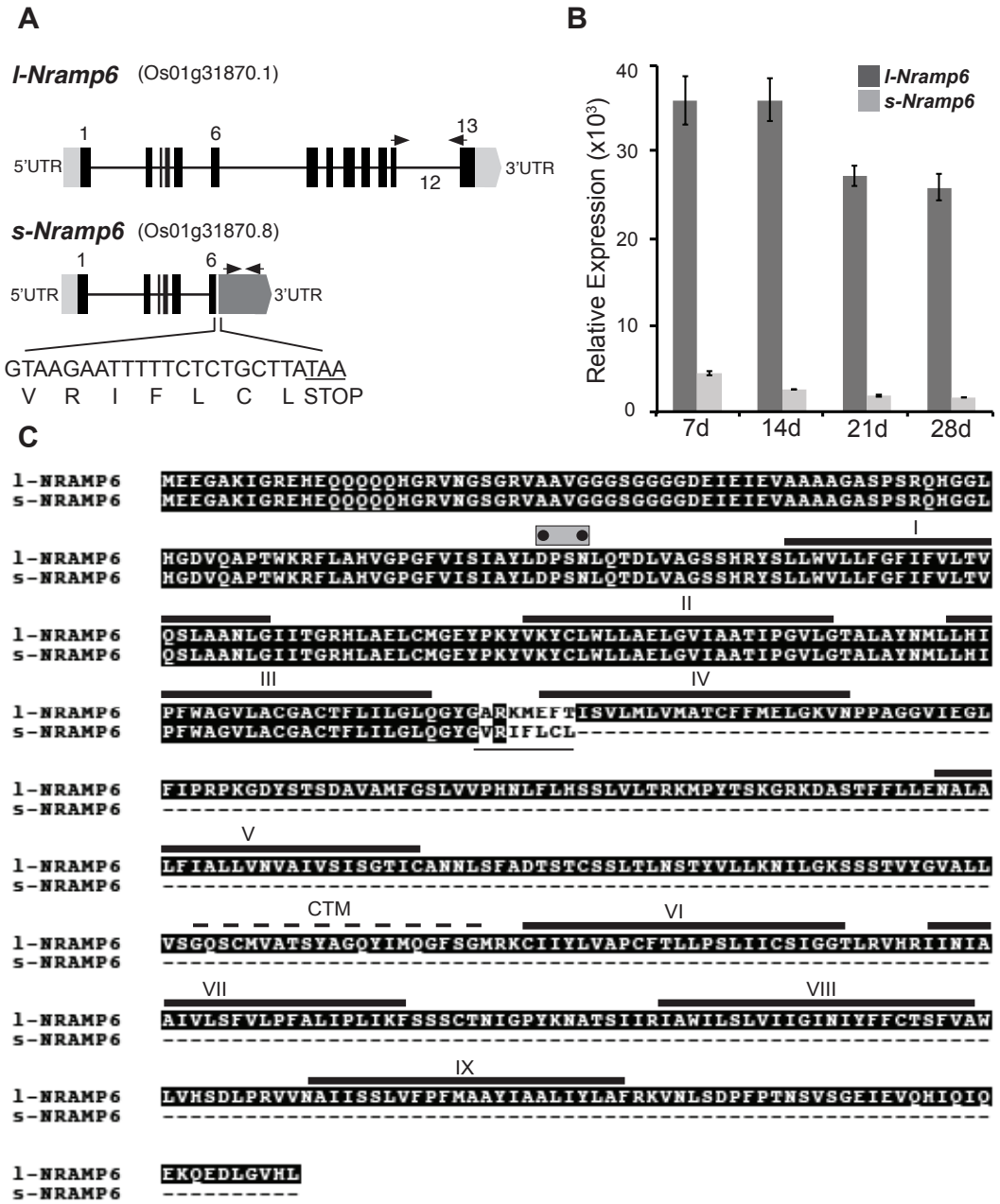

Figure CII.1. Gene structure, *OsNramp6* expression and amino acid sequences of OsNRAMP6. **(A)** Schematic representation of the *OsNramp6* gene. Black bars and lines represent exons and introns, respectively. Numbers indicate the first and last exon of each *l-Nramp6* and *s-Nramp6* transcript. The long *Nramp6* (*l-Nramp6*, Os01g31870.1) transcript encodes the full-length NRAMP6 protein and was taken as reference for intron/exon numbering. Miss-splicing of intron 6 produces a short Nramp6 (*s-Nramp6*, Os01g31870.8) transcript that introduces a stop codon 21 nucleotides downstream from the Exon6-Intron6 junction. In this way, *s-Nramp6* encodes a truncated OsNRAMP6 protein (amino acids 1–203) containing a C-terminal extension of 7 amino acids (showed in the figure). Arrows denote the position of primers used for expression analysis in B. Grey bars represent the 5' and 3' untranslated regions (UTR).

(B) Accumulation of the *l-Nramp6* and *s-Nramp6* transcripts in shoots of rice (*O. sativa* cv Nipponbare) plants at different developmental stages. RT-qPCR was carried out using the *OsUbiquitin1* (Os06g46770) gene as the internal control. Values represent the mean ± SE of three biological replicates.
(C) Alignment of l-NRAMP6 and s-NRAMP6 amino acid sequences. Transmembrane domains (I to IX) were predicted using the SOSUI program (Hirokawa *et al.,* 1998) and are indicated by thick solid lines above amino acid sequences. The 7 amino acid extension at the C-terminus of s-NRAMP6 (underlined) and the consensus transport sequence motif (CTM; dashed line) are shown. Dots indicate metal-coordinating residues within the metal binding site (solid grey bar; $D_{88}PSN_{91}$) based on the homology to the bacterial NRAMP ScaDMT transporter (these residues were reported to be involved in NRAMP metal binding and selectivity of ScaDMT) (Ehrnostorfer *et al.,* 2014; Pottier *et al.,* 2015).

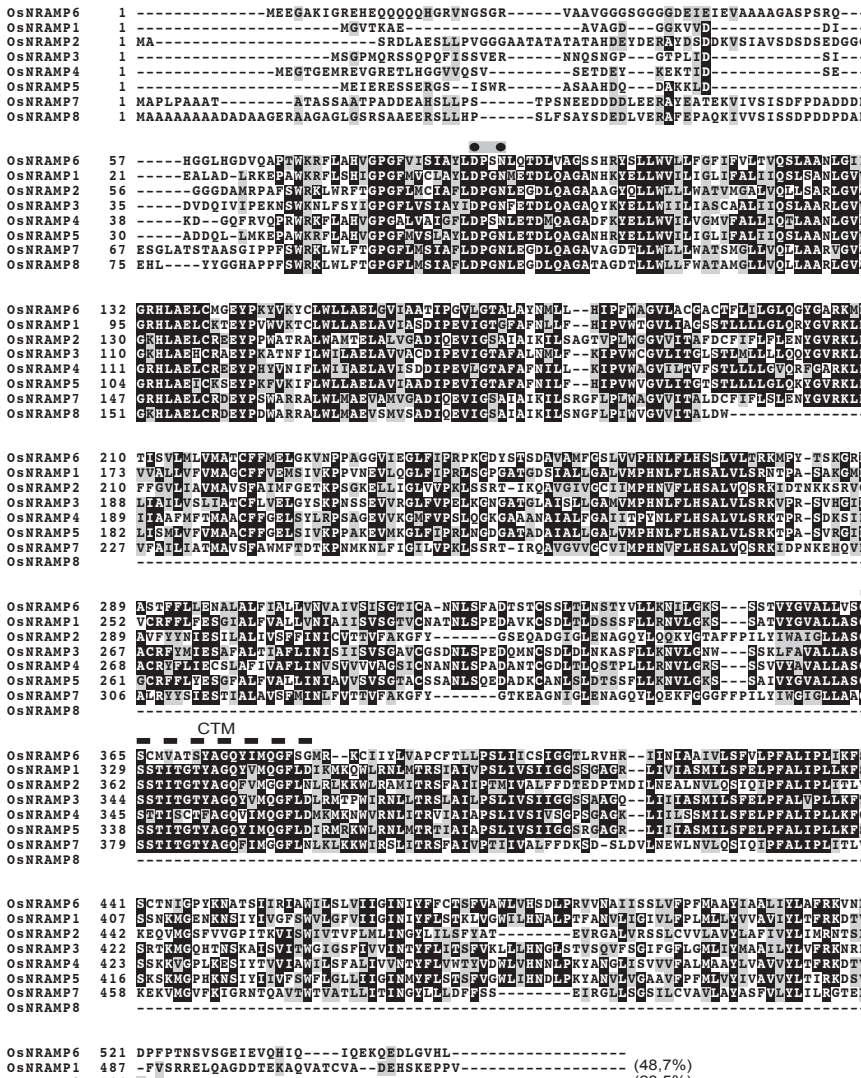

Figure CII.2. Alignment of the rice NRAMP family members.
The Clustal Omega and Boxshade programs were used for sequence alignment. The percentage of identity between OsNRAMP6 and other OsNRAMP proteins is shown in parenthesis. The consensus

transport motif (CTM) between the V and VI TM domains is indicated with a dashed line. Dots indicate metal-coordinating residues within the metal binding site (solid grey bar; $D_{88}PSN_{91}$) based on the homology to the bacterial NRAMP ScaDMT transporter (these residues were reported to be involved in NRAMP metal binding and selectivity of ScaDMT) (Ehrnostorfer *et al.,* 2014; Pottier *et al.,* 2015). The percentage of identity is indicated at the bottom right side.

Predicted tridimensional structure of OsNRAMP6

The crystal-structure of *Staphylococcus capitis* DMT (ScaDMT), a prokaryotic homolog of the NRAMP family also known as Solute Carrier 11 (SLC11), has been recently determined (Ehrnstorfer *et al.,* 2014). ScaDMT transports Mn^{2+}, Fe^{2+}, and Cd^{2+}. The ScaDMT structure defines the common architecture of the NRAMP family and contains 11 transmembrane helices forming two related halves interrupted by short loops, of which the first five α-helices are structurally related to the following five α-helices. The Asp (D) and Asn (N) residues at the metal binding-site are part of a conserved DPGN motif identified as a signature for the family (Figure CII.2). Mutations in metal binding residues have important effects on metal affinity and/or selectivity of this transporter (Ehrnstorfer *et al.,* 2014). The NRAMP6 protein shares 28.34% identity with the ScaDMT protein (Figure CII.3).

We have used the ScaDMT structure for homology-based modeling of the NRAMP6 protein. The tridimensional structure of OsNRAMP6 showed a high similarity to the ScaDMT structure (Figure CII.4A), the largest differences being observed in the N- and C-terminus. Residues contributing to ion binding in the ScaDMT are Asp49 and Asn52 (Asp88 and Asn91 in NRAMP6), as well as Ala223 and Met226 (Ser260 and Val263 in NRAMP6) (Figure CII.4B).

OsNRAMP6 is a plasma membrane protein

NRAMP proteins have been shown to localize in different subcellular compartments, including the plasma membrane, tonoplast, and vesicles. To investigate the subcellular localization of NRAMP6 in the plant cell, we transiently expressed an *OsNRAMP6-GFP* (green fluorescent protein) fusion gene in *Nicotiana benthamiana* leaf epidermal cell (Figure CII.5A). The subcellular localization of the l-NRAMP6 and s-NRAMP6 proteins was examined by CLSM.

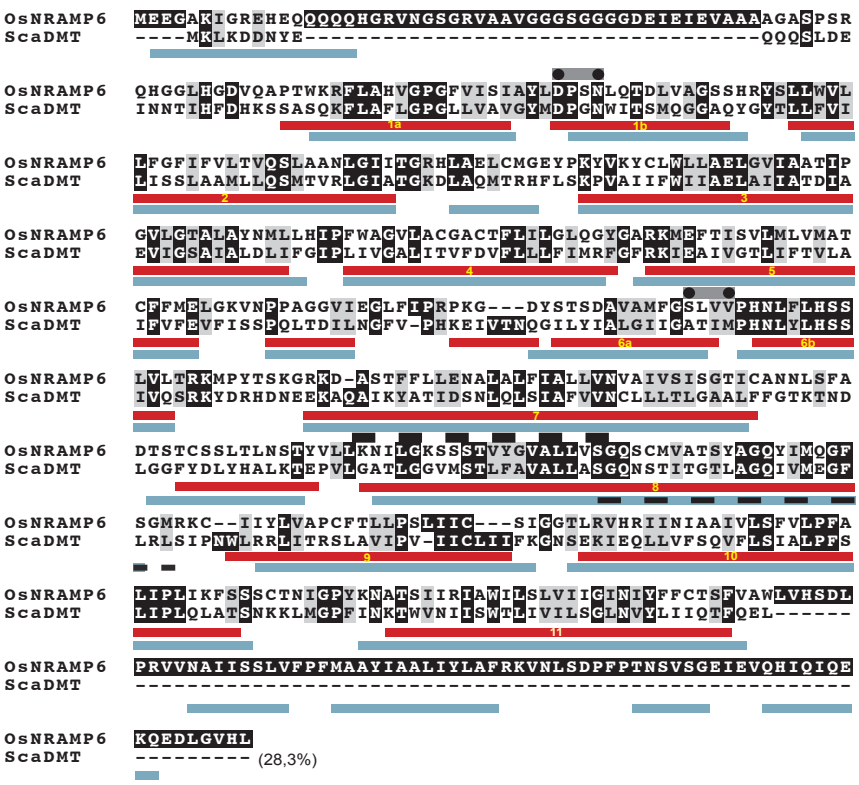

Figure CII.3. Alignment of the rice NRAMP6 and ScaDMT proteins.
Sequence alignment was carried out using the Clustal Omega and Boxshade programs. Residues involved in metal coordination are shown (D88 and N91 in NRAMP6, corresponding to D49 and N52 in ScaDMT; S260 and V263 in NRAMP6, corresponding to A223 and M226 in ScaDMT. TM domains in NRAMP6 and ScaDMT are indicated by red and blue bars, respectively. The percentage of identity is indicated.

Cells expressing the *l-NRAMP6-GFP* gene revealed a continuous labeling all along the cell periphery, likely the plasma membrane (Figure CII.5B). Discrete regions enriched in GFP fluorescence embedded in this subcellular compartment as well as vesicles exhibiting GFP fluorescence located near the cell periphery were occasionally observed (Figure CII.5B, arrows). A similar localization was observed in *N. benthamiana* cells expressing the *s-NRAMP6-GFP* gene (Figure CII.5C). However, in this case, the pattern of fluorescence was discontinuous along the cell periphery, with random distribution of discrete domains accumulating strong fluorescence (Figure CII.5C, right panel). *GFP*-expressing control cells showed a distribution of green fluorescence through the cytoplasm and the nucleus (Figure CII.5D).

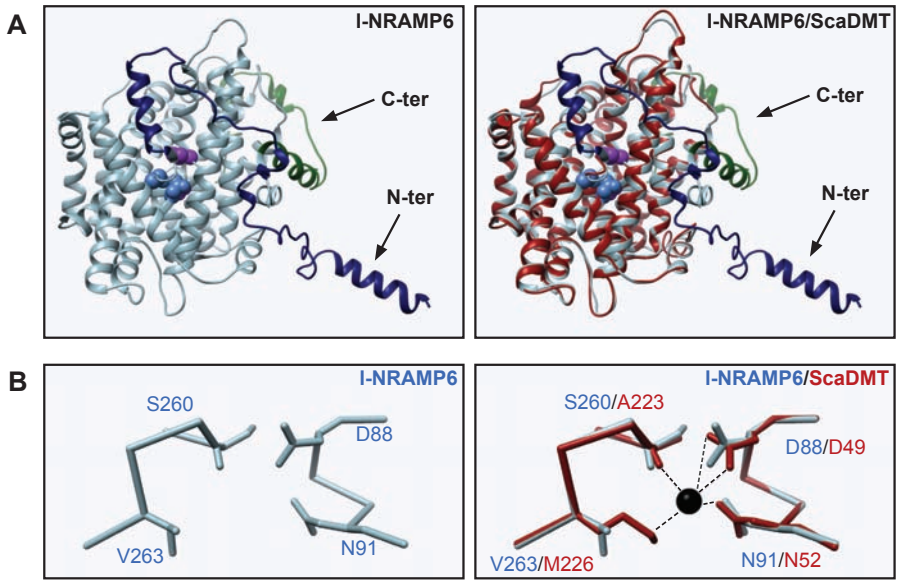

Figure CII.4. Molecular modeling of OsNRAMP6.
(A) Predicted three-dimensional structure of NRAMP6 (I-NRAMP6) by homology-based modeling. The model was generated by I- TASSER (Roy *et al.*, 2010) using the *Staphylococcus capitis* DMT (ScaDMT; Protein Data Back code 4WGW) structure as template (Ehrnstorfer *et al.,* 2014). Molecular graphics images were produced using the UCSF Chimera package (Pettersen *et al.,* 2004) (left panel). The NRAMP6 structure is colored in grey, with the N-terminal and C-terminal regions in blue and green, respectively. The structural superposition of NRAMP and ScaDMT (red) is shown on the right panel. Residues involved in metal coordination are shown as spheres: blue, D88 and N91 in NRAMP6 (corresponding to D49 and N52 in ScaDMT); purple, S260 and V263 in NRAMP6 (A223 and M226 in ScaDMT).
(B) Residues involved in metal coordination in NRAMP6 based on ScaDMT homology (left panel). Superposition of NRAMP6 and ScaDMT in the metal binding site. Interactions are indicated by dashed lines and the metal ion is shown as a black sphere.

To confirm the plasma membrane localization of the NRAMP6 protein, each *GFP*-tagged *NRAMP* gene was co-expressed with the plasma membrane marker *LTI6b (LOW-TEMPERATURE-INDUCED6b)* fused to the red fluorescent protein (*RFP-LTI6b*) (Kurup *et al.,* 2005). The continuous pattern of GFP fluorescence observed with the l-NRAMP6 perfectly co-localized with the RFP-tagged LTIB6b protein, thus confirming its plasma membrane localization (Figure CII.5E). The co-localization was uneven in cells expressing s-NRAMP6-GFP, indicating an irregular distribution in the plasma membrane (Figure CII.5F).

From these results, it is concluded that the two NRAMP6 protein isoforms, l-NRAMP6 and s-NRAMP6, localize at the plasma membrane. In addition, l-NRAMP6 also accumulated in vesicles in the vicinity of the plasma

membrane (not shown). Whether these vesicles represent an anterograde or retrograde trafficking of l-NRAMP6 remains to be determined. Most importantly, this study reveals that s-NRAMP6 has the molecular determinants that are required to target OsNRAMP6 to the plasma membrane.

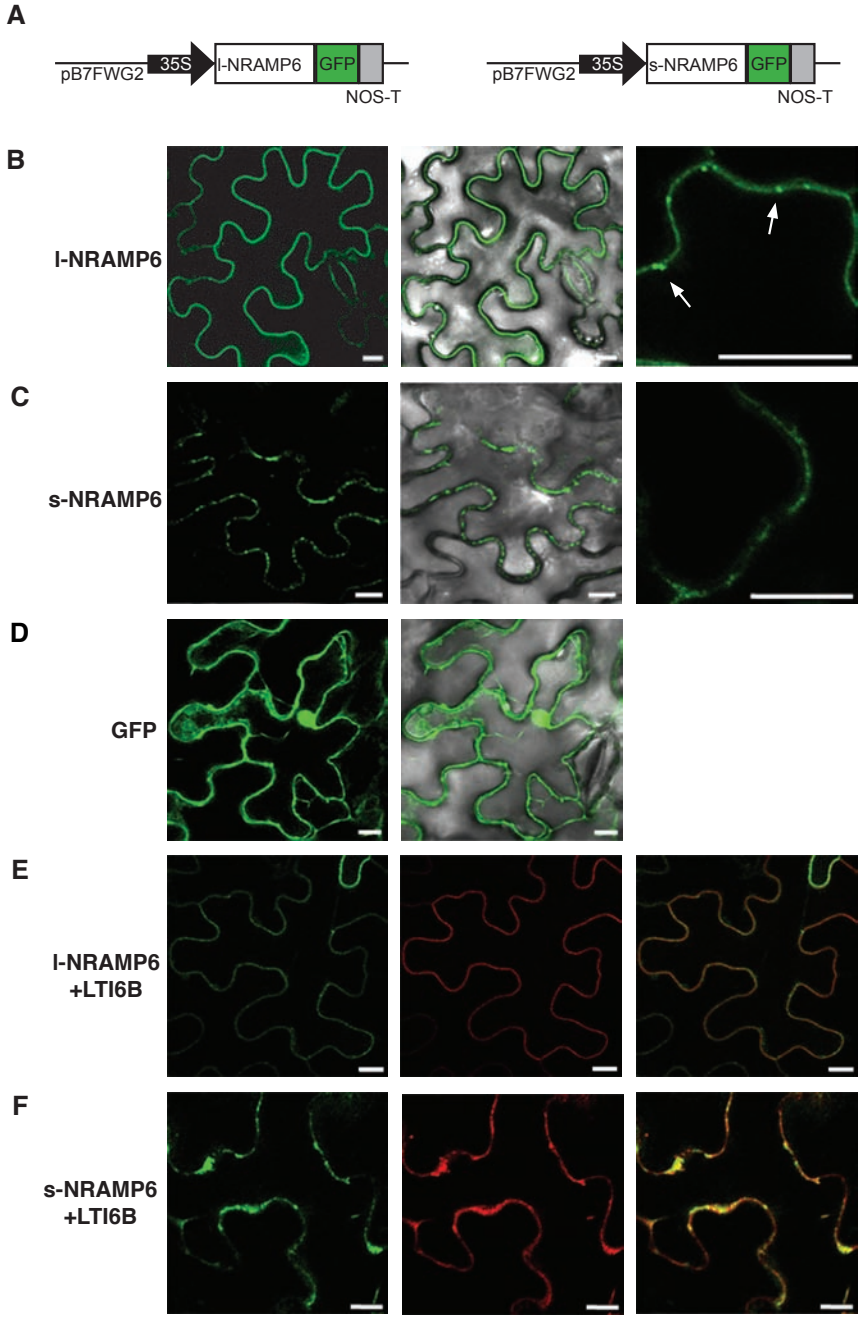

Figure CII.5. Plasma membrane localization of OsNRAMP6. Confocal fluorescence microscopy of *Nicotiana benthamiana* leaves transiently expressing *l-NRAMP6-GFP* or *s-NRAMP6-GFP* fusion genes.

(A) Schematic representation of the plasmids used for transient expression of *Nramp6-GFP* fusion genes. *OsNRAMP6* cDNAs were fused to the N-terminal of GFP and expressed under the control of the constitutive viral 35S promoter.
(B-C) Cells expressing *l-Nramp6-GF*P **(B)** or *s-Nramp6-GFP* **(C)**. From left to right: GFP channel; overlay of GFP and bright-field channel; magnification images. Arrows indicate discrete regions in the periphery and vesicles exhibiting strong GFP fluorescence.
(D) Cells expressing *GFP* .
(**E-F)** Cells co-expressing *l-Nramp6-GFP* **(E)** or the *s-Nramp6-GFP* **(F)** with the plasma membrane marker *RFP-LTI6b.* From left to right: GFP channel; RFP channel; overlay of GFP and RFP channel. Confocal images were taken at 52 h after agroinfiltration. Individual sections are shown. Scale Bars correspond to 10 µm.

OsNRAMP6 complements yeast mutants defective in Fe and Mn transport

Certain NRAMP proteins from higher plants have been shown to rescue the phenotype of yeast mutants impaired in metal transport activities (Curie *et al.,* 2000; Thomine *et al.*, 2000; Lanquar *et al.,* 2005; Cailliatte *et al.,* 2009; Xia *et al.,* 2010; Ishimaru *et al.,* 2012; Yang *et al.,* 2013; Yamaji *et al.,* 2013). We used this approach to investigate the metal transport activity of both l-NRAMP6 and s-NRAMP6. For expression in yeast, each cDNA was cloned under the control of the constitutive promoter *ADH1* into the pWS93 vector, which allows the production of hemaglutinin (HA) epitope-tagged proteins. Western blot analysis of protein extracts from wild-type cells were transformed with the HA-NRAMP constructs confirmed the accumulation of both l-NRAMP6 and s-NRAMP6 in the insoluble protein fraction, as expected for a plasma membrane localization (Figure CII.6A).

As a first step to investigate the metal transport activity of OsNRAMP6, the cDNA encoding either the long or the short NRAMP isoform was expressed in the *fet3 fet4* yeast mutant, which is defective in both high- and low-affinity iron uptake systems (Portnoy *et al.,* 2000). Due to reduced Fe uptake, this mutant requires addition of significant amounts of Fe to the medium for growth. We tested the ability of yeast transformants to grow on media supplemented with iron in a wide range of concentrations (5 to 100 µM Fe µM). For simplicity, only results obtained using 10 µM FeCl3 are presented (Figure CII.6B). As observed, expression of *l-OsNRAMP6* restored growth of the yeast *fet3 fet4* mutant on iron-limited medium, suggesting that l-NRAMP6 protein is an iron transporter. Interestingly, s-NRAMP6 also rescued growth of the *fet3 fet4* mutant at the same level (Figure CII.6B), supporting that the short NRAMP6 protein is a functional isoform.

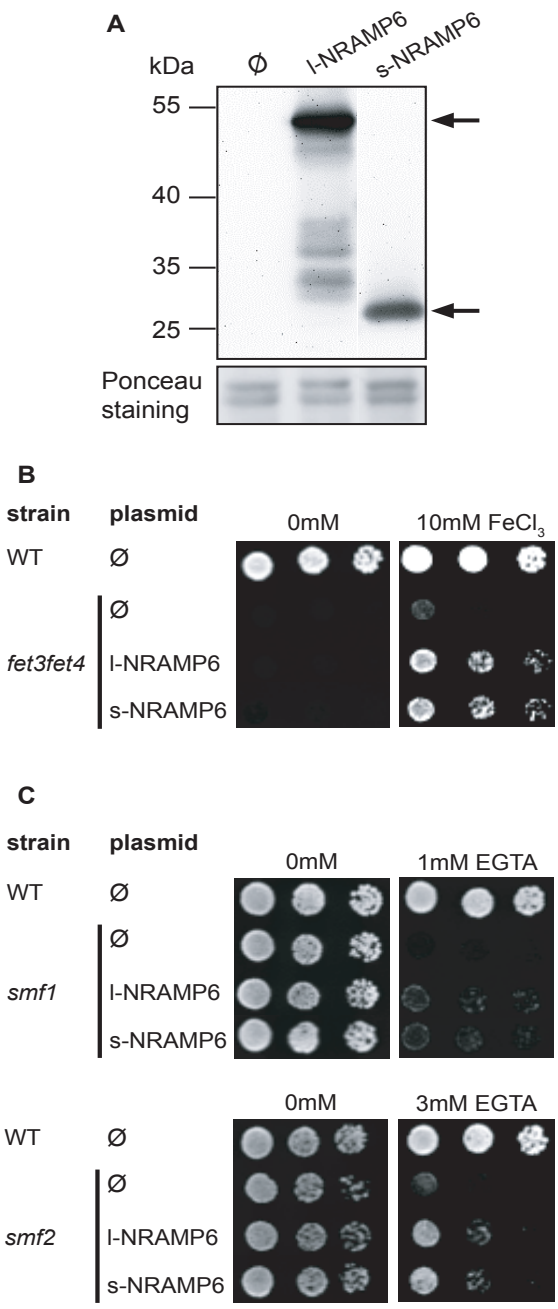

Figure CII.6. NRAMP6 functions in Fe and Mn transport in yeast.

(A) Production of NRAMP6 proteins in yeast. The wild type strain BY4741 was transformed with plasmid *pWS93/l-Nramp6* and *pWS93/s-Nramp6* which encoded HA-tagged l-NRAMP6 and s-NRAMP6 proteins, respectively, and grown on synthetic minimal medium (lacking uracil) until exponential phase. Cultures were taken, protein extracts prepared, and subjected to fractionation by centrifugation. Similar amounts of proteins from the insoluble fraction were subjected to 8% SDS-PAGE followed by immunoblotting using the anti-HA antibody. PageRuler pre-stained protein ladder (Thermo Fisher) was used. Arrows denote NRAMP6 proteins.

(B, C) Complementation assays in yeast mutant strains deficient in the uptake of iron (*fet3 fet4*) **(B)** or manganese transport (*smf1* and *smf2*) **(C)**. The wild type strain BY4741 (WT) and the yeast mutants transformed with pWS93 empty vectors were used as positive and negative controls, respectively.

(B) Complementation of *fet3fet4* was evaluated by spotting transformed yeast cells at an OD_{600} = 0.05, plus two 10-fold dilutions, on synthetic minimal medium (lacking uracil) agar plates without or with $FeCl_3$ supplementation. Growth was monitored after 3 days at 28°C.

(C) Complementation of the *smf1* and *smf2* deletions was scored on Low-YPD containing 50 mM MES (pH 6) agar plates supplemented with 1mM (*smf1*) or 3 mM (*smf2*) ethylene glycol tetra-acetic acid (EGTA). Growth was monitored after 3 days at 28°C.

Next, we investigated the ability of each NRAMP6 protein to transport Mn by functional complementation of the *smf1* and *smf2* yeast mutants. The SMFs proteins (Suppressor of Mitochondria import Function) are the yeast NRAMP homologues and act in manganese uptake. The growth of yeast *smf*

mutant strains and the isogenic wild-type, transformed with pWS93 vector (negative control), l-NRAMP6 or s-NRAMP6, was analyzed in Mn-limited medium, which was controlled by addition of increasing concentrations of the divalent cation chelator ethylene glycol tetraacetic acid (EGTA). Results obtained indicated that both l-NRAMP6 and s-NRAMP6 isoforms rescued, up to some extent, the growth of *smf1* and *smf2* yeast mutants under Mn-limited condition (Figure CII.6C).

Thus, yeast complementation studies indicated that the two protein isoforms, l-NRAMP6 and s-NRAMP6 are involved in Fe and Mn transport. Of interest, this study demonstrated that s-NRAMP6 is a functional protein that likely targets to the specific subcellular compartment in which l-NRAMP6 also localizes.

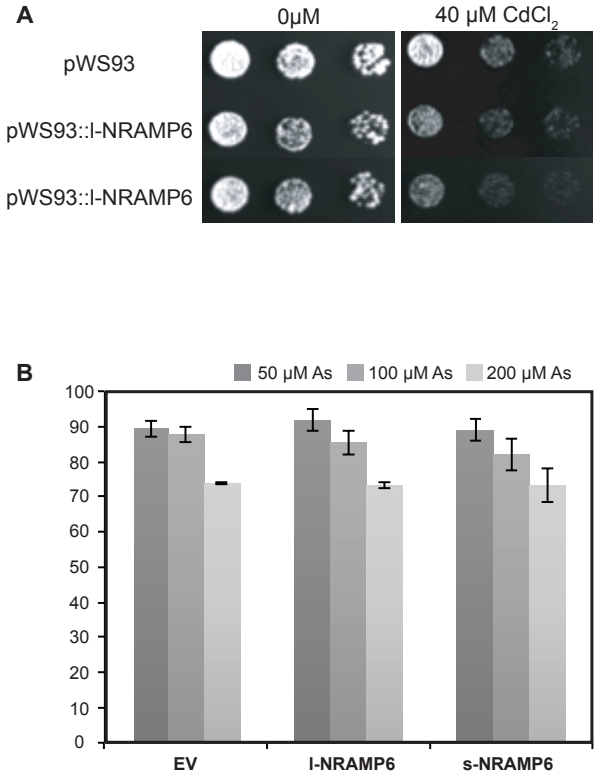

Figure CII.7. OsNRAMP6 does not contribute to Cd and As transport in yeast.
(A) Cultures of the wild type strain transformed with the empty plasmid (pWS93) or expressing *OsNramp6* were spotted at an OD_{600} = 0.05, plus two 10-fold dilutions, on synthetic minimal medium (lacking uracil) agar plates without (-$CdCl_2$) or with 40 µM $CdCl_2$. Growth was monitored after 3 days at 28°C.
(B) Yeast cells (OD_{660}= 0.006) were grown on YPD medium supplemented with Na_2HAsO_4 at the indicated final concentrations and the OD_{650} was determined after 20 hours at 28°C. The figure represents the % of growth at each As concentration with respect to the samples without As supplementation. Data are mean ± SD from three experiments.

Cadmiun (Cd) and arsenic (As) transport activities have been described for several NRAMP proteins (Thomine *et al.,* 2000; Cailliatte *et*

al., 2009; Takahashi *et al.,* 2011; Ishimaru *et al.,* 2012; Sasaki *et al.,* 2012; Tiwari *et al.,* 2014) Here, we tested the sensitivity to Cd and As in wild-type yeast cells expressing either *l-NRAMP6* or *s-NRAMP6*. No significant difference in toxicity was observed in synthetic medium supplemented with Cd or As (Figure CII.7), indicating that OsNRAMP6 does not mediate Cd or As transport in yeast.

Collectively, these results indicate that no significant differences in metal transport specificity and/or activity exist between l-NRAMP6 and s-NRAMP6 expressed in yeast.

Phylogenetic relationships between members of the rice and Arabidopsis NRAMP family

Knowing that OsNRAMP6 functions as a Fe and Mn transporter, we searched for similarities/differences in metal transport activity of NRAMP proteins in the context of their phylogenetic relationship. For this, a phylogenetic three was constructed using rice and Arabidopsis NRAMPs protein sequences. The Arabidopsis ETHYLENE INSENSITIVE2 (EIN2) protein shares sequence identity at its N terminus with NRAMP proteins (Alonso *et al.,* 1999; Jun *et al.,* 2004) and was included as an outroot.

Collectively, NRAMPs from rice and Arabidopsis form two distinct phylogenetic groups (Figure CII.8), which is consistent with results previously reported (Gross *et al.,* 2003; Victoria *et al.,* 2012). When examining the metal transport activity of Arabidopsis and rice NRAMPs, we noticed that closely related NRAMP proteins might have different activities. For instance, the rice OsNRAMP1 and OsNRAMP5 function as iron and Cd transporters, and clustered in Group I. Additionally, OsNRAMP5 (but not OsNRAMP1) is also involved in Mn transport, whereas OsNRAMP1 (but not OsNRAMP5) functions in Zn transport. Also in Group I, the closely-related AtNRAMP1 and AtNRAMP6 function in Cd transport, but only AtNRAMP1 functions as Fe and Mn transporter. Finally, in Group II, AtNRAMP3 and AtNRAMP4 proteins group together and are Fe, Mn and Cd transporters, but only AtNRAMP4 exhibits Zn transport activity. Altogether, these observations indicate that NRAMPs within a particular phylogenetic clade might have different metal transport properties and points to an important specialization of NRAMP proteins in metal transport activity. This fact makes difficult to predict transport activities

of NRAMPs solely based on protein sequence homology, and experimental evidence is needed to unravel the metal transport properties of individual NRAMP proteins.

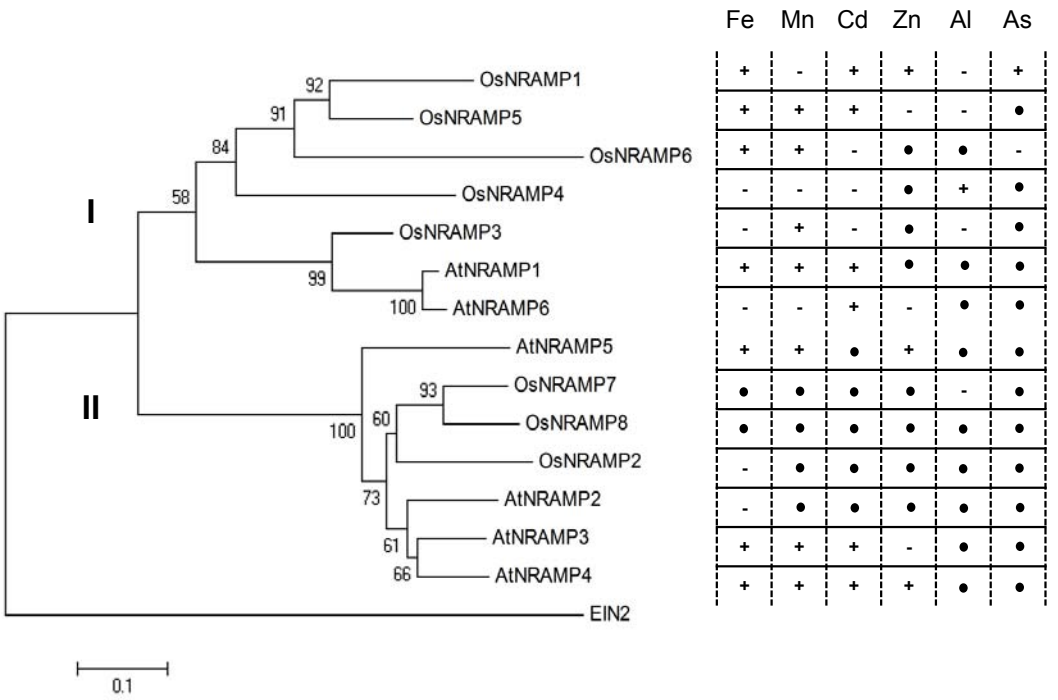

Figure CII.8. Phylogenetic analysis and metal transport activity of rice and Arabidopsis NRAMP proteins.

The phylogenetic tree of rice (Os) and *A. thaliana* (At) NRAMP proteins was constructed with Molecular Evolutionary Genetics Analysis (MEGA 4.0; Tamura *et al.,* 2007) using Blossum matrix and the neighbor-joining method (Saitou and Nei, 1987). Accession numbers are: *OsNramp1* (Os07g15460), *OsNramp2* (Os03g11010), *OsNramp3* (Os06g46310), *OsNramp4* (Os02g03900), *OsNramp5* (Os07g15370), *OsNramp6* (Os01g31870), *OsNramp7* (Os12g39180), *OsNramp8* (Os03g41070), *AtNramp1* (At1g80830), *AtNramp2* (At1g47240), *AtNramp3* (At2g23150), *AtNramp4* (At5g67330), *AtNramp5* (At4g18790), *AtNramp6* (At1g15960) and *AtEIN2* (At5g03280). The metal transport activity of the various NRAMPs was previously described: Curie *et al.,* 2000 (AtNRAMP1,2 and OsNRAMP1,2,3); Thomine *et al.,* 2000 (AtNRAMP1,2,3); Lanquar *et al.,* 2004 (AtNRAMP1,2,3); Vaughan *et al.,* 2001 (AtNRAMP5); Cailliatte *et al.*, 2009 (AtNRAMP6); Xia *et al.,* 2010 (OsNRAMP1,2,3,4,5,6,7); Takahasi *et al.,* 2011 (OsNRAMP1); Ishimura *et al.,* 2012 (OsNRAMP1,5); Sasaki *et al.,* 2012 (OsNRAMP5); Tiwari *et al.,* 2013 (OsNRAMP1); Yang *et al.,* 2013 (OsNRAMP3); Yamaji *et al.,* 2013 (OsNRAMP3). **+**, transport activity; **-**, no transport activity; ●, not determined).

Analysis of *Osnramp6* knockout mutant plants

To further investigate the role of OsNRAMP6 in rice, we searched for *OsNramp6* mutants in public mutant collections. A T-DNA insertion mutant produced in the Hwayoung genotype was identified in the POSTECH RISD database (2B-20317) (http://cbi.khu.ac.kr/). As it was previously found

in the Nipponbare rice cultivar, leaves of Hwayoung plants accumulated significantly higher levels of *l-Nramp6* transcripts compared to *s-Nramp6* transcripts (Figure CII.9).

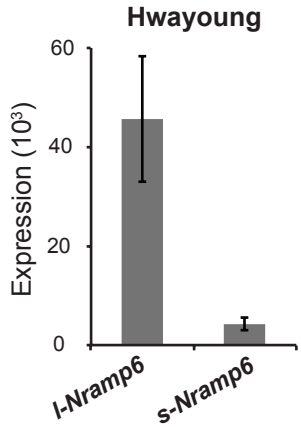

Figure CII.9. Accumulation of *l-Nramp6* and *s-Nramp6* transcripts in leaves of soil-grown rice plants (cv Hwayoung) at the 4 leaf stage.
RT-qPCR was carried out using the *OsUbiquitin1* (Os06g46770) gene as the internal control. Values represent the mean ± SD.

The 2B-20317 mutant has a T-DNA inserted in intron 12 of *OsNramp6* (Figure CII.10A). PCR analysis using combinations of gene-specific and T-DNA-specific primers followed by sequencing of PCR products confirmed the T-DNA insertional locus in this mutant (Figure CII.10A, lower panel). This mutant had a single copy of T-DNA inserted in its genome, as revealed by quantitative PCR (qPCR). Furthermore, *l-Nramp6* transcripts were found to be absent in the *nramp6* mutant supporting that this is a knock-out mutant for *l-Nramp6* (Figure CII.10B). Surprisingly, s-*Nramp6* transcripts accumulated at similar levels in *nramp6* mutant and wild-type (azygous, segregated from heterozygous) plants (Figure CII.10B). This observation points to the existence of co-transcriptional splicing events generating *s-Nramp6* transcripts during *OsNramp6* transcription.

Phenotypic analysis of *nramp6* knockout plants under Fe or Mn deficiency.

Knowing that NRAMP6 functions as a Fe and Mn transporter in yeast,

we performed a phenotypic analysis of the *nramp6* plants under metal deficiency. For this, *nramp6* and wild-type (azygous, segregated from heterozygous) plants were grown in hydroponic cultures under sufficient metal supply (control condition; half-strength Kimura B solution containing 10µM Fe-EDTA, 8 µM $MnCl_2$) or metal deficiency, either low Fe- (0.1 µM Fe-EDTA) or low Mn- (0.8 µM $MnCl_2$). After 14 days of treatment, the dry weight (DW) of roots, leaves and stems was determined.

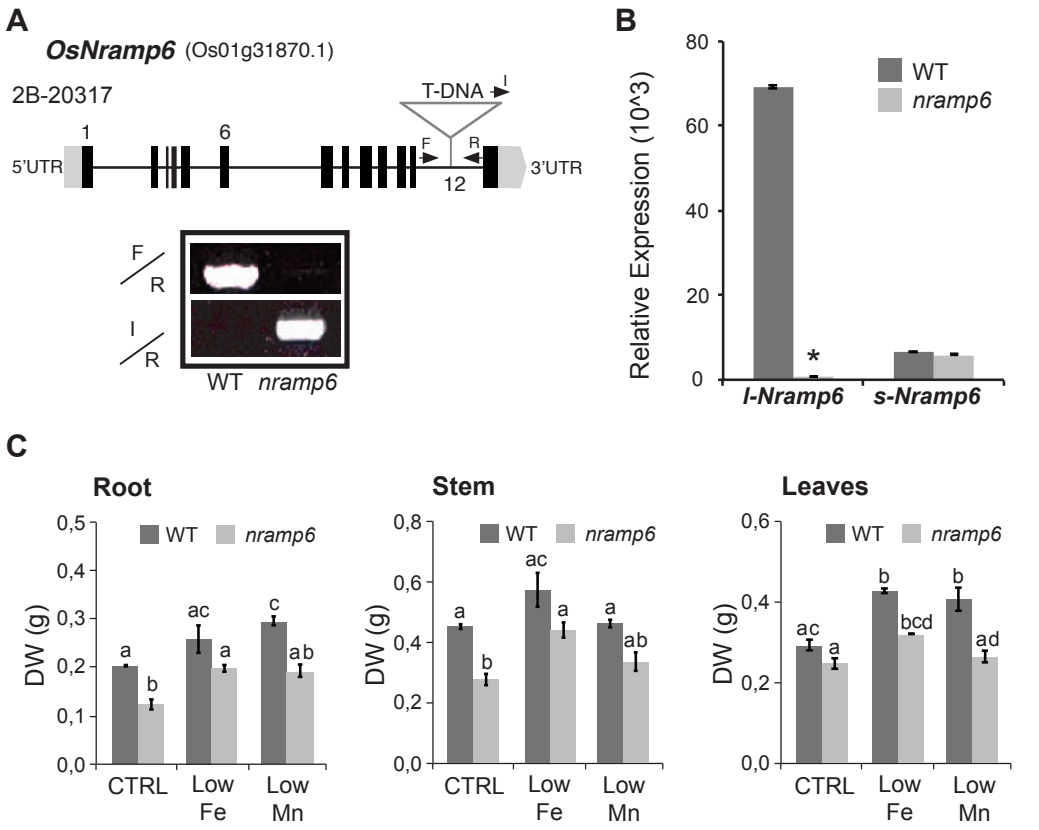

Figure CII.10. Characterization of the *nramp6* mutant.
(A) Schematic representation of the T-DNA insertion in *OsNramp6* (2B-20317, POSTECH collection). PCR analysis using combinations of gene-specific and T-DNA-specific primers (arrowheads) confirmed T-DNA insertion in the 12th intron of *OsNramp6* gene. Verification was done by sequencing of the amplification fragments. WT, segregated azygous plant.
(B) Accumulation of *l-Nramp6* and *s-Nramp6* transcripts in *nramp6* plants (Hwayoung background). RT-qPCR was carried out using the *OsUbiquitin1* gene (Os06g46770) as the internal control. Primers used for expression analysis are indicated in Figure CII.1 and Table CII.1. Error bars represent ± SE (* P ≤ 0.05). Data represent results obtained in two independent experiments that gave similar results.
(C) Biomass of *nramp6* and wild-type (segregated azygous) plants in sufficient and deficient metal supply conditions. Plants were grown in hydroponic cultures in half-strength Kimura B solution for two weeks (10 µM Fe-EDTA, 8 µM $MnCl_2$ control sufficient condition), and then shifted to low Fe (0.1 µM, Fe deficiency) or low Mn (0.08 µM $MnCl_2$, Mn deficiency). Control plants were maintained in half-strength Kimura B solution. Plants were allowed to continue growth for 2 more weeks. Roots, stem and leaves

from each genotype and condition were collected, pooled and dried. The dry weight of roots, stems, and leaves from wild type and *nramp6* mutant plants grown under sufficient metal supply, low Fe, or Low Mn concentrations are shown (left, middle and right panels, respectively). Data represents the means ± SE of two biological replicates (five plants per replicate). Statistically significant differences are denoted by different letters (ANOVA and post-hoc Tukey HSD test, $p < 0.05$).

When grown under sufficient metal supply, root and stem biomass of *nramp6* plants was reduced compared to wild-type plants, but remained unaltered when grown under low Fe condition (Figure CII.10C, left and middle panels). Regarding leaf biomass, no significant differences were observed, whereas the *nramp6* plants showed an important reduction in leaf biomass compared to wild-type plants when grown in low Fe condition (Figure CII.10C, right panel). On the other hand, biomass of *nramp6* plants in low Mn concentration was reduced compared to that of wild-type plants (roots, stems, leaves) (Figure CII.10, Low Mn).

Collectively, these results indicate that loss of *OsNramp6* function (e.g. knocking out *l-Nramp6* expression) results in biomass reduction when the plants were grown under sufficient metal supply, thus, supporting a role of *OsNramp6* in plant growth. However, when plants were grown under Fe-limiting conditions, there were no significant differences in root and stem biomass between *nramp6* plants and wild-type plants, pointing to compensation mechanisms in *nramp6* plants under limiting Fe conditions. Indeed, growth of *nramp6* plants in low Fe was comparable to that of wild-type plants grown under sufficient conditions.

Knocking-out *Nramp6* expression enhances disease resistance in rice

Results previously reported by our group revealed a miRNA-regulated accumulation of *s-Nramp6* transcripts in rice plants. Thus, miR7695 was found to negatively regulate the expression of *s-Nramp6* (Campo *et al.,* 2013). The recognition site for miR7695 locates at the 3' untranslated region of the *s-Nramp6* transcripts, a target site that is not present in *l-Nramp6* transcripts. This particular miRNA was discovered in a search for miRNAs that are regulated during infection of rice plants with the blast fungus *M. oryzae* (Campo *et al.,* 2013). It was then of interest to investigate whether *M. oryzae* infection is accompanied by alterations in the accumulation of *Nramp6* transcripts.

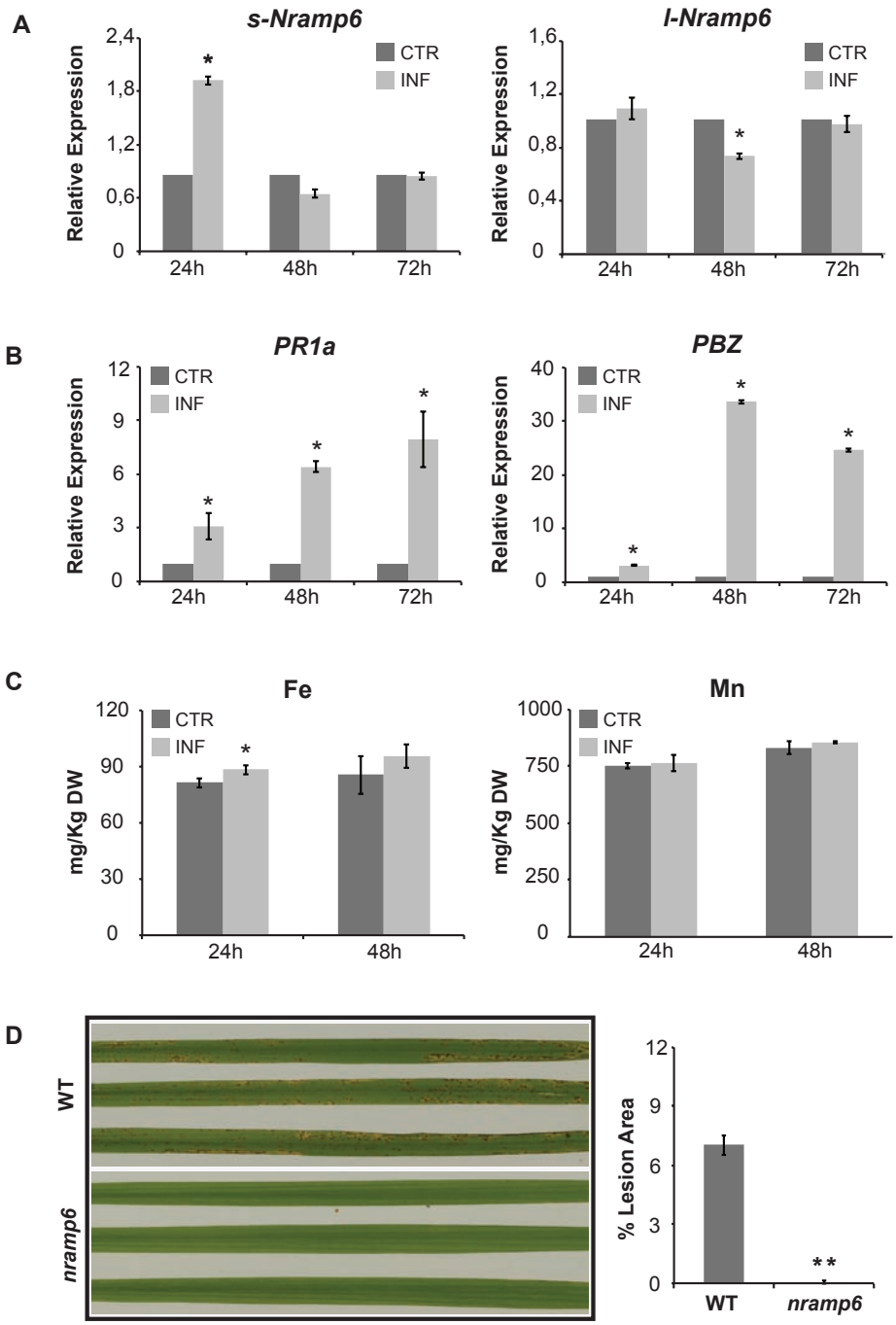

Figure CII.11. *OsNramp6* silencing confers enhanced resistance to infection by the rice blast fungus *M. oryzae*.

(A) Expression analysis of *l-Nramp6* and *s-Nramp6* in leaves of *M. oryzae*-inoculated and mock-inoculated rice plants at the indicated times after inoculation. RT-qPCR was carried out using the *OsUbiquitin1* gene (Os06g46770) as the internal control. Values represent the mean ± SE (t-student test; * P ≤ 0.05) of three biological replicates (pool of 5 plants/replicate). Primers used for *Nramp6* expression analyses are indicated in **Figure CII.1**.

(B) Expression analysis of the defense marker genes *PBZ1* and *PR1a*. Same RNA samples as in (A). **(C)** Iron and manganese content in *M. oryzae*-infected rice leaves. Fe **(**left**)** and Mn **(**right**)** content was determined by ICP-OES in leaves of *M. oryzae*-inoculated and mock-inoculated rice plants at the indicated times after inoculation. Three biological replicates were assayed, each one consisting of a pool of 15 leaves. Statistical significance was determined by t-Student (*, $P \leq 0.05$). **(D)** Resistance of *nramp6* mutant plants to *M. oryzae* infection. Leaves of soil-grown plants at the 4 leaf-stage were sprayed with a 10^5 spores/ml suspension (0.2 ml/plant). Disease symptoms at 7 d post-inoculation are shown (left panel). Results are from one out of three independent experiments (at least 6 plants/genotype), which gave similar results. The percentage of leaf area affected by blast lesions was determined by image analysis (APS Assess 2.0 PROGRAM) (right panel). Histograms show the mean ± SE (t-Student; *, $P \leq 0.05$; ** $P \leq 0.01$).

Expression analysis of *OsNramp6* revealed a clear and transient increase in the accumulation of *s-Nramp6* transcripts at 24 hpi, whereas *l-Nramp6* transcripts remained at a normal level by this time of infection (Figure CII.11A). At a later stage, however, *l-Nramp6* expression was slightly, but significantly down-regulated in *M. oryzae*-infected plants compared to mock-inoculated plants, and *s-Nramp6* transcripts returned to normal levels (Figure CII.11A, 48 hpi). To assess the effectiveness of the infection treatment and the correct activation of the plant defense response to pathogen infection, we examined the expression of the defense marker genes *PBZ1* (*Probenazole-inducible 1*) and *PR1a* (*Pathogenesis-Related1a*) (Midoh and Iwata, 1996; Agrawal *et al.,* 2001). As expected, fungal infection strongly induced *OsPBZ1* and *PR1a* expression (Figure CII.11B).

Having established that fungal infection has an effect on *OsNramp6* expression we examined whether *M. oryzae* infection is accompanied by alterations in iron and/or manganese content in wild-type rice. Metal content of leaves of *M. oryzae*-infected and mock-inoculated plants was determined by Inductively Coupled Plasma optical emission spectrometry (ICP-OES). This analysis revealed a small but significant increase in iron content at 24h post-inoculation with *M. oryzae* (Figure CII.11C, left panel). Fungal infection, however, does not appear to have an important effect on Mn accumulation, at least at the time points assayed in this work (Figure CII.11C, right panel).

Next, we examined the development of disease in *nramp6* plants upon challenge with the fungal pathogen *M. oryzae.* As clearly shown in Figure CII.11D, *nramp6* plants displayed enhanced disease resistance to *M. oryzae* infection relative to control azygous plants.

Collectively, this study revealed that *l-Nramp6* and *s-Nramp6* expression is regulated during infection of rice plants with the fungal pathogen *M. oryzae*. During the infection process, alterations in *s-Nramp6* expression occur earlier than those in *l-Nramp6*. Moreover, infection assays in the *nramp6* mutant revealed that *OsNramp6* is a positive regulator of disease resistance in rice.

DISCUSION

We report the functional characterization of *OsNramp6*, a member of the rice NRAMP gene family that mediates pathogen resistance. Whereas the crucial role of NRAMP proteins in plant developmental processes and/or detoxification of heavy metals is well demonstrated, the involvement of NRAMPs in disease resistance is understudied. Work carried out in the model system of Arabidopsis revealed that *AtNRAMP3* and *AtNRAMP4* regulate basal resistance to the bacterial pathogen *Erwinia chrysanthemi* (syn. *Dickeya dadantii*) (Segond *et al.,* 2009). In other studies, *MtNramp1* was shown to function in rhizobia-infected nodule cells in *Medicago truncatula* (Tejada-Jiménez *et al.,* 2015). Additionally, the pathogen-regulated expression of *Nramp* genes has been reported in different plant species, including rice and Arabidopsis (Zhou and Yang, 2004; Segond *et al.,* 2009). These pieces of evidence support the idea that *Nramp* genes might be important players in plant-microbe interactions.

Historically, the rice *NRAMP* family of metal transporters has been considered to contain 7 members, but the rice genome contains an additional *Nramp* gene, *OsNramp8*, that encodes a shorter, still uncharacterized NRAMP protein. The metal transport activity has been reported for certain rice NRAMPs, such as OsNRAMP1 (Fe, Cd, Zn, As, but not Mn), OsNRAMP3 (Mn, but not Fe or Cd), OsNRAMP4 (Al, but not Fe, Mn or Cd), and OsNRAMP5 (Fe, Mn, Cd, but not Zn). As for OsNRAMP6, the results here presented support that both the full-length (l-NRAMP6) and the shortest (s-NRAMP6) NRAMP6 proteins function as Fe and Mn transporters, as revealed by functional complementation of yeast mutants defective in iron (*fet3 fet4*) or Mn (*smf1* and *smf2*) transport. Both l-NRAMP6 and s-NRAMP6

localize at the plasma membrane. In general, plasma membrane-targeted NRAMP proteins are associated with metal uptake, whereas those located in intracellular membranes contribute to metal mobilization (Thomine *et al.,* 2003; Lanquar *et al.,* 2005; Sasaki *et al.,* 2012; Ishimaru *et al.,* 2012). In the case of OsNRAMP3, this protein localizes at the plasma membrane and contributes to Mn distribution in rice (Yang *et al.,* 2013; Yamaji *et al.,* 2013).

Determination of the crystal structure of ScaDMT, a prokaryotic orthologue of NRAMPs, provides the opportunity to advance understanding of metal transport activities in this protein family. Homology-based modeling revealed that the overall structure of NRAMP6 and ScaDMT is conserved, except for the N-terminal and C-terminal region. The Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} and Pb^{2+} ions occupy the same binding site in the ScaDMT protein (Ehrnstorfer *et al.,* 2014), and mutations of ion-coordinating residues (e.g D49, N52 and M226) have an important effect in the binding properties of ScaDMT (Ehrnstorfer *et al.,* 2014). Whereas the binding-site aspartate and asparagine are shared by NRAMP6 and ScaDMT (D88/D49 and N91/N52; **Figure CII.4**), the alanine and methionine residues in the metal binding site of ScaDMT (A223, M226) are replaced by serine and valine in NRAMP6 (S260, V263). Changes in the metal-binding residues might explain differences in transport activities between NRAMP6 and ScaDMT. For instance, ScaDMT functions as a Cd transporter (Ehrnstorfer *et al.,* 2014), whereas NRAMP6 has no Cd transport activity (present work). It is also possible that NRAMP6 transports other metals not assayed in this work. The intriguing question arises from the observation that s-NRAMP6, which lacks S260 and V263, is functional in transporting Fe^{2+} and Mn^{2+}. There is the possibility that s-NRAMP6 forms active dimers or multimers. Here, it is worthwhile to recall that the ScaDMT structure consists of two symmetrically oriented subdomains (inverted repeats of 5 helices, helices 1-5 and helices 6-10), and that the two related halves gives rise to the ion channel in the center of the membrane (Ehrnstorfer *et al*., 2014). The observation of fluorescent aggregates in the plasma membrane of *s-Nramp6-GFP* expressing *N. benthamiana* cells points to the formation of s-NRAMP6 dimers/multimers. Further studies are required to clarify the structural/mechanistic features underlying the observed metal transport activity of s-NRAMP6.

We show that *l-Nramp6* and *s-Nramp6* transcripts coexist in leaves of rice plants during vegetative growth. The accumulation of both *l-Nramp6* and *s-Nramp6* transcripts is regulated during infection with *M. oryzae*, although with different trend and kinetics. Definitive prove for a role of *OsNramp6* in rice immunity came from disease resistance assays showing that knockout *nramp6* plants exhibited enhanced resistance to *M. oryzae* infection. This observation suggests that *OsNramp6* is a negative regulator of resistance to *M. oryzae* infection in rice. Here, it is worthy to recall that whereas *l-Nramp6* expression is abolished in the *nramp6* mutant, *s-Nramp6* expression is not affected in this mutant (see **Figure CII.10B**). In earlier studies we reported that down-regulation of *s-Nramp6* transcripts through overexpression of miR7695 also confers resistance to infection by the rice blast fungus (Campo *et al.,* 2013). Thus, *l-Nramp6* knock-out (present work) and down-regulation of *s-Nramp6* (miR7695 overexpressor rice plants; Campo *et al.,* 2013) confer enhanced resistance to pathogen infection. The relative abundance of each one NRAMP6 isoform might then be important to ensure proper Fe and Mn levels in the rice plant compatible with normal development and immune responses. Disturbance in *l-Nramp6* or *s-Nramp6* expression levels might trigger still unknown signaling processes mediating defense responses in rice plants. Whether unbalanced *Nramp6* transcript isoforms are directly or indirectly responsible of alterations in Fe content remains to be investigated.

Recently, Liu *et al.,* (2016) provided evidence for alternative splicing of two rice transcription factors of the WRKY family involved in resistance to the rice blast fungus *M. oryzae* and a dominant-negative function of the truncated splice variants on the function of full-length OsWRKY proteins was reported. In this respect, transcriptome-wide analysis of alternative splicing events in plants revealed that alternative splicing is highly pervasive, although the functions of most splice variants are unknown (Reddy *et al.,* 2013). It will then be of interest to investigate whether alternative splicing-mediated feedback regulation mechanism occurs for the OsNRAMP6 proteins. On the other hand, the observation that accumulation of *Nramp6* transcripts is abolished (*l-Nramp6*) or unaltered (*s-Nramp6*) in *nramp6* plants, points to a co-transcriptional production of *s-Nramp6* transcripts during *OsNramp6* transcription. In this context, although limited information is currently available

regarding co-transcriptional splicing in plant cells, studies in yeast, Drosophila, human and mouse cells, demonstrated that the majority of splicing events take place co-transcriptionally (Brugiolo *et al.,* 2013).

The *nramp6* mutant plants had less biomass than wild-type plants when grown with sufficient Fe and Mn supply. Therefore, in addition to its role in disease resistance, *OsNramp6* might also play a role in controlling plant growth as it has been demonstrated for other *Nramp* genes (Lanquar *et al.,* 2005; Cailliatte *et al.,* 2010; Ishimaru *et al.,* 2012; Sasaki *et al.,* 2012; Yamaji *et al.,* 2013). When examining the behavior of *nramp6* mutant plants in response to Fe or Mn deficiency, we observed that growth inhibition in *nramp6* plants was partially rescued by decreasing Fe supply. Presumably, compensation mechanisms might allow the *nramp6* plants to escape from *l-Nramp6* inactivation under unfavorable conditions. Given the high number of metal transporter families present in rice, it is plausible to consider that compensatory mechanisms operate in *OsNramp6*-silenced plants. Candidates for compensation by other transporters are the closely related OsNRAMP1 and OsNRAMP5 (also Fe and Mn transporters), as well as OsIRT1 and OsYSL (implicated in Fe transport) (Inoue *et al.,* 2009; Ishimaru *et al.,* 2006, 2010). In favor of this hypothesis, previous studies have shown that OsNRAMP5, OsNRAMP1, OsIRT1 and OsYSL15 function jointly in Fe uptake in rice (Ishimaru *et al.,* 2012). Such compensation mechanisms would be more effective to restore normal growth of *nramp6* plants under Fe deficiency than under Mn deficiency.

As previously mentioned, metal micronutrients are essential elements for plant growth, but are toxic when present in excess. In the context of plant-pathogen interactions, metal homeostasis must be tightly controlled as host and pathogen compete for the available metals. The host plant must arrest pathogen invasion with minimal interference on normal growth and development. Plants might counteract pathogen infection by sequestering essential micronutrients away from the invading pathogen or by creating a controlled, localized accumulation of metals acting as antimicrobial agents. Indeed, the term “nutritional immunity” is used in human health and disease to define the dynamic interaction between pathogens and hosts as a non-specific host immune response against invading pathogens.

Being a foliar pathogen, *M. oryzae* entirely depends on the host for supply of mineral nutrients. This fungus has a hemibiotrophic lifestyle that maintains an initial biotrophic relationship with its host, followed by a necrotrophic lifestyle (Kankanala *et al.,* 2007; Wilson and Talbot, 2009; Campos-Soriano *et al.,* 2012). Iron acquisition is a key step during the initial infection process in *M. oryzae*, and the synthesis of high-affinity iron chelators is related to fungal virulence (Hof *et al.,* 2007). In this work, we observed alterations in *s-Nramp6* and *l-Nramp6* expression during the *M. oryzae* biotrophic colonization phase (24 - 48hpi). During this period the fungus has no, or limited, access to intracellular metals which are often sequestered in the vacuole for the plant's own use. Under this scenario, a signaling function of NRAMP6-mediated alterations in host metal content regulating rice immunity should be considered.

Finally, we show that infection by *M. oryzae* is accompanied by an increase in Fe content in rice leaves (at 24 hpi) whereas the Mn level appears not to be affected during fungal infection (at least at the time points here assayed). Iron is a potent generator of ROS, and signaling via ROS is widely regarded to be central to disease resistance in plants (Torres *et al.,* 2006). A model to explain how targeted alterations in Fe homeostasis mediate plant defense responses in the wheat/*Blumeria graminis f. sp. tritici* pathosystem was proposed (Liu *et al.,* 2007). According to this model, pathogen recognition elicits the accumulation of Fe^{+3} in cell wall appositions at the infection site. The accumulated apoplastic Fe mediates oxidative burst with the production of apoplastic H_2O_2, thus inducing the expression of defense-related genes. Since excessive accumulation of Fe can be dangerous to the plant cell, this localized accumulation of Fe avoids toxic effects to the host plant. Now, it will be of interest to determine whether alterations in *OsNramp6* expression is accompanied by a localized and targeted accumulation of Fe in *M. oryzae*-infected rice leaves which, in turn, might modulate still unknown rice defense responses. In other studies, the impact of metals (e.g. Zn, Fe, Cu) in pathogenesis during plant/bacteria interactions has been reported (Fones and Preston, 2012).

Collectively, our results support that *Nramp6* plays a role in rice immunity. Since the rice blast fungus *M. oryzae* is one of the primary causes of rice

losses worldwide, a better understanding of the mechanisms involved in Fe and Mn homeostasis in which *Nramp6* participates will help in designing novel strategies to improve disease resistance in rice.MATERIAlS AND METHODS

MATERIALS AND METHODS

Plant material and growth conditions

Rice plants were grown at 28ºC with a 14h/10h light/dark cycle. The T-DNA insertion line for *OsNramp6* (Os01g31870) and its wild-type genotype (*O. sativa* cv Hwayoung, *japonica* type) were obtained from the POSTECH Rice Insertion Database (RISD) (http://cbi.khu.ac.kr/). For hydroponics assays, the rice seeds (homozygous lines) were sterilized and germinated in half-strength Kimura B solid medium (8g/l Plant Agar, Duchefa Biochem). The composition of the nutrient solution was 0.18 mM $(NH_4)_2SO_4$, 0.27 mM $MgSO_4 \cdot 7H_2O$, 0.09 mM KNO_3, 0.18 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.09 mM KH_2PO_4, 8 µM $MnCl_2 \cdot 4H_2O$, 3 µM H_3BO_3,1 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.4 µM $ZnSO_4 \cdot 7H_2O$, 0.2 µM $CuSO_4 \cdot 5H_2O$, 10 µM Fe-EDTA (pH 5.6) (Sasaki *et al.,* 2012). Seven-day old seedlings were hydroponically grown in half-strength Kimura B solution for one week. To assess the effect of Fe or Mn deficiency, plants were transferred to the same nutrient condition with a lower Fe (0.1 µM Fe-EDTA) or Mn (0.08 µM $MnCl_2 \cdot 4H_2O$) concentration. The nutrient solution was renewed every 3 days. After two weeks of treatment, plants were harvested, and their roots, stem and leaves dissected and dried (70°C, 3 d).

Phylogenetic analysis

The cDNA and genomic sequences were retrieved from the Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/). Alignment of NRAMP amino acid sequences was carried out with ClustalW using default settings (http://clustalw.ddbj.nig.ac.jp/) and Boxshade (http://www.ch.embnet.org/software/BOX_doc.html). The phylogenetic tree was constructed using the neighbor-joining algorithm by MEGA4 software (http://megazoftware.net/) (Tamura *et al.,* 2007). Transmembrane domains were predicted with SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) (Hirokawa *et al.,* 1998).

Structure modeling

The 3D structure of long and short-NRAMP6 were performed using as a template the ScaDMT protein (PDB code 4WGV) (Ehrnstorfer *et al.,* 2014). The three-dimensional model was built directly using I-Tasser and then evaluated using the ProSA program (Sippl, 1993; Wiederstein and Sippl, 2007). Molecular graphics images were produced using the UCSF Chimera package (Pettersen *et al.,* 2004).

RNA extraction and Reverse Transcription quantitative PCR (RT-qPCR).

Total RNA was extracted from plant tissues using the Trizol reagent (Invitrogen). The first complementary DNA was synthesized from DNase-treated total RNA (1 µg) (High Capacity cDNA Reverse Transcription kit, Life Technology). RT-qPCR was performed on optical 96-well plates (Light Cycler®480; Roche Diagnostics, Mannheim, Germany) using the following program: 95ºC for 5 minuntes, 40 cycler of 95ºC 30 seconds, 60ºC 10 seconds 72ºC 10 seconds and 72ºC for 10 minutes. The 10 µl reaction mixture contained 10 µl of SYBR Green Master Mix (2X) (Roche), 2 µl diluted cDNA (1:5), and 300 nM of each gene-specific. Data were normalized using the OsUbiquitin1 (Os06g46770) gene. All oligonucleotides used in this work are listed in Table CII.1. Three independent biological replicates and three technical replicates per sample were analyzed.

Transient expression of *OsNramp6* in *Nicotiana benthamiana* leaves

The cDNA sequence encoding the full-length (Os01g31870.1) or the short (Os01g31870.8) NRAMP6 proteins were obtained from the Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/; J013135E06 and J013164K10 clones, respectively). Each *OsNramp6* cDNA sequence was cloned into the pB7FWG2,0 plant expression vector designed for the production of GFP fusion proteins under the control of the Cauliflower Mosaic Virus (CaMV) promoter (Karimi *et al.,* 2002). The *OsNramp6* cDNA sequence corresponding to *l-Nramp6* or *s-Nramp6* was amplified using the gw_l-Nramp6 and gw_s-Nramp6 primers (for *l-Nramp6* and *s-Nramp6*, respectively; Table CII.1). PCR products were cloned into the pENTR™/D-TOPO, using the

gateway procedure according to the manufacturer's instructions (Invitrogen) and then into the pB7FWG2,0 plasmid to obtain a C-terminal GFP-tagged plant expression vector. The plasmid construct containing either the *l-Nramp6-GFP* or the *s-Nramp6-GFP* fusion gene was introduced into the *Agrobacterium tumefaciens* EHA105 strain.

Agrobacterium-mediated transient expression assays were carried out in *Nicotiana benthamiana* leaves (*rdr6i* mutant; Schwach *et al.*, 2005). Experiments for co-expression with the fluorescently-labelled plasma membrane marker LTI6B-mRFP (Kurup *et al.,* 2005) were also carried out. For this, the pUBN-RFP-LTI6b plasmid was used (kindly provided by Dr. K. Schumaker, Heidelberg, Germany). The subcellular localization of the fusion proteins was determined by confocal laser scanning microscopy (CLSM) at 52 h after agroinfiltration in an Olimpus FV1000 microscope. The excitation wavelength was 488 nm for GFP and 543 nm for RFP. The emission window was set at 500–530 nm for GFP and 570-670 for RFP.

Yeast functional complementation

The cDNA sequence encoding either the l-NRAMP6 or the s-NRAMP6 protein were amplified by PCR using primers BglII_l-Nramp6 and SalI_l-Nramp6 (for *l-Nramp6*), or the BglII_s-Nramp6 and SalI_s-Nramp6 primers (for *s-Nramp6*) (Table CII.1). The PCR products were cloned into the pGEM T-Easy vector and then inserted into the pWS93 yeast vector for N-terminal HA-tagged expression under the control of the *ADH1* promoter.

S. cerevisiae strains were grown in YPD medium (10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter dextrose) or, when carrying plasmids, in synthetic minimal medium lacking uracil (Adams *et al.,* 1997). Growth assays on agar plates were performed as described previously (Posas *et al.,* 1995). In some experiments, Low-YPD medium (2.5 g/liter yeast extract, 5 g/liter peptone, and 20 g/liter dextrose) was employed. Wild type *S. cerevisiae* strains BY4741 and its isogenic derivatives *smf1::kanMX4*, *smf2::kanMX4* and *fet3::kanMX4* were obtained from the EUROSCARF collection. Strain ASC64 (BY4741 *fet3::kanMX4 fet4::nat1*) was constructed by transformation of the *fet3* strain with a 1.25-kbp *fet4::nat1* disruption cassette amplified from plasmid pAG25 (Goldstein and McCusker, 1999) with oligonucleotides FET4_5'_NAT and FET4_3'_NAT (Table CII.1).

Table CII.1. Sequences of oligonucleotides used.

Primer name	Primer sequences	
BglII_l-Nramp6	For	5'-GAGATCTGTATGGAGGAGGGGGCGAAGATC-3'
SalI_l-Nramp6	Rev	5'-CCGTCGACCTCATAAATGTACACCTAAATC-3'
BglII_s-Nramp6	For	5'-GAGATCTGTATGGAGGAGGGGGCGAAG-3'
SalI_s-Nramp6	Rev	5'-CCGTCGACCTTATAAGCAGAGAAAAATTCT-3'
gw_l-Nramp6	For	5'-CACCATGGAGGAGGGGGCGAAGATCGGG-3'
	Rev	5'-TAAATGTACACCTAAATCTTCCTGCTTC-3'
gw_s-Nramp	For	5'-CACCATGGAGGAGGGGGCGAAGATC-3'
	Rev	5'-TAAGCAGAGAAAAATTCTTACCCCAT-3'
HY22	For	5'-GCACGTCCCTCATTCTCTCAG-3'
	Rev	5'-GGACTATCATGCTTATAGCTTC-3'
	Internal (T-DNA)	5'-GGTGAATGGCATCGTTTGAA-3'
l-Nramp6	For	5'-CATAGCTGCGATTGTACTATCATTTG-3'
	Rev	5'-CGCGATTCGGATAATAGATGTTG-3'
s-Nramp6	For	5'-TCTCCGTCCGACATCCGTA-3'
	Rev	5'-TCTCCGTCCGACATCCGTA-3'
OsUbi1	For	5'-TTCCCCAATGGAGCTATGGTTT-3'
	Rev	5'-AAACGGGACACGACCAAGG-3'
OsPR1a	For	5'-GGAAGTACGGCGAGAACATC-3'
	Rev	5'-GGCGAGTAGTTGCAGGTGAT-3'
OsPBZ	For	5'-CCGGGCACCATCTACACC-3'
	Rev	5'-CCTCGATCATCTTGAGCATGT-3'
FET4_NAT	For	5'-AAACCTAATAAGACCCTTATTATTAAACATTCGTTAATCACGTACGCT-GCAGGTCGAC-3'
	Rev	5'-ACGATCGGCCCAATTATGTGCTTGCAACAAAACTAGCAAGATCGAT-GAATTCGAGCTCG-3'

Western blot analysis of yeast protein extracts

For the preparation of yeast protein extracts, 10 ml of cell cultures were collected by centrifugation (4 min, 546 x g), washed with cold double-distilled water, and the pellet was stored at -80°C. Cells were re-suspended in 300 µl of disruption buffer and fractionation of the extract to separate soluble from precipitable (membrane-bound) material were performed as described in Pérez-Valle *et al.,* (2007). Pellets were re-suspended in 100 µl of loading buffer, heated at 95ºC (3 min) and loaded on SDS-PAGE gels (20 µl of each sample). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The NRAMP6 proteins were immunodetected using a mouse monoclonal anti-HA anti-body (12CA4; 1:1,000 dilution; Roche). The

secondary anti-mouse IgG–horseradish peroxidase (GE Healthcare) was used at a 1:20,000 dilution, and immunoreactive proteins were visualized with the ECL Select kit (GE Healthcare).

Analysis of *nramp6* plants

Genotyping of *nramp6* plants was carried out by PCR on genomic DNA using *OsNramp6*-specific primers (HY22_F and HY22_R) in combination with a T-DNA-specific primer (HY22_I, located at the left border of the T-DNA) (Supplementary Table S1). Homozygous and azygous plants were identified. *Nramp6* expression was examined in mutant plants by RT-qPCR as described above. The T-DNA copy number was evaluated by real-time quantitative PCR using the Sucrose Phosphate Synthase gene as the endogenous reference gene (Yang *et al.*, 2005).

Blast resistance assay

The fungus *M. oryzae* (strain Guy-11) was grown in CMA medium (9 cm plates, containing 30 mg/L chloramfenicol) for two weeks at 28ºC under a 16h/8h light/dark photoperiod condition. *M. oryzae* spores were prepared as previously described (Campo *et al.,* 2013). Infections were carried out by spraying the leaves of soil-grown plants (3-4 leaf stage) with a spore suspension (10^5 spores/ml). Plants were maintained in the dark under high humidity conditions overnight. The percentage of leaf area affected by blast lesions was determined by using the APS Assess 2.0 programme, typically at 7 days post-inoculation.

Metal content determination

Rice tissues were lyophilized, and 80-100 mg (dry weight) and digested in 2 ml of 65% HNO_3 and 1 ml of H_2O_2 overnight at 100ºC. Samples were diluted in deionized water and the Mn and Fe content was measured by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) at the Scientific and Technological Center from the University of Barcelona. Concentrations were calculated by comparison with metal standards. At least, three biological replicates for each condition tested were assayed.

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GENERAL DISCUSSION

Plants are constantly exposed to a wide range of pathogens, however, diseases rarely occur. To cope with potential pathogens, plants have developed a sophisticated recognition- and-defense system: the PAMP-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI). Historically, PTI and ETI have been considered as protein-based defense mechanisms. However, recent studies support the role of miRNAs as regulators of plant innate immunity through posttranscriptional regulation of their target transcripts. In our group, we previously identified a set of miRNA candidates from small RNA libraries obtained from rice tissues treated with *M. oryzae* elicitors (Campo *et al.*, 2013). Among them was miR7695, which was predicted to target a short splice variant of the *Nramp6* gene. The *Nramp6* gene produces up to eight transcript variants encoding different NRAMP6 isoforms, and only the shortest transcript variant (*s-Nramp6* or *OsNramp6.8*) contains the target site for miR7695 (Campo *et al.*, 2013).

In this work, we show that the miR7695/*Nramp6* pair is involved in rice immunity against *M. oryzae*. As miRNA negatively regulates the expression of their targets, one might expect similar phenotypes in miRNA overexpressor and target mutant plants. We report that accumulation of miR7695 in rice (with the concominant down-regulation of its target transcript *OsNramp6.8)* enhances resistance to *M. oryzae.* Similarly, knock-out *nramp6* mutants showed reduced blast lesions. Here, it is worthy to recall that, in the *nramp6* mutant, the expression of the long *Nramp6* transcript variant (*l-Nramp6*) is compromised, whereas expression of the shorter transcript variant (*s-Nramp6*, containing the miR7695 target site) is not affected. Therefore, *l-Nramp6* knock-out (*nramp6* mutant) and *s-Nramp6* down-regulation (*MIR7695* transgenic rice) results in enhanced resistance to pathogen infection. In line with this, we show that *l-Nramp6* and *s-Nramp6* transcripts are regulated during infection with *M. oryzae*, although with different trend and kinetics. This finding further supports that both *l-Nramp6* and *s-Nramp6* are involved in rice immunity against *M. oryzae.* Therefore, a proper rice response might require an equilibrium between both NRAMP6 isoforms. Disturbance in *l-Nramp6* or *s-Nramp6* accumulation might trigger still unknown signaling processes mediating defense responses in rice plants.

We show that the two NRAMP6 isoforms, l-NRAMP6 and s-NRAMP6,

are plasma membrane proteins capable of transporting Fe^{2+} and Mn^{2+} in yeast cells. The intriguing question arises from the observation that s-NRAMP6, which is considerably shorter, retains the same l-NRAMP6 transport capabilities even lacking two out of the four amino acid residues implicated in ion binding. There is the possibility that s-NRAMP6 forms active dimers or multimers that might form an ion channel. In favor of this hypothesis, ScaDMT, a prokaryotic orthologue of NRAMPs, adopts a tridimensional structure with two subdomains of five transmembrane domains each (the first five α-helices in ScaDMT are structurally related to the following α-helices by rotation around an axis located in the center of the membrane) (Ehrnstorfer *et al.*, 2014). The observation of fluorescent aggregates in the plasma membrane of *s-Nramp6-GFP* expressing *N. benthamiana* cells points to the formation of s-NRAMP6 dimers/multimers. Further studies are required to clarify the structural/mechanistic features underlying the observed metal transport activity of s-NRAMP6. In any case, the relative abundance of each NRAMP6 isoform in *MIR7695*-accumulating (*s-Nramp6* altered) and *nramp6* (*l-Nramp6* altered) mutant plants might be important to ensure proper Fe and/or Mn levels in the rice plant, which might be needed for the production of the most appropriated immune responses. In this context, we show that infection by *M. oryzae* is accompanied by alterations in *l-Nramp6* and *s-Nramp6* transcripts, as well as an increase in Fe content in rice leaves (at 24 hpi). The Mn level appears not to be affected during fungal infection (at least at the time points here assayed). Whether unbalanced *Nramp6* transcript isoforms are directly or indirectly responsible of alterations in Fe content remains to be investigated.

Iron, is essential for plant and fungal growth. However, when in excess, is toxic. Thus, iron homeostasis in plants needs to be tightly controlled to ensure proper plant development while arresting pathogen growth. Here, we show that a set of iron homeostasis-related genes were down-regulated in *MIR7695* activation tagged (*MIR7695*-Ac) lines in reponse to *M. oryzae* infection. All of them, were described to be induced under Fe deficiency in plants (Bashir *et al.*, 2014; Kobayashi and Nishizawa, 2014). The iron-deficiency response comprises a set of induced processes that facilitate iron uptake and mobilization to ensure a proper iron homeostasis (Kobayashi

and Nishizawa, 2012). Thus, *MIR7695*-Ac plants might respond to pathogen infection by activating mechanisms to withhold iron from invading pathogens, or by exposing them to an excess of iron, which will be toxic. A model to explain how targeted alterations in Fe homeostasis mediate plant defense responses was proposed in the wheat/*Blumeria graminis f. sp. tritici* pathosystem (Liu *et al.*, 2006). According to this model, pathogen recognition elicits the accumulation of Fe^{+3} in cell wall appositions at the infection site. The accumulated apoplastic Fe mediates oxidative burst with the production of apoplastic H_2O_2, thus inducing the expression of defense-related genes (Liu *et al.*, 2006). This observation is in agreement with results obtained in the present work, were down-regulation of iron-homeostasis genes is accompanied by the up-regulation of the *OsPR1a* in infected *MIR7695-Ac* plants. These results also suggest that Fe might accumulate in the fungal-infected leaves of *MIR7695* plants. It will be of interest to determine Fe and ROS content in fungal infected wild-type and *MIR7695*-Ac plants, as well as to examine the expression of defense-related rice genes other than *OsPR1a*.

Moreover, iron acquisition in fungi is a key step during the infection process. Since *M. oryzae* is a foliar pathogen, it entirely depends on the host for iron supply. To uptake the iron, *M. oryzae* synthetizes intracellular siderophores which play a role in the early infection stages (Hof *et al.*, 2007, 2009).However, fungal siderophores not only act as iron chelators to capture iron in a plant-induced iron deficiency situation, but also provide protection against ROS produced by plant “Fe burst” (Haas *et al.*, 2008). Further studies are required to characterize the iron-dependent pathway in *M. oryzae*-infecting *MIR7695-Ac* plants.

Collectively, results here presented support the existence of links between iron homeostasis and immunity in rice plants, a feature that has been previously observed in other graminaceous and Arabidopsis plants. For instance, as previously mentioned, in the wheat-powdery mildew pathosystem, basal resistance-linked H_2O_2 production is always associated with a “Fe^{3+} burst” at the pathogen site (Liu *et al.*, 2006). In Arabidopsis, the iron status controls the outcome of the infection with the bacterial pathogen *Dickeya dadantii* by controlling both the pathogen’s virulence and the host’s defense (Kieu *et al.*, 2012). More recently, it was reported that an adequate

iron nutritional status suppresses infection by *Colletotrichum graminicola* in maize (Ye *et al.*, 2014). These findings reinforce the notion that miRNAs contribute to plant immunity.

To conclude, results here presented further support that miRNAs and their corresponding target genes can be considered an integral part of the rice response to *M. oryzae* infection. Most probably, the miR7695/*OsNramp6* gene pair contributes to disease resistance through modulation of metal homeostasis, particularly Fe homeostasis. Considering that rice is one of the most important cereal crops, and that the rice blast fungus *M. oryzae* causes important crop losses worldwide, the knowledge gained in these studies might be important in designing new strategies to improve disease resistance in rice.

CONCLUSIONS

1. The *MIR7695* represents a novel, recently evolved miRNA-generating locus that is processed by DCL4.

2. *MIR7695* is expressed in both shoot and roots. However, *miR7695* precursor processing, and subsequent accumulation of mature miR7695, occurs only in shoot tissues. Transcripts corresponding to the full length and the shortest transcript variant generated by alternative splicing of *Nramp6* primary transcripts (*l-Nramp6* and *s-Nramp6*, respectively) co-exist in rice leaves.

3. Infection by *Magnaporthe oryzae* is accompanied by alterations in the accumulation of *l-Nramp6* and *s-Nramp6* transcripts in the rice leaves, although with a different kinetics.

4. Accumulation of miR7695 in *MIR7695* overexpressor and *MIR7695* activation-tagged mutant rice plants reduces the accumulation of *s-Nramp6* transcripts, thus, supporting that miR7695 targets the short alternatively spliced transcript variant of the *OsNramp6* gene. These findings provide evidence on the existence of a regulatory network that integrates miRNA function and mRNA processing in plant immunity.

5. Accumulation of miR7695 in *MIR7695* overexpressor and *MIR7695* activation-tagged mutant rice plants confers resistance to *Magnaporthe oryzae*. Altogether, these observations indicate that miR7695 positively regulates resistance to infection by the rice blast fungus *Magnaporthe oryzae*.

6. The observed phenotype of disease resistance in *MIR7695* activation-tagged mutant plants might result, at least in part, from a stronger activation of host defense responses during pathogen infection, as observed for *PR1a* gene expression.

7. Iron homeostasis-related genes were found to be strongly down-regulated in *MIR7695* activation-tagged mutant plants, supporting a link between miR7695 functioning and iron homeostasis exists.

8. The NRAMP6 protein functions as an iron and manganese transporter, as revealed by functional complementation of yeast mutants defective in Fe or Mn transport. Consistent with the metal transport properties of NRAMP6, molecular modeling of the rice NRAMP6 protein revealed a high similarity to the three-dimensional structure of the *Staphylococcus capitis* DMT, a member of the NRAMP (Scl11) family that has been structurally and functionally characterized. The short NRAMP6 protein is a functional protein involved in Fe and Mn transport.

9. The NRAMP6 proteins (l-NRAMP6 and s-NRAMP6 isoforms) localize at the plasma membrane, implying that this protein might function in the import/export of cellular iron and/or Mn.

10. Knock-out mutant plants for *OsNramp6* in which *l-Nramp6* accumulation was abolished (whereas *s-Nramp6* levels remain unchanged) enhances resistance to *Magnaporthe oryzae*. Thus, changes in the expression of either *l-Nramp6* (knock-out *nramp6* mutant) or *s-Nramp6* (transgenic lines overexpressing *MIR7695*) has an impact on disease resistance in rice plants.

11. During infection by the rice blast fungus there is a small increase in iron content. Knowing that NRAMP6 functions as an iron transporter it can be hypothesized that alterations in *OsNramp6* expression might contribute to modulation of iron content in *M. oryzae*-infected leaves, which in turn, might have an impact on blast resistance.

12. Loss of *OsNramp6* function in *nramp6* rice plants affects rice growth and biomass production suggesting that *OsNramp6* might also play a role in rice development.

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ANNEX I

Research

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Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the *Nramp6* (*Natural resistance-associated macrophage protein 6*) gene involved in pathogen resistance

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Summary

• Plants have evolved efficient defence mechanisms to defend themselves from pathogen attack. Although many studies have focused on the transcriptional regulation of defence responses, less is known about the involvement of microRNAs (miRNAs) as post-transcriptional regulators of gene expression in plant immunity. This work investigates miRNAs that are regulated by elicitors from the blast fungus *Magnaporthe oryzae* in rice (*Oryza sativa*).

• Small RNA libraries were constructed from rice tissues and subjected to high-throughput sequencing for the identification of elicitor-responsive miRNAs. Target gene expression was examined by microarray analysis. Transgenic lines were used for the analysis of miRNA functioning in disease resistance.

• Elicitor treatment is accompanied by dynamic alterations in the expression of a significant number of miRNAs, including new members of annotated miRNAs. Novel miRNAs from rice are proposed. We report a new rice miRNA, osa-miR7695, which negatively regulates an alternatively spliced transcript of *OsNramp6* (*Natural resistance-associated macrophage protein 6*). This novel miRNA experienced natural and domestication selection events during evolution, and its overexpression in rice confers pathogen resistance.

• This study highlights an miRNA-mediated regulation of *OsNramp6* in disease resistance, whilst illustrating the existence of a novel regulatory network that integrates miRNA function and mRNA processing in plant immunity.

Introduction

Plants possess an innate immune system that efficiently detects potential microbial pathogens. The initiation of plant defence responses depends on the recognition of pathogen epitopes, known as pathogen-associated molecular patterns (PAMPs), or elicitors, by host-encoded surface receptors (Chisholm *et al.*, 2006; Jones & Dangl, 2006; Boller & He, 2009). This recognition activates a complex process in which different signalling cascades operate, leading to a massive reprogramming of the transcriptome. The regulation of immune response genes has been studied mostly at the transcriptional level. However, several recent studies have indicated that plants additionally use post-transcriptional regulation of defence responses against pathogens and that host endogenous small RNAs appear to be essential in this gene expression reprogramming process (Ruiz-Ferrer & Voinnet, 2009; Katiyar-Agarwal & Jin, 2010; Staiger *et al.*, 2012).

Small RNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), regulate gene expression in a sequence-specific manner (Baulcombe, 2004; Xie *et al.*, 2005; Jones-Rhoades *et al.*, 2006; Voinnet, 2009). Plant miRNAs are produced from precursors with unique stem–loop structures which are sequentially processed by the RNase III DICER-like 1 (DCL1) to give rise to an miRNA–miRNA* duplex. Alternative pathways for miRNA biogenesis involving DCL4 or DCL3 have also been described (Rajagopalan *et al.*, 2006; Vazquez *et al.*, 2008). The miRNA–miRNA* duplex intermediates are translocated to the cytoplasm, where the miRNA guide strand is selectively incorporated into an ARGONAUTE1 (AGO1)-containing RNA-induced silencing complex (RISC) (Baumberger & Baulcombe, 2005; Xie *et al.*, 2005; Jones-Rhoades *et al.*, 2006; Vaucheret, 2008). miRNAs direct post-transcriptional gene silencing by triggering the cleavage or translational repression of the target transcripts (Llave *et al.*, 2002; Brodersen *et al.*, 2008).

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They have been proven to influence temporal changes in target gene expression in developmental processes, as well as in abiotic stress tolerance and nutrient starvation (Palatnik *et al.*, 2003; Mallory *et al.*, 2004; Sunkar *et al.*, 2008; Jagadeeswaran *et al.*, 2009; Ruiz-Ferrer & Voinnet, 2009; Jeong *et al.*, 2011). Evidence for miRNAs controlling pathogen resistance came first in Arabidopsis plants, where perception of the bacterial flagellin flg22 causes an increase in miR393 accumulation which negatively regulates transcripts for F-box auxin receptors. Repression of auxin signalling results in bacterial resistance (Navarro *et al.*, 2006). More recently, miRNAs that guide the cleavage of disease resistance (*R*) genes in Solanaceae and Leguminosae species have been described (Zhai *et al.*, 2011; Li *et al.*, 2012; Shivaprasad *et al.*, 2012).

Most of the miRNAs that were discovered in early reports are highly conserved throughout the plant kingdom and have conserved functions in the regulation of developmental processes (Jones-Rhoades *et al.*, 2006). It is also generally assumed that plants express species-specific miRNAs that might play a regulatory role in a time- and/or spatial-restricted manner, or in specific biological processes (Rajagopalan *et al.*, 2006; Fahlgren *et al.*, 2007; Cuperus *et al.*, 2011). One important challenge now is to identify novel species-specific miRNAs, and to elucidate their biological function, in crop species that undergo major environmental stresses.

Rice (*Oryza sativa*) is a species of evident interest for miRNA analysis, not only because of its worldwide agricultural importance, but also because it represents the model plant for research in monocotyledonous species with a sequenced genome. Moreover, rice has a long history of natural selection and selective breeding, thus providing an excellent system for studies on the molecular evolution and selection of plant miRNAs. In the present study, we sequenced small RNA libraries from rice tissues (leaves, roots) that had been treated with elicitors obtained from the rice blast fungus *Magnaporthe oryzae*, the causal agent of the rice blast disease (Talbot, 2003). A diverse set of known miRNAs, both conserved and nonconserved, was found to be responsive to elicitor treatment. We also describe novel miRNA candidates. In particular, we report the functional characterization of a novel miRNA from rice, osa-miR7695, which negatively regulates an alternatively spliced transcript of the *OsNramp6* (*Natural resistance-associated macrophage protein 6*) metal transporter gene. Overexpression of the newly identified miRNA results in enhanced resistance to pathogen infection in rice plants. Finally, we show that this novel miRNA experienced natural and domestication selection events during rice evolution.

Materials and Methods

Plant material and elicitor treatment

Rice plants (*Oryza sativa* L. cv Nipponbare) were grown at $28 \pm 2°C$ with a 16 h : 8 h light : dark cycle. Elicitors from the *M. oryzae* strain 18.1 were prepared as described by Casacuberta *et al.* (1992) and used at a final concentration of 300 $\mu g\ ml^{-1}$. In all experiments, mock inoculations were performed. Cultivated rice varieties from different geographical locations, as well as wild rice accessions, were assayed. They were: *Oryza sativa* (21 accessions, including eight temperate *japonica*, six tropical *japonica* and seven *indica* accessions), *Oryza glaberrima* (two accessions) and wild rice species (24 accessions) (Supporting Information Table S1). Rice seeds were obtained from the International Rice Research Institute (IRRI, http://beta.irri.org/seeds/).

Small RNA library construction and sequencing

Total RNA was extracted from tissues (leaves, roots; 30 min and 2 h each tissue) of 15-d-old seedlings using TRIzol reagent (Invitrogen). Three biological replicates were analysed. Each library represented a pool of *c.* 50 rice plants. The construction of small RNA libraries has been reported previously (Donaire *et al.*, 2009). Amplicons were prepared by adaptor ligation in which the 5′ adaptor contained a 'barcode' for each sample. The same quantity of DNA amplicon from each library was pooled and sequenced using 454 Life Sciences Technology. A total of 383 397 row reads was produced. Computational analysis of reads containing recognizable adaptor sequences yielded 271 487 reads. The leaf libraries included 155 465 sequences: controls, 40 480 and 45 812 sequences (30 min and 2 h, respectively); elicitor-treated, 31 357 and 37 816 sequences (30 min and 2 h, respectively). The root libraries included 116 022 sequences: controls, 37 477 and 16 138 (30 min and 2 h, respectively); elicitor-treated, 28 677 and 33 730 (30 min and 2 h, respectively). A total of 96 069 unique small RNA sequences that perfectly matched the rice genome was identified in our libraries. The small RNA sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30583).

Data mining of the small RNA pool and prediction of new miRNAs

Small RNA sequences were parsed from FASTA-formatted files from 454 sequencing and assigned to each one of the eight specific libraries through identification of the small RNA/adaptor boundaries and barcode analysis. Small RNA sequences were mapped to the rice genome (*Oryza sativa*, version 5.0; http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules/info.shtml) using BLASTn. The unique RNA sequences that perfectly matched the genome were subjected to subsequent analysis. Reads showing identical sequences to known miRNAs from the miRBase database (http://www.mirbase.org, release 18.0, November 2011) were collected. The remaining sequences were considered for computational prediction and the identification of novel miRNAs. For this, genomic sequences spanning the putative miRNA, 1500 nucleotides upstream and downstream, were extracted and used for fold-back secondary structure prediction employing the RNAfold from the Vienna package version 2.0.0 with default parameters (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Criteria for the recognition of candidate miRNA precursors have been outlined elsewhere (Meyers *et al.*, 2008). The same procedure was used to search for orthologous sequences in the genome of different plant species. Sequences with up to

three mismatches were retrieved and subjected to fold-back secondary structure prediction as described above.

RNA analyses

For northern blot analysis of rice miRNAs, the low-molecular-weight fraction was obtained from total RNA by PEG8000/NaCl precipitation. RNAs were fractionated in a 17.5% denaturing polyacrylamide gel containing 8 M urea, transferred to nylon membranes and probed with [γ^{32}P]ATP-labelled oligonucleotides (Table S2). Hybridization signals were detected using a Phosphorimager (Bio-Rad). Synthetic RNA oligonucleotides were loaded as size markers.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in optical 96-well plates (Roche Light Cycler® 480; Roche Diagnostics, Mannheim, Germany) using SYBR Green I dye and the primers listed in Table S2. Data were normalized with *OsUbi1* (AK121590) as internal control. Three independent biological samples and three technical replicates per sample were analysed. When appropriate, Student's *t*-test/ANOVA was used to determine the statistical significance of the differential expression patterns ($P \leq 0.05$). Semi-quantitative RT-PCR of miR156 precursors and amplification of the osa-miR7695 precursor transcript are detailed in Methods S1.

Transient expression assays in *Nicotiana benthamiana* leaves and rice transformation

The DNA fragment containing the *osa-MIR7695* precursor was amplified by PCR from rice (*O. sativa* cv Nipponbare) genomic DNA, and used in transient expression assays in *N. benthamiana* leaves. To avoid the transgene-derived production of siRNAs, the *rdr6IR-N. benthamiana* line was used in these studies (Schwach *et al.*, 2005). Details on construct preparation, agroinfiltration and expression analysis of osa-miR7695-related small RNAs are indicated in Methods S1. Transgenic rice plants were produced by *Agrobacterium*-mediated transformation of embryogenic calli derived from mature embryos (Methods S1).

Target prediction

Target prediction for rice miRNAs was performed using the psRNATarget program (http://bioinfo3.noble.org/psRNATarget) with default parameters. Target sequences were searched for matching in the *O. sativa* cDNA set provided by The Institute for Genomic Research (TIGR, Rice Annotation Release 5.0). All predicted target genes were evaluated by scoring, and the criteria used were as follows: each G : U wobble pairing was assigned 0.5 points, each indel was assigned 2.0 points and all other noncanonical Watson–Crick pairings were assigned 1.0 point each. A penalty score of ≤ 3.0 points was considered in our analysis.

Microarray analysis

The GeneChip® rice genome array (Affymetrix, Santa Clara, CA, USA) was used for transcript profiling. Expression studies were performed in leaf tissues, treated or not with elicitors of *M. oryzae* (30 min and 2 h of treatment). In each case, total RNA was isolated from three different biological replicates, and each replicate was independently hybridized to the rice microarray. The statistical analysis of microarray data is presented in Methods S1. Genes with $P \leq 0.05$ and fold changes ≥ 1.2 in all replicates were considered. Microarray data have been deposited in the NCBI GEO database (GSE30583).

Blast resistance assays

Resistance to infection by the rice blast fungus *M. oryzae* strain Guy11 (courtesy of Ane Sesma; Sesma & Osbourn, 2004) was determined using the detached leaf assay, as described previously (Coca *et al.*, 2004). Further experimental details can be found in Methods S1.

Results

Deep sequencing of small RNA populations from rice leaves and roots

To obtain a genome-wide comprehensive survey of miRNAs in rice showing responsiveness to fungal elicitors, we constructed small RNA libraries from leaves and root tissues that had been treated, or not, with elicitors prepared from the rice blast fungus *M. oryzae* (30 min or 2 h of elicitor treatment). Libraries containing unique barcodes were combined and subjected to pyrosequencing (454 Life Sciences™; Roche). After trimming the adaptor sequences, distinct sequences perfectly matching the rice genome were identified. The size distribution of small RNAs was determined in each tissue on the basis of both total abundances and unique sequences. In terms of total sequence abundance, the 24-nucleotide class of small RNAs was the most abundant class in leaves, whereas the 21-nucleotide class was predominant in roots (Fig. 1a, left panel). When the size distribution of unique small RNA sequences was determined, we found that the 24-nucleotide sequences prevailed in both tissues (Fig. 1a, right panel).

A sequence similarity search of the sequencing data against the central miRBase registry allowed us to identify up to 114 known miRNAs or miRNAs* representing 63 miRNA families in our rice libraries, which included both conserved and nonconserved miRNAs (Table 1). By sorting the reads according to the barcode added in 454 primers, we determined the accumulation of each miRNA in each tissue (roots, leaves) (Fig. 1b and Table S3). miR168a and miR167defghij were the most abundant miRNAs in rice leaves (Fig. 1b). A good correlation occurred between the frequency observed for miRNAs in the 454 dataset and their expression level, as determined by northern blot analysis of selected miRNAs in rice leaves (Fig. S1). However, caution should be taken when interpreting the expression levels for those miRNAs with a low number of reads in the 454 sequencing dataset.

This study also revealed the presence of miRNA sequences representing new members of known miRNA families, namely

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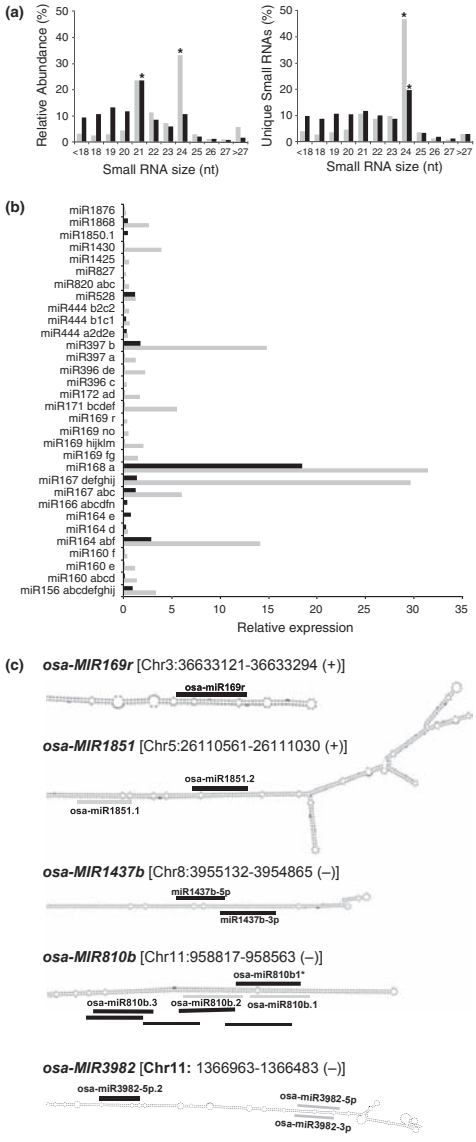

Fig. 1 Abundance of small RNA sequences, expression profiling of known microRNAs (miRNAs) from rice (*Oryza sativa*) and novel members of known miRNA families identified in this work. (a) Total abundance of small RNA sequences for each size class (left panel) and size distribution of unique small RNAs (right panel). Asterisks denote the predominant small RNA class. nt, nucleotide. (b) Expression profiling of miRNAs. Reads retrieved from the 454 sequencing dataset for each miRNA were normalized against the total count of reads obtained in the corresponding library. Only miRNAs showing 20 or more reads are presented. In (a, b), grey bars represent leaf tissue and black bars denote root tissue. (c) Precursor fold-back structures of novel members of known miRNA families. Grey bars denote the annotated sequences in the miRBase (miRBase version 18.0; November 2011). Black bars indicate the newly identified miRNAs in either leaf (thick bars) or root (thin bars) tissues. The nucleotide sequence of these precursor structures is shown in Supporting Information Fig. S2.

miR169r, miR1851.2, miR1437b, miR810b and miR3982 (Figs 1c, S2). Concerning the miR810 and miR3982 precursor structures, the annotated sequence for each precursor is extended relative to the annotated sequence in the miRBase.

Computational identification and experimental validation of novel miRNAs

Criteria for the annotation of novel plant miRNAs are based on both computational and experimental data. They include the excision from a stem–loop precursor structure, *dcl* dependence and conservation among species of both the stem–loop secondary structure and the mature miRNA sequence (Meyers *et al.*, 2008). In the absence of genetic tools (i.e. *dcl* mutants), sequencing of both miRNA and miRNA* is recommended. Nonetheless, some rice miRNAs are registered solely on the basis of computational prediction or with little evidence of expression, and their authenticity is not certain.

To identify novel miRNAs from rice, we scanned the rice genome for stem–loop hairpin structures comprising the small RNA sequences identified in our libraries. For each small RNA sequence that had a perfect match in the rice genome, we determined the ability of the surrounding genomic sequences to fold into stem–loop hairpin structures. By using a maximum length of 3 kb, 219 loci that fulfilled the hairpin structure criterion for miRNA precursors were identified (Figs S3, S4; Table S4). The stem region for most of these precursor structures showed a high degree of complementarity, a feature characteristic of young recently evolved *MIR* genes (Vazquez *et al.*, 2008). In those cases in which several small RNAs mapped at different positions along the same fold-back structure, the various small RNAs were consolidated into a single prospective *MIRNA* locus. In this respect, the production of two or more miRNAs from an miRNA precursor has been described already in rice and Arabidopsis (Zhu *et al.*, 2008; Zhang *et al.*, 2010). However, although we provide bioinformatics evidence for the 219 miRNA precursor structures supported by small RNA profiling, these miRNA precursor candidates should still be considered as miRNA candidates (accordingly, the names of the stem–loop precursors identified in this work are hyphenated to distinguish them from annotated miRNAs).

A search in the genome of different plant species revealed that 20 of the 219 miRNA candidates from rice have orthologue sequences in the genome of at least one of the other plant species tested (Table 2). In each plant species, the genomic regions surrounding the small RNA sequence also possessed intramolecular

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Table 1 Known microRNAs (miRNAs) present in small RNA libraries from rice (*Oryza sativa*) leaves and roots

			Monocot				Dicot				
Family	miRNA	miRNA*	*Zm*	*Sb*	*Ta*	*Hv*	*Mt*	*Pt*	*At*	*Gm*	*Vv*
156	miR156abcdefghij, miR156d	miR156i*, miR156hj*	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
159	miR159ab, miR159f	miR159a.1*	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
160	miR160abcd,miR160e, miR160f		(+)	(+)	(+)	(–)	(+)	(+)	(+)	(+)	(+)
162	miR162a, miR162b		(+)	(+)	(–)	(–)	(+)	(+)	(+)	(+)	(+)
164	miR164abf, miR164c, miR164d, **miR164e**		(+)	(+)	(+)	(–)	(+)	(+)	(+)	(+)	(+)
166	miR166abcdfn, miR166gh, miR166m	miR166c*, miR166n* miR166g*	(+)	(+)	(–)	(+)	(+)	(+)	(+)	(+)	(+)
167	miR167abc, miR167defghij, miR167e, miR167f		(+)	(+)	(+)	(–)	(+)	(+)	(+)	(+)	(+)
168	miR168a		(+)	(+)	(–)	(+)	(+)	(+)	(+)	(+)	(+)
169	miR169a, miR169bc, miR169 fg, miR169hijklm, miR169no		(+)	(+)	(–)	(+)	(+)	(+)	(+)	(+)	(+)
	miR169r[a]										
	miR169i.2		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
171	miR171bcdef, miR171f, **miR171h**, miR171i	miR171f*	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
172	miR172ad		(+)	(+)	(–)	(–)	(+)	(+)	(+)	(+)	(+)
319	**miR319a.2**[a]		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
390	miR390	miR390*	(+)	(+)	(–)	(–)	(+)	(+)	(+)	(+)	(+)
393	miR393, miR393b	miR393b*	(+)	(+)	(–)	(–)	(+)	(+)	(+)	(+)	(+)
394	miR394		(+)	(+)	(–)	(–)	(–)	(+)	(+)	(+)	(+)
396	**miR396ab**, miR396c, miR396de		(+)	(+)	(–)	(–)	(+)	(+)	(+)	(+)	(+)
397	miR397a, miR397b	miR397b*	(+)	(+)	(–)	(+)	(–)	(+)	(+)	(–)	(+)
399	miR399d		(+)	(+)	(+)	(–)	(+)	(+)	(+)	(–)	(+)
408	miR408	miR408*	(+)	(+)	(+)	(–)	(–)	(+)	(+)	(–)	(+)
435	miR435	miR435*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
444	miR444a2d2e, miR444b1c1, miR444b2c2, miR444d3		(–)	(–)	(+)	(+)	(–)	(–)	(–)	(–)	(–)
528	miR528		(+)	(+)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
529	miR529[b]		(+)	(+)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
535	miR535		(–)	(–)	(–)	(–)	(–)	(+)	(–)	(–)	(+)
810	miR810b1, miR810b2, miR810b[a]	miR810b1*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
812	miR812ghij		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
820	miR820abc	miR820c*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
827	miR827ab		(+)	(–)	(–)	(–)	(–)	(+)	(+)	(–)	(–)
1317	miR1317-5p, miR1317-3p		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1318	miR1318		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1423	miR1423, miR1423b		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1425	miR1425		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1427	miR1427		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1429	miR1429-3p		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1430	miR1430		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1437		miR1437*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1849		miR1849*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1850	miR1850.1		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1851	miR1851.2[a]		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1856	miR1856		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1861	**miR1861hj**		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1862	miR1862d, **miR1862e**	miR1862d*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1863	miR1863	miR1863*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1865	miR1865-5p		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1868	miR1868	miR1868*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1870	**miR1870**		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1871	miR1871		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1873	miR1873		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1876	miR1876		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1878	miR1878		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1879	miR1879		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1883	miR1883ab	miR1883a*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1884	**miR1884b**		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
2863	miR2863c		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
2869		miR2869*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)

Table 1 (Continued)

Family	miRNA	miRNA*	Monocot				Dicot				
			Zm	*Sb*	*Ta*	*Hv*	*Mt*	*Pt*	*At*	*Gm*	*Vv*
2871		miR2871a*	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
2873	miR2873		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
2877	miR2877		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
3979	**miR3979-3p**		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
3982	miR3982-3p		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
	miR3982-5p.2[a]										
5144	miR5144-5p	miR5144-3p	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
5150	miR5150-5p	miR5150-3p	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
5508	miR5508		(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)

Annotated miRNAs or miRNAs* whose sequences were represented at least three times in one or more of the rice libraries are included. miRNA sequences found in root, but not in leaf, tissues are shown in bold. Conservation among different plant species is shown (+ and –, identical and nonconserved sequences, respectively). *Zm, Zea mays; Sb, Sorghum bicolor; Ta, Triticum aestivum; Hv, Hordeum vulgare; Mt, Medicago truncatula; Pt, Populus trichocarpa; At, Arabidopsis thaliana; Gm, Glycine max; Vv, Vitis vinifera.*

[a]New member of the indicated family.

[b]This sequence was annotated as miR529* in miRBase, but was proposed to be the functional miRNA (Zhu *et al.*, 2008).

Table 2 Nucleotide sequences and chromosomal locations of previously uncharacterized miRNAs from rice (*Oryza sativa*) that are conserved in other plant species

Precursor	Sequence	Organism		Chr	Start	End	Strand
		Monocot	Dicot				
miR-2	CGGAGCCGGUGGUGGCGGUGG	*Sb*		Chr1	2201435	2201515	(+)
miR-35	AGAUAAAUGGUCAAACAUAUGAGA		*Vv*	Chr3	34134892	34134650	(–)
miR-38	AUGACACCGUUGACUUCUUGACCA	*Zm*	*Mt*	Chr4	435880	435638	(–)
miR-40	ACUUUUGGAUAUGAUGUUUGACCA		*Pt*	Chr4	16475306	16475525	(+)
miR-58	AAGACAAGUGGUCAAAUAUUGCAA		*Pt*	Chr6	10118138	10117881	(–)
miR-65	AGUAGGUAGCAUAUAAGUAUGAGA		*Mt*	Chr6	22195674	22195395	(–)
miR-68	AGAAUAAGACGAAUGGUCAAACG	*Sb*, *Zm*		Chr6	27252346	27252589	(+)
miR-73	GACUUACAUGUUUGACCGUUCGUC	*Sb*		Chr7	22740067	22739835	(–)
miR-82	AAGACAGAUGGUCAAACGUUGGAA	*Sb*		Chr8	17061486	17061639	(+)
miR-83	AUACGAAUGGUCAAACAUGUAAGA	*Sb*	*Vv*	Chr8	18542561	18542802	(+)
miR-87	AUAAGACGGGUGAUCAAAGUUGGG	*Sb*, *Zm*		Chr9	10650766	10650539	(–)
miR-117	ACGGGUUUUGAUAGUUGAGGGAUC	*Sb*		Chr1	7261342	7261710	(+)
miR-122	AAGACAUGUGUAUAUGAUAGGUGA AGACAUGUGUAUAUGAUAGGUGAG	*Zm*	*Mt*	Chr1	32569375	32569766	(+)
miR-157	CCCUUGGCUGUGGAGAGAGAGA GCGGGGGUGAGCUGCGAUGGG	*Sb*	*Pt*	Chr5	1410235	1410880	(+)
miR-163	GGAGUCUGACAUGCGUGCGAGUC GAGUCUGACAUGCGUGCGAGUC GAGUCUGACAUGCGUGCGAGUCGA UGUCGGGACCCGAAAGAUGGU UCUAGUAGCUGGUUCCCUCCG		*At*, *Mt*	Chr6	8365563	8366436	(+)
miR-167	AGAGACCGGGAUGACACAUGCGAA	*Sb*		Chr6	27547369	27546632	(–)
miR-171	ACAGUCGAACAAGUAUGAGGACCU	*Sb*		Chr7	30019510	30020256	(+)
miR-182	UGUAGUCUGCAAGGAGAAGGC		*Mt*	Chr9	15008303	15009268	(+)
miR-207	GGGAAAUCACGUGAAAGUUAUGAG		*Pt*	Chr2	23602921	23603940	(+)
miR-218	UGGCGCGGAGGCCGCGGCGGUG	*Sb*, *Zm*		Chr8	5990432	5988987	(–)

The sequences given represent the small RNA sequences identified in the 454 dataset obtained from the leaf and root libraries whose precursor sequences have the capacity to adopt hairpin structures in rice as well as in the other plant species, monocots or dicots. A search for miRNA sequence homology was performed by BLASTN against National Center for Biotechnology Information (NCBI) genomes by allowing zero to three nucleotide substitutions. For those sequences mapping in the genome of any other species, the surrounding genomic sequences were analysed to confirm their ability to form fold-back structures. Their precursor structures are shown in Supporting Information Figs S3 and S4 (boxed in blue colour). *At*, *Arabidopsis thaliana*; *Mt*, *Medicago truncatula*; *Pt*, *Populus trichocarpa*; *Sb*, *Sorghum bicolor*; *Vv*, *Vitis vinifera*; *Zm*, *Zea mays*).

folding capacities, thus indicating that these hairpin-forming precursors might well represent previously uncharacterized miRNAs from rice.

In this work, six of the 219 hairpin-forming structures were selected for experimental validation. Northern blot analysis demonstrated that small RNAs mapping to all six selected precursor

structures accumulated in one or another rice tissue, some of these precursor structures generating various small RNAs (Fig. 2). The names assigned in the miRBase registry for the novel miRNAs identified in this work were osa-miR7692, osa--miR7693, osa-miR7694 and osa-miR7695. Three of the new miRNAs (osa-miR7692, osa-miR7694 and osa-miR7695) were found in leaf libraries, whereas osa-miR7693 appeared in leaf and root libraries. As for the two other experimentally validated miRNAs (osa-miR5150 and osa-miR5144), Chen *et al.* (2011) identified these particular miRNAs in a population of small RNAs from *in vitro*-cultured rice embryogenic calli. These two miRNAs were also present in our small RNA libraries from vegetative rice tissues (leaves, roots), and their accumulation has been validated experimentally in this work (Fig. 2).

Elicitor responsiveness of rice miRNAs

Further analysis of the sequencing data revealed that treatment with fungal elicitors is accompanied by alterations in the accumulation of a repertoire of rice miRNAs, including miRNAs that are recognized as major regulators of gene expression in developmental processes (Fig. 3a,b). In some cases, the elicitor responsiveness of a miRNA was in the same direction in the two tissues (e.g. miR164abf and miR168a), whereas, in other cases, a different response to elicitors occurred depending on the tissue (e.g. miR156a-j and miR160abcd). Moreover, a different response could be observed at one or another time of elicitor treatment for particular miRNAs (e.g. miR159ab, Fig. 3a). Of note, the expression of miR168 was found to be up-regulated by fungal elicitors in the two rice tissues (elicitor responsiveness of miR168 was further confirmed by northern blot analysis; Fig. S5). Knowing that miR168 controls AGO1 homeostasis (Vaucheret *et al.*, 2006), this finding anticipates an important role of miR168 functioning in the rice response to fungal elicitors.

This analysis also revealed different expression patterns among members of a particular miRNA family (Fig. 3). In those cases in which several members of a family share the same mature miRNA sequences (i.e. miR156abcdefghij, miR160abcd or miR171bcdej), the 454 sequencing data do not provide information on which specific member is under tissue-specific

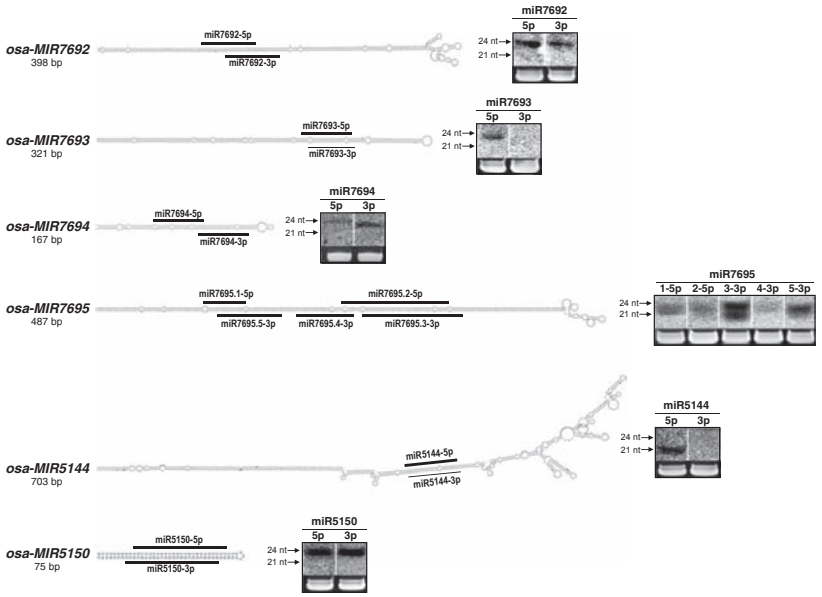

Fig. 2 Predicted hairpin structures and experimental validation of novel microRNA (miRNA) candidates from rice (*Oryza sativa*). Small RNA sequences recovered from 454 sequencing data mapping into these structures are represented by black bars (thick bars, leaf libraries; thin bars, root libraries). Following miRNA nomenclature (Meyers *et al.*, 2008), we named the small RNA sequences within the hairpin structure as miR-*x*.*y*, where *x* denotes a number for a particular miRNA precursor and *y* specifies the position of each sequenced small RNA along the precursor starting from the 5′ end (the suffix -5p or -3p was used to refer to the mapping arm within the stem–loop). Northern blot analysis of small RNAs, and corresponding ethidium bromide staining, is shown on the right side. The small RNA fraction obtained from 100 to 350 μg of total RNA, depending on the miRNA, was probed with synthetic oligonucleotide sequences complementary to the indicated sequences. The experimental validation of two miRNAs previously identified in small RNA libraries from *in vitro*-cultured rice embryogenic calli (Chen *et al.*, 2011), osa-miR5144 and osa-miR5150, is also shown.

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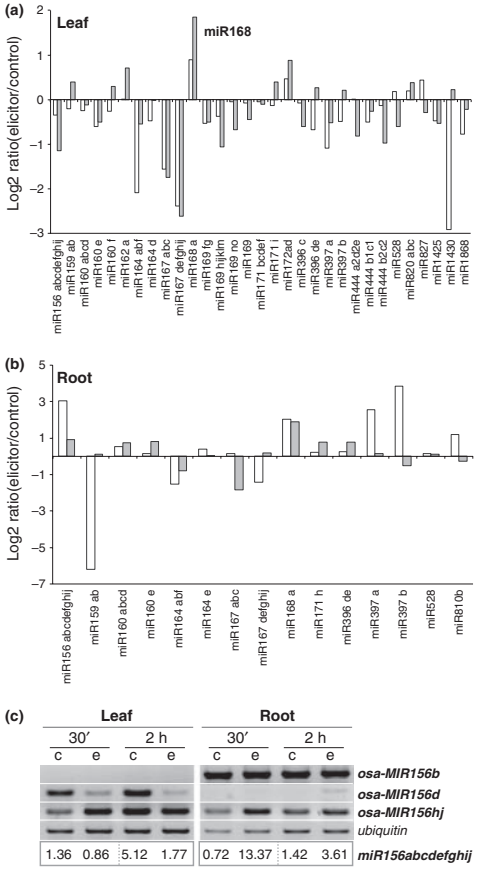

Fig. 3 Expression and elicitor responsiveness of known microRNAs (miRNAs) from rice (*Oryza sativa*). (a, b) Fold change of miRNAs in elicitor-treated leaves (a) and roots (b) relative to nontreated tissues at 30 min or 2 h of treatment (light and dark bars, respectively). Fold change was calculated on the basis of normalized reads + 1 (treated vs untreated tissues). Only miRNAs represented by 20 or more reads in the datasets are shown. (c) Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) of *MIR156* precursors generating the same mature miRNA sequence (miR156abcdefghij) (c, control; e, elicitor-treated). Numbers in the lower part indicate the normalized relative abundance in the 454 sequencing data in each tissue and condition.

control and/or exhibits elicitor responsiveness. In this work, we examined the expression of miRNA precursors for individual members of the *MIR156* family. Thus, *MIR156* has nine paralogous genomic loci that generate an identical mature miRNA sequence (miR156abcdefghij, miR156h and miR156j derive from the same locus). Semi-quantitative RT-PCR showed that the *osa-MIR156b* precursor is only expressed in roots, whereas *osa-MIR156d* is preferentially expressed in leaves (Fig. 3c). Although *osa-MIR156b* does not respond to elicitor treatment in roots, a significant reduction in *osa-MIR156d* accumulation occurred in elicitor-treated leaves relative to control leaves. Likewise, *osa-MIR156hj* exhibited a transient increase in elicitor-treated tissues (30 min of treatment) (Fig. 3c). Precursors for the other members of this family either accumulated at similar levels or were barely detected (data not shown). Reasonably, the reduction in miR156-related sequencing reads which was observed in elicitor-treated leaves relative to nontreated leaves might reflect the down-regulation of *osa-MIR156d* in this tissue. Likewise, the increase in the number of reads found in elicitor--treated roots might result from variations in the expression of *osa-MIR156hj*. Overall, these findings revealed a differential and dynamic regulation of the accumulation of an important number of rice miRNAs in response to treatment with fungal elicitors, which could also be observed among members of a particular miRNA family.

Expression of elicitor-regulated miRNAs from rice correlates inversely with the expression of their target genes

Global transcript profiling was carried out to investigate whether changes in the accumulation of elicitor-responsive miRNAs are accompanied by inverse trends in target gene expression. This microarray analysis identified genes whose expression was affected significantly (up- and down-regulation) in response to elicitors ($P \leq 0.05$; fold changes $\geq$ 1.2-fold). Moreover, the examination of expression profiles of microarray data and 454-sequencing datasets revealed that a substantial number of validated target genes for known miRNAs exhibited an opposite response to elicitor treatment with respect to that of their corresponding regulatory miRNA (Table 3). Among them were miRNAs that target genes involved in protection against oxidative stress (e.g. miR528 and miR1879, targeting laccase and catalase, respectively) as well as genes that control plant development and hormone signalling (i.e. miR156, miR160, miR169 and miR393). This observation supports a functional interaction between these miRNAs and their corresponding target genes during the rice response to elicitor treatment. Consistent with the observed up-regulation of miR168 (Figs 3a, S5), microarray analysis confirmed the down--regulation of *AGO1* genes in response to elicitor treatment (Table 3). Presumably, a pathogen-regulated adjustment of miR168 levels would contribute to the maintenance of the appropriate levels of AGO1, and accordingly of miRNA functioning, during the plant response to fungal elicitors.

The target genes for four of the experimentally validated miRNAs (osa-miR7692, osa-miR7693, osa-miR7694 and osa-miR7695) were predicted using the psRNATarget program (http://bioinfo3.noble.org/psRNATarget). Several predicted mRNA targets possessed a function coherent with plant response to pathogen infection, that is, genes involved in protection against oxidative stress and detoxification, disease resistance and receptor protein kinase genes (Table S5). Moreover, a search in the Genevestigator database (http://www.genevestigator.com) revealed a pathogen-associated expression for most predicted

Table 3 Elicitor responsiveness of rice (*Oryza sativa*) microRNAs (miRNAs) and their target genes

		Direction of miRNA expression				
	miRNA	30 min	2 h	Target gene		Fold change
156	miR156abcdefghij	Down	Down	SBP domain-containing protein (OsSPL4) (SBP TF)[c,d,e]	LOC_Os02g07780	+1.65 (30′)
160	miR160abcd, miR160e	Down	Down	Auxin response factor (ARF10) (ARF TF)[c,d,e]	LOC_Os06g47150	+1.20 (30′)
	miR160f	–	Up	Auxin response factor (ARF16) (ARF TF)[c,d,e]	LOC_Os10g33940	−1.37 (2 h)
164	miR164abf, miR164c, miR164d	Down	Down	NAC domain-containing protein (NAC TF)[c,d,e]	LOC_Os06g23650	+1.33 (2 h)
				NAC domain-containing protein (NAC TF)[c,d,e]	LOC_Os12g41680	+1.25 (30′)
166	miR166m	Down	–	START domain-containing protein (HB TF)[c,d,e]	LOC_Os03g01890	+1.33 (30′)
167	miR167abc, miR167defghij	Down	Down	Retinol dehydrogenase 14 [c]	LOC_Os06g03830	+1.31 (30′)
168	miR168a	Up	Up	ARGONAUTE1 protein (AGO1)[c,e]	LOC_Os04g47870	−1.44 (2 h)
				ARGONAUTE1 protein (AGO1)[c,e]	LOC_Os02g58490	−1.32 (2 h)
169	miR169a	Up	Up	Nuclear transcription factor Y subunit (NF-YA)[c,d,e]	LOC_Os02g53620	−1.24 (30′)
	miR169bc, miR169 fg, miR169hijklm	Down	Down	Nuclear transcription factor Y subunit (NF-YA)[c,d,e]	LOC_Os12g42400	+1.26 (2 h)
	miR169no, miR169r [a]	Down	Down	Nuclear transcription factor Y subunit (NF-YA)[c,d,e]	LOC_Os12g42400	+1.26 (2 h)
171	miR171i	Up	Up	SCARECROW gene regulator (SCL) (GRAS TF)[c,d,e]	LOC_Os06g01620	−1.23 (2 h)
390	miR390	Down	Down	STRUBBELIG-RECEPTOR FAMILY 6	LOC_Os03g51040	+1.25 (2 h)
				Wall-associated receptor kinase-like 10	LOC_Os04g30060	+1.91 (2 h)
				Leucine-rich repeat (LRR) family protein	LOC_Os04g45170	+1.46 (30′)
393	miR393b	Down	Down	Transport inhibitor response 1 protein (TIR1)[c,d,e]	LOC_Os05g05800	+1.36 (30′)
394	miR394	–	Up	RNA polymerase sigma factor rpoD[e]	LOC_Os05g51150	−1.31 (2 h)
396	miR396c	Down	Down	Growth-regulating factor (GRF TF)[c,d,e]	LOC_Os06g10310	+1.25 (30′)
				Growth-regulating factor (GRF TF)[c,d,e]	LOC_Os03g51970	+1.20 (2 h)
				Deaminase[c]	LOC_Os06g29430	+1.17 (30′)
444	miR444b1c1, miR444b2c2	Down	Down	MADS-box transcription factor (MADS TF)[c,d,e]	LOC_Os02g49840	+1.28 (30′)
528	miR528	Up	Down	Laccase	LOC_Os01g62600	−1.20 (30′)
				Copper ion binding protein	LOC_Os01g03620	−1.26 (30′)
				Copper ion binding protein	LOC_Os01g03640	+1.20 (2 h)
529	miR529[b]	Down	Down	SBP-box gene family member	LOC_Os02g07780	+1.65 (30′)
820	miR820abc	Up	Up	Cellulose synthase like C12	LOC_Os11g13650	−1.22 (30′)
827	miR827ab	Up	Down	OsWAK receptor-like protein kinase	LOC_Os02g56370	+1.72 (2 h)
1425	miR1425	Down	Down	Protein kinase[c]	LOC_Os01g49614	+1.33 (2 h)
1430	miR1430	Down	Up	ASYMMETRIC LEAVES 2 (MYB TF)	LOC_Os05g34450	−1.23 (2 h)
				myb/SANT domain protein	LOC_Os03g13790	+1.20 (30′)
1850	miR1850.1	Up	Down	Pectinesterase inhibitor domain	LOC_Os08g04650	+1.20 (2 h)
1865	miR1865-5p	Down	–	Aspartate aminotransferase	LOC_Os02g14110	+1.27 (30′)
1876	miR1876	Up	Down	Esterase/lipase/thioesterase	LOC_Os02g31200	+1.48 (2 h)
1879	miR1879	Up	Up	Catalase isozyme B	LOC_Os06g51150	−1.60 (2 h)

Expression of known rice miRNAs in response to elicitor treatment, as determined by 454 sequencing (–, no alteration). The elicitor responsiveness of target genes was determined by microarray analysis. The fold change (elicitor-treated vs control nontreated tissue, $P \leq 0.05$) for the target gene(s) for each miRNA family is shown.

[a]New member of the indicated family.

[b]This sequence was annotated as miR529* in miRBase, but was proposed to be the functional miRNA (Zhu *et al.*, 2008).

[c,d,e]The target genes for these miRNAs have been described in the literature: [c]Li *et al.* (2010); [d]Wu *et al.* (2009); [e]Zhou *et al.* (2010). The remaining target genes are predicted but not experimentally validated. TF, transcription factor.

targets, including infection by *M. oryzae* (Table S5). It is also true that, although microarray analysis demonstrated opposite trends in the expression of known, highly conserved miRNAs relative to their corresponding target transcripts, we could not observe such opposite expression patterns between novel miRNA candidates and their predicted target genes. However, the comparative analysis of data generated by transcriptome analysis and small RNA profiling excludes target genes that are under translational repression by their regulatory miRNA or target genes that exhibit a dynamic response to elicitor treatment (transcription profiles were determined at only two time points of elicitor treatment).

osa-miR7695, a novel DCL4-processed miRNA from rice

In this work, osa-miR7695 was characterized in more detail. Initially, we demonstrated that the entire *osa-MIR7695* precursor structure is transcribed as a single transcriptional unit that comprises all five sequenced small RNAs mapping to this precursor (Fig. 4a,b). Moreover, *osa-MIR7695*-related small RNAs were consistently detected in leaves, but not in roots, of rice plants at different developmental stages (14-, 28- and 90-d-old rice plants; Fig. 4c).

Next, we examined the accumulation of *osa-MIR7695*-related species in the loss-of-function *Osdcl1* and *Osdcl4* genetic backgrounds (Liu B *et al.*, 2005, 2007). Although rice *dcl2, dcl3* and

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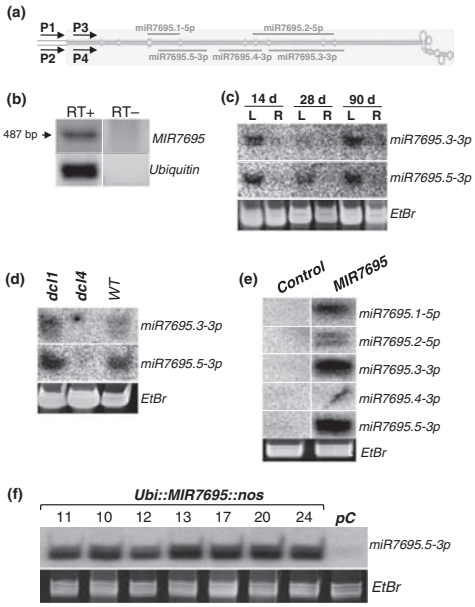

Fig. 4 Processing of the *osa-MIR7695* precursor and the production of transgenic rice (*Oryza sativa*) lines. (a) Structure of the *osa-MIR7695* precursor. Arrows indicate the primers used for PCR amplification in (b). (b) Detection of the *osa-MIR7695* precursor by nested reverse transcription polymerase chain reaction (RT-PCR). Sequencing of the 487-bp DNA fragment confirmed the specific amplification of the entire *osa-MIR7695* precursor, whilst revealing that *osa-MIR7695* is transcribed as a single transcriptional unit that comprises all five sequenced small RNAs. Control reactions without addition of the reverse transcriptase enzyme were included (RT–). (c) Accumulation of *osa-MIR7695*-related small RNAs (osa-miR7695.3-3p and osa-miR7695.5-3p) in leaves (L) and roots (R) of rice plants at different developmental stages (14, 28 and 90 d). Corresponding ethidium bromide (EtBr)-stained gels served as loading controls. (d) Northern blot analysis of *osa-MIR7695*-derived small RNAs in *dcl1* and *dcl4* rice mutants. The production of *osa-MIR7695*-related small RNAs is impaired in the *dcl4* mutant. (e) Functional analysis of the *osa-MIR7695* precursor in *Nicotiana benthamiana* leaves (*rdr6IR* line). The small RNA sequences produced from this precursor were detected by agroinfiltration of *N. benthamiana* leaves, followed by Northern blot analysis using oligonucleotides complementary to the five sequenced small RNAs produced by the *osa-MIR7695* precursor. No signals were detected in control leaves transformed with the empty pCAMBIA vector for any of the probes (control). (f) Accumulation of osa-miR7695.5-3p in leaves of independently generated rice lines overexpressing the *osa-MIR7695* precursor (*ubi::MIR7695::nos*) and control plants expressing the empty vector (pC). Results obtained for representative transgenic lines are presented. The same RNAs stained with EtBr are shown in the lower panel.

rdr knockdown rice mutants have been reported (Urayama *et al.*, 2010), this material was unavailable for our study. It is generally assumed that canonical 21-nucleotide miRNAs are generated by DCL1, and that, during evolution of *MIR* genes, a progressive shift in DCL usage from young to old *MIR* genes occurs, namely from DCL4/DCL3 to DCL1 (Voinnet, 2009). Of interest, the accumulation of osa-miR7695 species was found to be severely compromised in the *dcl4* mutant, but remained unaffected in the *dcl1* mutant (Fig. 4d). These findings, together with the observation that *osa-MIR7695* shows a high degree of complementarity in the stem–loop precursor structure, supports the notion that *osa-MIR7695* represents a novel, recently evolved miRNA-generating locus that is processed by DCL4 to produce multiple unique small RNAs (possible miRNAs and/or miRNA-like RNAs). Similar results have been reported previously for young miRNAs in other plant species (Rajagopalan *et al.*, 2006; Ben Amor *et al.*, 2009).

osa-miR7695 down-regulates the expression of an alternatively spliced transcript of the *Nramp6* gene

In order to identify the target gene(s) for osa-miR7695, we generated transgenic rice lines overexpressing the *osa-MIR7695* precursor. For this, the DNA fragment containing the *osa-MIR7695* fold-back structure was PCR amplified from genomic DNA and cloned into a plant expression vector. Before rice transformation, we confirmed that the cloned *osa-MIR7695* sequence was actually a functional source of *osa-MIR7695*-derived small RNAs. Indeed, transient expression assays in *N. benthamiana* leaves confirmed the processing of the *osa-MIR7695* precursor and the production of all five expected small RNAs (Fig. 4e; details on transient expression assays in *N. benthamiana* are given in Methods S1).

Transgenic rice was produced by *Agrobacterium*-mediated transformation. As controls, transgenic rice plants were transformed with the empty vector (pCAMBIA 1300). Northern blot confirmed that the transgenic lines accumulated higher levels of *osa-MIR7695*-related small RNAs (Fig. 4f). T2 homozygous progeny plants were obtained which were phenotypically indistinguishable from wild-type plants.

Based on target gene prediction, the osa-miR7695.3-3p and osa--miR7695.5-3p small RNAs showed extensive sequence complementarity with two rice genes: *Nramp6* (*Natural resistance-associated macrophage protein 6*) gene (Os01g31870) and a lectin--like receptor kinase gene (Os08g03002) (see Table S5). Concerning *OsNramp6*, cDNA data indicated that eight transcript variants were produced from this gene by alternative splicing (Fig. 5a). Among the various *OsNramp6* splice variants, only the shortest transcript variant Os01g31870.8 contained complementary sites for *osa-MIR7695*-derived small RNAs (osa-miR7695.5-3p and osa-miR7695.3-3p), which were located at the 3′ untranslated region of this transcript variant (Fig. 5b). Interestingly, the accumulation of short transcripts of *OsNramp6* (Os1g31870.8 splice variant) was found to be drastically reduced in transgenic rice lines overexpressing the *osa-MIR7695* precursor, which was indicative of an osa-miR7695-mediated regulation of this transcript variant (Fig. 5c). By contrast, no significant alterations were observed in the level of nontarget transcripts of *OsNramp6* (Os1g31870.4 splice variant) between transgenic and control plants (Fig. 5d). The accumulation of other *OsNramp6* transcript variants could not be accurately determined because of their extremely low level of expression in rice leaves. In addition, no significant alterations were observed in the expression of other genes that were predicted for *osa-MIR7695*-related small RNAs, such as the lectin-like receptor

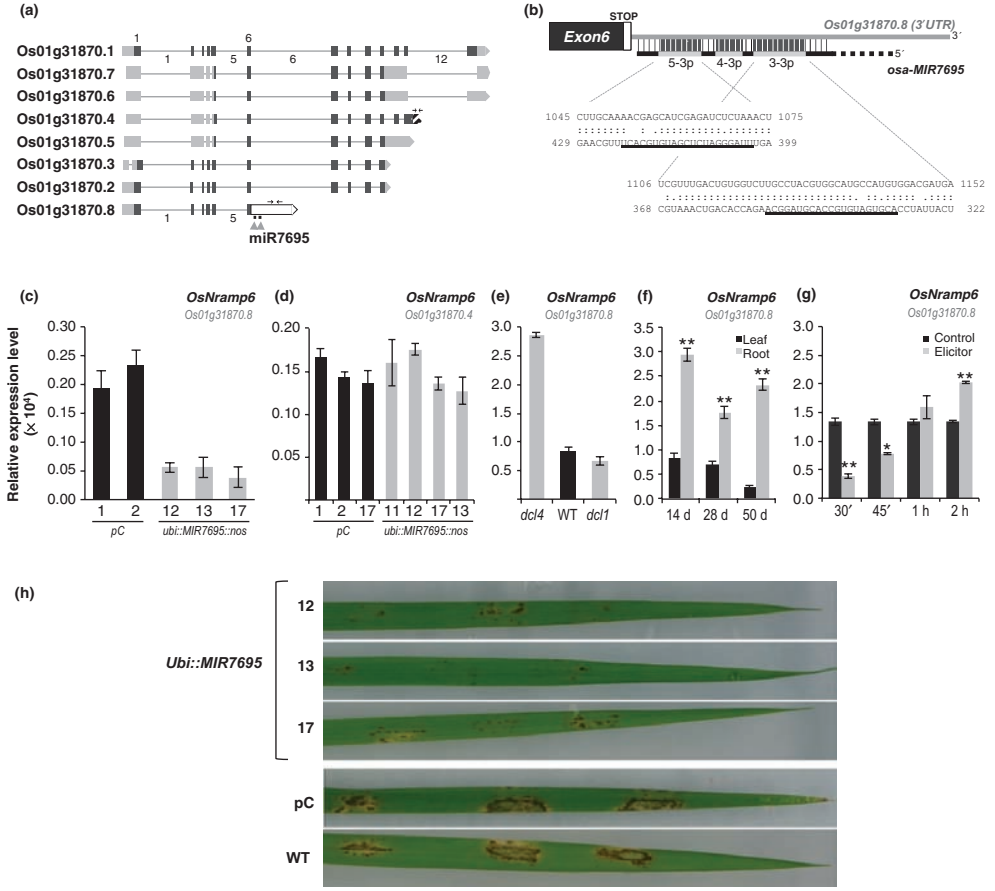

Fig. 5 osa-miR7695 targets an alternatively spliced transcript of the *OsNramp6* (*Natural resistance-associated macrophage protein 6*) gene. (a) Alternative splicing transcript variants of *OsNramp6*. The Os01g31870.1 transcript variant encodes the full-length protein and was taken as reference for intron/exon numbering. Only the short variant contains the target sites for osa-miR7695 (black bars at the 3′ untranslated region (UTR) of Os01g31870.8). Arrows in the 3′ region of Os01g31870.8 and Os01g31870.4 denote the primers used for expression analyses. (b) Complementarity of osa-miR7695.3p-related small RNAs with the 3′ UTR region of Os01g31870.8 transcripts. (c) Accumulation of short *OsNramp6* transcripts (Os01g31870.8) in leaves of rice plants overexpressing the *osa-MIR7695* precursor (*ubi::MIR7695::nos*) and vector control (pC) lines. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out using the *ubiquitin 1* (*Ubi1*) gene as the internal control. (d) Accumulation of *Os01g31870.4 OsNramp6* transcripts in transgenic rice lines overexpressing the *osa-MIR7695* precursor. (e) Accumulation of short *OsNramp6* transcripts (Os01g31870.8) in *dcl4* and *dcl1* mutants. (f) Accumulation of Os01g31870.8 *OsNramp6* transcripts in leaves and roots of wild-type rice (cv Nipponbare) plants. (g) Accumulation of short *OsNramp6* transcripts (Os01g31870.8) in control and elicitor-treated leaves of wild-type rice (cv Nipponbare) plants (black and grey bars, respectively). Each RNA was prepared from a pool of leaves from 50 rice plants. Differences in the accumulation levels were statistically significant (**, $P \leq 0.001$; *, $P \leq 0.05$). (h) Resistance of rice plants overexpressing the *osa-MIR7695* precursor to infection by the rice blast fungus *Magnaporthe oryzae*. Five independent osa-miR7695 transgenic lines, four independent vector control plants as well as Nipponbare (WT) plants were assayed with similar results. Leaves were locally inoculated with a *M. oryzae* spore suspension (10^5 spores ml^{-1}). Disease symptoms of leaves at 4 d post-infection are shown. Representative results from one of three independent experiments that produced similar results are presented. Error bars in (c–g) represent ± SD.

kinase (Os08g03002, predicted target gene for osa-miR7695.3-3p and osa-miR7695.5-3p), nucleotide-binding site–leucine-rich repeat (NBS-LRR) resistance (Os12g18360, predicted target gene for osa-miR7695.2-5p) and lipase (Os12g01030, predicted target gene for osa-miR7695.2-5p) genes, in any of the *osa-MIR7695* transgenic lines relative to control plants (data not shown).

Knowing that the *osa-MIR7695* precursor was processed in a DCL4-dependent manner, we assayed whether genetic inactivation of *DCL4* also had an effect on the accumulation of target transcripts of osa-miR7695. As shown in Fig. 5(e), qRT-PCR analysis revealed that short *OsNramp6* transcripts accumulated at significantly higher levels in *dcl4* plants relative to wild-type rice plants, indicating that suppression of *osa-MIR7695* precursor processing, and the subsequent production of *osa-MIR7695*-derived small RNAs, is accompanied by a higher accumulation of short *OsNramp6* transcripts. In contrast, the accumulation of short *OsNramp6* transcripts was not affected in the *dcl1* rice mutant relative to wild-type plants, which was consistent with the observation that DCL1 is not required for the processing of the *osa-MIR7695* precursor. In agreement with the finding that osa-miR7695 accumulates at high levels in leaves relative to roots in wild-type rice (see Fig. 4c), the short transcripts of *OsNramp6* (Os01g31870.8) accumulated at significantly higher levels in roots relative to leaves at the different developmental stages assayed in this work (Fig. 5f). Collectively, these results strongly suggest that osa-miR7695 down-regulates transcript levels of the short transcript variant of *OsNramp6* (Os01g31870.8).

Finally, we investigated the effect of elicitor treatment on the accumulation of short *OsNramp6* transcripts in rice leaves. Although these transcripts accumulated to almost identical levels in control untreated leaves, elicitor treatment was accompanied by changes in the accumulation of short *OsNramp6* transcripts during the period of elicitor treatment assayed in this work (Fig. 5g).

osa-miR7695 overexpression confers resistance to pathogen infection

To obtain further insights into the biological function of osa-miR7695, transgenic plants overexpressing the *osa-MIR7695* precursor were tested for resistance to infection with the fungal pathogen *M. oryzae*. Five independent T2 homozygous lines overexpressing *osa-MIR7695* were assayed. All the transgenic lines displayed enhanced disease resistance to pathogen infection relative to control plants (empty vector transgenic lines and wild-type plants) (representative results are presented in Fig. 5h). In agreement with the visual inspection, the inoculated leaves from *osa-MIR7695* transgenic lines exhibited a lower percentage of diseased leaf area relative to inoculated leaves from nontransformed plants (Fig. S6). Depending on the line, the leaves from *osa-MIR7695* transgenic lines exhibited 1.04–5.53% of their area affected by blast lesions at 4 d after inoculation. Under the same experimental conditions, leaves of control plants were affected in 15.21% of their area (Fig. S6). From this, it is concluded that osa-miR7695 accumulation positively regulates resistance to infection by the fungal pathogen *M. oryzae*.

osa-miR7695 occurs in *japonica*, but not *indica*, subspecies of cultivated rice

To investigate whether osa-miR7695 is conserved in plant species, its expression was analysed in other monocotyledonous species, as well as in dicotyledonous species. As shown in Fig. 6(a), osa-miR7695 could not be detected in any of the other plant species assayed here, suggesting that this miRNA may be specific to rice.

The genus *Oryza* comprises two cultivated and 22 wild species (http://www.gramene.org). The two cultivated species are *O. sativa* and *O. glaberrima*. Most of the cultivated rice varieties belong to the *O. sativa* group, which includes *japonica* and *indica* subspecies. *Oryza sativa japonica* is further differentiated into temperate *japonica* (*japonica*) and tropical *japonica* (*javanica*) varieties (Garris *et al.*, 2005). In the present study, a collection of cultivated *O. sativa* and *O. glaberrima* cultivars was surveyed for osa-miR7695 expression. This collection included temperate *japonica* (eight accessions), tropical *japonica* (six accessions) and *indica* (seven accessions) subspecies from *O. sativa*, as well as two *O. glaberrima* cultivars. Interestingly, *osa-MIR7695*-related small RNAs were detected in all the *japonica* subspecies of *O. sativa*, both temperate and tropical *japonica* subspecies, but remained below northern blot detection limits in all the *indica* subspecies analysed here (Fig. 6b). In *O. glaberrima* cultivars, only osa-miR7695.5-3p small RNAs (the 24-nucleotide species) were found to accumulate in the leaves, whereas osa-miR7695.3-3p could not be detected in these cultivars. When examining osa-miR7695 accumulation in wild rice species, and similar to that observed in cultivated *O. glaberrima*, only the 24-nucleotide species of osa-miR7695.3-3p could be detected in some, but not all, wild rice species, and none accumulated osa-miR7695.5-3p (Fig. 6c).

Finally, the observed accumulation of osa-miR7695 in *japonica* subspecies (temperate and tropical *japonica*) correlated well with a lower level of *OsNramp6* target transcripts (Os01g31870.8) relative to that observed in *indica* subspecies of *O. sativa* (Fig. 6d). Similarly, lower levels of *OsNramp6* target transcripts occurred in wild rice species in which osa-miR7695 could be detected relative to those in which osa-miR7695 could not be detected (Fig. 6e).

Altogether, these results suggest that osa-miR7695 might represent a rice-specific miRNA that is produced in *japonica,* but not in *indica*, subspecies from the *O. sativa* genus. Moreover, differences in the processing of the *osa-MIR7695* precursor appear to occur between the two groups of cultivated *Oryza* species, namely *O. sativa* and *O. glaberrima*. Processing of the *osa-MIR7695* precursor in cultivated *O. glaberrima* subspecies resembles that of wild rice accessions (rather than cultivated *O. sativa japonica* subspecies).

Discussion

In this study, we have shown that treatment with fungal elicitors is accompanied by dynamic alterations in the accumulation of a set of miRNAs from rice, both conserved and nonconserved miRNAs. Opposite expression patterns occur between elicitor-regulated known miRNAs and their corresponding target genes, suggesting that the observed elicitor-induced alterations in this set of miRNAs have an impact in shaping the plant transcriptome. The target genes for elicitor-regulated miRNAs are known to be involved in a variety of biological processes, such as stress responses, hormone

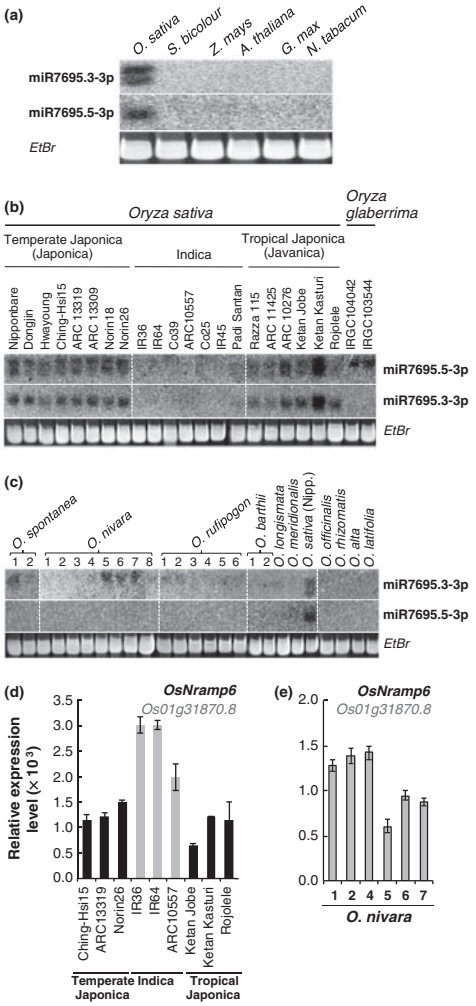

Fig. 6 Northern blot analysis of osa-miR7695 in plant species and rice varieties. (a) Monocotyledonous and dicotyledonous species. (b) Cultivated varieties of the genus *Oryza* (*Oryza sativa* and *O. glaberrima*). (c) Wild species of the genus *Oryza*. (d) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of short *OsNramp6* transcripts (Os01g31870.8) in cultivated rice varieties. Differences between *indica* and *japonica* cultivars were statistically significant ($P \leq 0.001$). (e) qRT-PCR analysis of short *OsNramp6* transcripts (Os01g31870.8) in wild species (*O. nivara* cultivars). Differences between *O. nivara* 1, 2, 4 and *O. nivara* 5, 6, 7) cultivars were statistically significant ($P \leq 0.001$). Error bars in (d–e) represent ± SD.

regulation and development, or miRNA functioning. In particular, our data indirectly support a role for the miR168/*AGO1* pair, and hence of the miRNA pathway, in coordinating the response of rice plants to fungal elicitors. Because miRNAs provide the quantitative regulation of target gene expression, rather than on–off regulations, the observed dynamic responses on miRNA accumulation could provide the fine-tuning of gene expression in different physiological processes. This, in turn, could enhance the plant's ability to escape from, resist or compensate for disease. This work also provides a rich source of expression data for a set of potential novel miRNAs showing elicitor responsiveness. Although we cannot rule out the possibility that some of the predicted miRNAs and target genes represent false-positive predictions, the possibility that some are young, recently evolved miRNAs, or even derive from proto-*MIR* genes, that exist without actual targets should be considered (Cuperus *et al.*, 2011). Future experimental work will determine whether these miRNA candidates, and their predicted targets, are genuine miRNA/target gene partners.

osa-miR7695 represents a recently evolved miRNA that experienced natural and/or domestication selection during rice evolution

A large proportion of *MIR* genes appear to be generated by inverted gene duplication events that give rise to new *MIR* genes. The transcription of such young miRNA genes produces fold-back structures that are processed by DCL4 which, through the accumulation of mutations, lead to a switch to *DCL1* processing (Vazquez *et al.*, 2008; Cuperus *et al.*, 2011). In this way, young *MIR* genes have stem structures with few bulges, are often processed imprecisely and give rise to miRNAs of variable length. By contrast, ancient *MIR* genes show reduced similarity in the fold-back arms and produce canonical miRNAs. In this work, we have shown that the processing of the osa-miR7695 precursor is largely dependent on DCL4. This finding, together with the long extensive base pairing within the stem region of *osa-MIR7695*, supports the hypothesis that this miRNA is probably an evolutionarily recent *MIR* gene. Moreover, *osa-MIR7695*-related small RNAs were detected in rice and not in any of the other monocotyledonous or dicotyledonous species analysed here, suggesting that osa-miR7695 evolved either after the divergence of the monocotyledonous and dicotyledonous lineages and/or during rice domestication.

The history of rice domestication remains an issue of debate. Although it is generally assumed that *O. glaberrima* originated from its wild ancestor *O. barthii* (Linares, 2002), controversy still exists about the wild ancestor for *O. sativa* species. It has been proposed that *japonica* and *indica* subspecies of *O. sativa* are the products of separate domestication events from pre-differentiated ancestral *O. rufipogon* populations (Londo *et al.*, 2006). The detection of osa-miR7695 in *japonica*, but not in *indica*, subspecies is consistent with the idea that the two groups, *japonica* and *indica*, originated from different wild populations. In line with this, our results show that *osa-MIR7695*-related small RNAs are detected in certain wild rice varieties, but not in others. In addition, osa-miR7695 appears to occur in both temperate *japonica* and tropical

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japonica, which is consistent with the already described genetic relationship between the two *japonica* subgroups of *O. sativa*.

Finally, that *osa-MIR7695* precursor processing is under molecular evolution is supported by the observation that the accumulation of *osa-MIR7695*-derived small RNAs differs in the two types of cultivated rice, *O. sativa* and *O. glaberrima*. Indeed, *osa-MIR7695* is imperfectly processed in *O. glaberrima*, its processing pattern being more closely related to that occurring in wild rice (instead of that occurring in *O. sativa* cultivars). Moreover, osa-miR7695.3-3p and osa-miR7695.5-3p small RNAs are produced in *japonica* subspecies, both temperate and tropical *japonica* rice, whereas only osa-miR7695.5-3p accumulates in *O. glaberrima* subspecies, which might indicate that *osa-MIR7695* precursor processing evolved during the domestication of *japonica* rice. In line with this, a role for miRNA genes as one of the driving forces in rice domestication has been proposed recently (Wang *et al.*, 2012). Clearly, the availability of whole genome sequences for an increasing number of wild and cultivated rice species will greatly facilitate the reconstruction of the evolutionary history of newly identified miRNAs from rice.

osa-miR7695, a novel miRNA targeting an alternatively spliced transcript of *OsNramp6* that contributes to pathogen resistance

We have reported that osa-miR7695 negatively regulates the accumulation of an alternatively spliced transcript of the *Nramp6* gene. Several lines of evidence support this conclusion: first, the overexpression of the *osa-MIR7695* precursor in transgenic rice, and the subsequent increase in the accumulation of *osa-MIR7695*-derived small RNAs, results in a drastic reduction in *OsNramp6* transcripts; second, the accumulation of both osa-miR7695 and *OsNramp6* target transcripts was affected in the *dcl4* rice mutant; and third, the accumulation of short *OsNramp6* transcripts is regulated by treatment with fungal elicitors. In addition, a good anti-correlation in osa-miR7695 and short *OsNramp6* transcripts is observed in the different rice species and cultivars analysed in this work. Collectively, these results support the existence of a regulatory mechanism that integrates both miRNA function and mRNA processing for the control of *OsNramp6* gene expression in rice plants. In this respect, a recent study using annotated gene models and publicly available high-throughput RNA sequencing data led the authors to propose that alternative splicing events might represent a mechanism for the attenuation of miRNA-mediated gene regulation in Arabidopsis plants (Yang *et al.*, 2012). The results presented here fully support this notion and add another layer of complexity to the already known mechanisms in plant immunity based on miRNA- and mRNA processing-based regulation of gene expression. From an evolutionary perspective, a course of osa-miR7695 and *OsNramp6* co-evolution can be reasoned in which *OsNramp6* expression could escape direct repression by osa-miR7695 through alternative splicing events and the selective production of target and nontarget *OsNramp6* transcripts. Interestingly, alternative splicing of *R* genes has been shown previously to play a role in pathogen defence, the production of these alternative splicing forms being required for full resistance. Some examples are the tobacco *N* gene conferring resistance to tobacco mosaic virus, and the *RPS4* (*RESISTANCE TO PSEUDOMONAS SYRINGAE 4*) gene conferring resistance to *P. syringae* expressing *AvrRps4* (Dinesh-Kumar & Baker, 2000; Zhang & Gassmann, 2003).

However, a role for distinct miRNAs in plant immunity has been documented. It was first described for miR393, an miRNA functioning in antibacterial defence by the repression of auxin signalling (Navarro *et al.*, 2006; Staiger *et al.*, 2012). More recently, miRNA regulation of innate immune receptors has been reported (Zhai *et al.*, 2011; Li *et al.*, 2012; Shivaprasad *et al.*, 2012). In this study, we have shown that the overexpression of osa-miR7695 contributes to a phenotype of resistance to infection by the rice blast fungus *M. oryzae*. This finding supports a positive role for osa-miR7695 in disease resistance, most probably by controlling the accumulation of an alternative splicing variant of the *OsNramp6* gene.

Concerning the biochemical function of *NRAMP* proteins, they are known to be involved in the transport of divalent metals and in the maintenance of metal homeostasis in a wide range of organisms, including plants (Cellier *et al.*, 1996). Although several Arabidopsis NRAMP proteins have been shown to function as iron transporters (Curie *et al.*, 2000; Thomine *et al.*, 2000; Gross *et al.*, 2003), very few have been assigned a physiological function. AtNramp3 and, to a lesser extent, AtNramp4 participate in iron mobilization in Arabidopsis and appear to be involved in resistance against the bacterial pathogen *Erwinia chrysanthemi* (Lanquar *et al.*, 2005; Segond *et al.*, 2009). Iron is an essential element for most living organisms, but, when in excess, iron induces the production of hydroxyl radicals which can cause multiple damage to cellular structures, eventually leading to death. Therefore, iron homeostasis must be tightly regulated in plant cells. Moreover, during pathogen infection, there is a competition between the host and the microorganism for iron. Of interest, a link between iron homeostasis and the expression of defence responses to pathogen attack has been documented in graminaceous species (Liu G *et al.*, 2007; Lemanceau *et al.*, 2009) and, more recently, in Arabidopsis plants (Kieu *et al.*, 2012). Clearly, to be cost-effective, plant defence mechanisms need to be tightly regulated during pathogen infection. Transient alterations in osa-miR7695 accumulation would provide a flexible mechanism by which *OsNramp6* could contribute to disease resistance. Further studies are needed to determine whether osa-miR7695/*OsNramp6* functioning plays a role in the modulation of iron homeostasis. It would also be of interest to decipher the biological significance of alternative splicing in *OsNramp6* in the context of both plant development and innate immunity, an aspect that remains to be investigated.

To conclude, the results presented here on miRNA functioning for the control of gene expression in disease resistance might also have broad implications in rice breeding programmes. Taking into account that rice has been adopted as the model species in cereal genomics, efforts to identify gene regulation networks that integrate miRNA functioning and alternative splicing events at target genes in rice will improve our understanding of the adaptation to pathogen infection in cereal species of agricultural importance.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Accumulation of miR168, miR160 and miR390 in rice (*Oryza sativa*) leaves.

Fig. S2 Nucleotide sequences of precursors containing novel miRNAs from previously known miRNA families from rice (*Oryza sativa*).

Fig. S3 Hairpin-forming structures representing potential novel miRNAs from rice (*Oryza sativa*).

Fig. S4 Nucleotide sequences of the 219 hairpin-forming structures representing potential novel miRNAs from rice (*Oryza sativa*).

Fig. S5 Northern blot analysis of miR168a in control and elicitor-treated rice (*Oryza sativa*) leaves.

Fig. S6 Percentage of leaf area affected by blast lesions in leaves of control and transgenic rice (*Oryza sativa*) plants at 4 d post-inoculation with *Magnaporthe oryzae* spores.

Table S1 *Oryza* species surveyed in this study, their genome type, accession number and geographical region of cultivation

Table S2 List of oligonucleotides used as probes for northern blot analysis of rice (*Oryza sativa*) miRNAs, and primers used for expression analyses of miRNA precursors and rice genes by reverse transcription polymerase chain reaction (qRT-PCR) and semi-quantitative RT-PCR

Table S3 Nucleotide sequences and expression of known miRNAs from rice (*Oryza sativa*) tissues (leaves and roots) that have been treated, or not, with fungal elicitors

Table S4 Nucleotide sequences of small RNAs identified in the 454 sequencing dataset mapping into the 219 hairpin-forming structures representing potential novel miRNAs from rice (*Oryza sativa*)

Table S5 Predicted target genes for the four experimentally validated miRNAs from rice (*Oryza sativa*)

Methods S1 Gene expression analysis, cloning of the *osa-miR7695* precursor, rice transformation and blast resistance assays.

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ANNEX II

Table Annex II.1. Genes differentially expressed in *MIR7695-Ac* plants in comparision to wild-type (Azygous), in control conditions.

RAPDB locus	Gene description	logFC	logCPM	PValue	FDR
		11,70	3,88	1,84E-8	5,72E-6
OS04G0572400	Dehydration-responsive element-binding protein 1E	8,76	1,00	2,97E-7	5,56E-5
OS06G0268500	Uncharacterized protein	7,53	0,11	3,27E-7	6,05E-5
OS12G0113600	Embryogenesis transmembrane protein	6,17	0,98	2,10E-7	4,09E-5
OS05G0103800	Conserved hypothetical protein.	6,06	3,32	2,61E-10	1,71E-7
OS02G0677300	Dehydration-responsive element-binding protein 1G	5,03	1,99	1,31E-5	1,29E-3
OS11G0119900	Hypothetical protein	4,93	2,55	3,52E-8	9,35E-6
OS01G0310100	Phospholipase D	4,67	7,70	5,79E-8	1,38E-5
OS09G0522100	Similar to C-repeat binding factor 3-like protein.	3,84	1,04	4,22E-5	3,26E-3
OS11G0151400	Cytochrome P450 family protein.	3,59	0,08	1,46E-5	1,42E-3
OS01G0310500	Conserved hypothetical protein.	3,36	8,26	8,01E-9	2,83E-6
OS01G0310800	OsWAK4 (OsWAK-RLCK)	3,18	6,50	1,25E-7	2,70E-5
OS02G0757100	Phi-1-like phosphate-induced protein	3,13	0,43	1,35E-4	7,94E-3
OS02G0770800	Nitrate reductase	3,07	6,28	6,07E-10	3,44E-7
OS01G0631200	Siroheme synthase	3,02	6,61	7,51E-11	5,99E-8
OS02G0540700	U-box domain containing protein	2,76	3,76	6,74E-6	7,78E-4
OS01G0597600	Transmembrane amino acid transporter protein	2,71	4,49	4,68E-8	1,19E-5
OS02G0759400	Zinc finger, RING-type domain containing protein.	2,56	4,56	3,49E-5	2,86E-3
OS04G0514600	Unknown protein	2,51	5,23	3,76E-8	9,85E-6
OS04G0571600	Multi antimicrobial extrusion protein MatE family protein	2,45	3,81	9,37E-6	1,02E-3
OS10G0521900	OsRhmbd17 - Putative Rhomboid homologue	2,42	3,51	2,18E-8	6,56E-6
OS04G0301500	OsRERJ1 - JA-responsive bHLH protein	2,39	1,33	3,30E-8	9,18E-6
OS01G0783700	EF-hand Ca2+-binding protein	2,34	0,46	1,00E-4	6,58E-3
OS08G0482600	Plastocyanin-like domain containing protein	2,30	5,80	2,86E-4	1,41E-2
OS01G0905200	Exo70 exocyst complex subunit family protein.	2,24	3,77	2,82E-8	8,08E-6
OS02G0629000	DUF584 family protein.	2,19	1,53	1,33E-4	7,90E-3
OS06G0543601	Unknown protein	2,17	4,39	9,86E-4	3,49E-2
OS01G0113650	Unknown protein	2,17	2,17	1,87E-7	3,69E-5
OS02G0667300	Alcohol dehydrogenase superfamily, zinc-containing protein.	2,17	0,71	2,70E-4	1,36E-2
OS02G0756800	Phosphate-induced protein 1 conserved region family protein.	2,15	4,42	1,05E-10	8,05E-8
OS07G0680600	Conserved hypothetical protein.	2,14	6,62	2,58E-5	2,26E-3
OS02G0703600	Abscisic acid 8'-hydroxylase 1	2,12	4,60	6,81E-6	7,81E-4
OS11G0559600	Hypothetical protein	2,06	3,68	1,03E-4	6,59E-3
OS03G0247600	Amino acid-binding ACT domain containing protein.	2,03	3,18	3,00E-12	3,74E-9
OS06G0255900	Exo70 exocyst complex subunit family protein.	2,02	4,08	1,85E-10	1,26E-7
OS03G0773000	Stress induced protein	2,01	3,59	1,01E-4	6,59E-3
OS03G0301200	COBRA-like protein 7 precursor.	2,01	3,64	2,26E-6	3,13E-4
OS02G0748300	OsFBK10 - F-box domain and kelch repeat containing protein	1,99	5,57	5,34E-8	1,31E-5
OS04G0175600	0-methyltransferase	1,98	4,52	1,68E-12	2,37E-9
OS02G0736900	Unknown protein	1,95	2,80	3,35E-5	2,79E-3

OS03G0609500	Similar to LOB domain protein 39.	1,92	5,73	1,75E-7	3,60E-5
OS01G0705700	bHLH protein	1,91	1,87	1,70E-4	9,41E-3
OS05G0447700	Ferritin/ribonucleotide reductase-like family protein.	1,91	3,67	4,40E-7	7,77E-5
OS01G0113200	OsRLK8 receptor serine/threonine kinase	1,89	2,49	3,10E-6	3,95E-4
OS01G0905300	Exo70 exocyst complex subunit family protein.	1,88	1,18	6,03E-4	2,47E-2
OS10G0555100	OsGGT - Glycogenin glucosyltransferase	1,84	4,16	2,12E-6	3,04E-4
OS01G0834900	Hypothetical protein.	1,82	2,85	1,51E-11	1,54E-8
OS05G0495250	Unknown protein	1,79	3,14	8,18E-6	9,10E-4
OS03G0152000	Heavy metal transport/detoxification protein domain containing protein.	1,79	4,11	1,03E-3	3,60E-2
OS02G0548700	U box domain containing protein.	1,79	0,96	1,17E-4	7,25E-3
OS01G0862800	NAC transcription factor-like protein	1,77	3,37	4,10E-5	3,18E-3
OS10G0469100	Conserved hypothetical protein.	1,76	2,19	5,08E-5	3,76E-3
OS03G0739700	Uncharacterized protein UPF0114 family protein.	1,76	3,95	5,76E-12	6,22E-9
OS07G0486000	mutS family domain IV containing protein	1,76	2,66	5,56E-10	3,29E-7
OS01G0760000	Dynein light chain 1	1,74	3,92	1,16E-6	1,87E-4
OS03G0181100	Os-JAZ10	1,73	3,99	1,78E-7	3,60E-5
OS04G0586500	FERONIA receptor-like kinase	1,71	3,84	8,13E-5	5,56E-3
OS03G0302800	Long cell-linked locus protein	1,70	1,06	2,07E-4	1,10E-2
OS08G0232700	OsEXO70FX4 - Leucine zipper protein-like	1,69	5,93	1,70E-8	5,43E-6
OS02G0818900	Heavy metal transport/detoxification protein domain containing protein.	1,69	1,98	8,35E-5	5,66E-3
OS05G0495700	Glycerol-3-phosphate dehydrogenase-like protein	1,67	3,90	7,22E-4	2,78E-2
OS01G0113300	receptor kinase ORK10	1,67	2,56	1,37E-5	1,35E-3
OS01G0699600	NPKL4	1,66	1,78	7,34E-5	5,16E-3
OS10G0392400	OsJAZ12	1,64	3,12	3,30E-7	6,05E-5
OS11G0570000	Receptor kinase-like protein	1,64	3,56	3,42E-8	9,22E-6
OS05G0127300	TKL_IRAK_CR4L.4 - The CR4L subfamily has homology with Crinkly4	1,62	3,12	3,35E-6	4,12E-4
OS03G0741100	bHLH domain containing protein.	1,62	4,48	1,14E-9	5,66E-7
OS01G0699500	NPKL3	1,61	1,84	2,70E-4	1,36E-2
OS01G0816100	OsNAC4	1,61	4,34	3,05E-9	1,27E-6
OS02G0789600	DEM protein	1,60	3,59	4,93E-10	3,02E-7
OS01G0863300	MYB family transcription factor	1,59	2,64	6,38E-4	2,56E-2
OS01G0908301	Unknown protein	1,56	3,38	1,15E-4	7,17E-3
OS03G0247900	MAP Kinase.	1,56	3,44	9,11E-7	1,52E-4
OS04G0497000	NADP-dependent oxidoreductase	1,54	6,05	3,87E-5	3,08E-3
OS10G0543400	CHIT15 - Chitinase family protein precursor	1,53	1,63	1,69E-4	9,41E-3
OS03G0191900	AP2/EREBP transcription factor superfamily protein	1,53	3,23	1,05E-4	6,69E-3
OS02G0661100	Trehalose-6-phosphate phosphatase.	1,52	3,77	5,59E-11	4,66E-8
OS05G0474800	OsWRKY70	1,50	5,10	3,24E-6	4,05E-4
OS01G0975000	Protein of unknown function DUF966	1,49	2,59	2,99E-4	1,45E-2
OS03G0315400	MYB family transcription factor	1,49	3,34	2,06E-5	1,86E-3
OS08G0100300	Non-protein coding transcript, uncharacterized transcript.	1,47	1,89	9,19E-4	3,32E-2
OS02G0739100	Actin-binding FH2 domain containing protein.	1,47	5,06	7,42E-6	8,45E-4
OS02G0791500	Nucleotide sugar epimerase-like protein	1,46	4,61	2,07E-5	1,86E-3
OS11G0572200	Expressed protein	1,46	1,81	1,36E-3	4,46E-2

OS09G0388400	Hydrolase, HAD superfamily, Cof family	1,45	1,75	1,06E-5	1,10E-3
OS08G0356800	Protein of unknown function DUF247	1,44	3,09	3,74E-4	1,72E-2
OS03G0334200	Conserved hypothetical protein.	1,44	5,25	1,03E-7	2,27E-5
OS02G0806400	OsGATA8 TF	1,42	5,39	3,47E-12	3,98E-9
OS04G0640850	Non-protein coding transcript	1,42	1,33	9,12E-4	3,31E-2
OS07G0114000	Protein phosphatase 2C-like.	1,41	4,04	1,43E-3	4,63E-2
OS01G0108400	bHLH domain containing protein	1,41	3,98	1,17E-3	3,97E-2
OS02G0181300	OsWRKY71	1,41	5,94	2,78E-9	1,20E-6
OS02G0205500	Fatty acid elongase 1	1,39	5,40	1,23E-7	2,69E-5
OS04G0209300	ABC transporter family protei	1,38	5,24	9,18E-7	1,52E-4
OS11G0548600	Putative uncharacterized protein	1,37	1,83	2,17E-4	1,14E-2
OS07G0559700	Monosaccharide transporter 3.	1,37	7,14	8,40E-9	2,91E-6
OS12G0181600	OsAAP11B - Amino acid/polyamine transporter II family protein.	1,37	5,26	8,08E-13	1,23E-9
OS07G0661400	Early response to dehydration 15-like protein	1,36	5,81	7,57E-5	5,30E-3
OS01G0826400	OsWRKY24	1,35	6,89	1,15E-5	1,18E-3
OS01G0575200	bHLH protein	1,35	4,45	1,72E-8	5,43E-6
OS03G0236200	Glutamate decarboxylase isozyme 3	1,34	3,49	6,01E-7	1,05E-4
OS03G0285800	Metallothionein.	1,33	6,20	4,65E-6	5,57E-4
OS07G0241500	UDP-glucuronosyl/UDP-glucosyltransferase family protein.	1,32	2,26	3,03E-5	2,56E-3
OS02G0125300	Bax inhibitor-1 (BI-1) (OsBI-1).	1,32	6,01	1,33E-10	9,78E-8
OS12G0218300	RALFL45 - Rapid ALkalinization Factor RALF family protein precursor,	1,31	2,57	8,40E-5	5,66E-3
OS01G0135700	OsCML16 - Calmodulin-related calcium sensor	1,31	4,55	1,47E-4	8,43E-3
OS03G0723700	Conserved hypothetical protein.	1,31	3,44	8,11E-5	5,56E-3
OS03G0815100	NAC transcription factor	1,30	6,14	3,10E-5	2,61E-3
OS02G0165100	Protein kinase-like domain containing protein	1,29	4,37	2,29E-6	3,13E-4
OS02G0806300	Unknown protein	1,29	4,46	2,82E-9	1,20E-6
OS09G0484900	Sodium/sulphate symporter family protein.	1,29	6,36	1,20E-5	1,21E-3
OS01G0570800	Calmodulin-binding protein	1,28	7,41	9,47E-6	1,02E-3
OS10G0570200	Similar to RIR1b protein precursor	1,27	5,04	4,02E-10	2,54E-7
OS04G0310400	Unknown protein	1,26	4,14	2,27E-5	2,02E-3
OS01G0295700	Protein phosphatase-2C.	1,26	5,31	7,75E-7	1,32E-4
OS12G0556200	Calmodulin binding protein	1,26	6,84	1,79E-7	3,60E-5
OS04G0578000	ACC synthase	1,24	2,93	1,65E-5	1,55E-3
OS03G0335200	WRKY44	1,23	4,26	1,33E-4	7,90E-3
OS04G0619400	Protein kinase-like domain containing protein	1,22	5,93	2,34E-6	3,18E-4
OS08G0445700	Trehalose-6-phosphate synthase	1,22	2,94	5,13E-4	2,19E-2
OS10G0570001	Unknown protein - Ximilar to RiR1	1,22	2,70	1,78E-4	9,74E-3
OS08G0526100	UDP-glucuronate 4-epimerase	1,21	2,06	3,94E-4	1,80E-2
OS05G0537100	OsWRKY7	1,21	5,29	5,42E-9	2,16E-6
OS01G0821600	OsWRKY21	1,21	4,17	1,02E-3	3,58E-2
OS07G0545800	CIGR1 - Chitin-inducible gibberellin-responsive protein.	1,20	6,17	1,17E-6	1,87E-4
OS04G0372700	Quinone-oxidoreductase QR1	1,20	3,15	2,88E-6	3,78E-4
OS01G0841700	Os-FIERG2 (elicitor-responsive protein)	1,20	6,27	6,06E-5	4,45E-3

OS03G0429000	Proteinase inhibitor I25, cystatin domain containing protein.	1,19	3,95	9,17E-8	2,08E-5
OS04G0598900	OsWAK50 (WAK-RLK)	1,18	3,16	3,66E-6	4,48E-4
OS06G0318800	Non-protein coding transcript, unclassifiable transcript.	1,18	5,22	3,44E-7	6,25E-5
OS01G0332100	Neutral invertase-like protein	1,18	8,04	9,79E-8	2,19E-5
OS01G0675800	No apical meristem (NAM) protein domain containing protein.	1,16	3,17	1,25E-4	7,56E-3
OS08G0248100	Receptor-like protein kinase 2	1,16	1,57	1,48E-3	4,76E-2
OS01G0837000	Ankyrin repeat containing protein.	1,15	4,95	2,52E-11	2,32E-8
OS02G0572400	Riboflavin biosynthesis protein ribA, chloroplast precursor	1,15	6,30	1,02E-5	1,07E-3
OS01G0742400	Receptor-like protein kinase	1,14	6,10	1,65E-9	7,74E-7
OS02G0251800	VQ protein	1,13	4,43	2,70E-4	1,36E-2
OS02G0585100	Heavy metal transport/detoxification protein domain containing protein.	1,12	3,85	3,17E-6	4,01E-4
OS08G0386200	OsWRKY69	1,12	6,69	5,65E-6	6,68E-4
OS05G0322900	OsWRKY45	1,12	7,58	3,09E-6	3,95E-4
OS03G0180800	TIFY protein	1,11	1,98	3,67E-4	1,70E-2
OS02G0654700	Ethylene-responsive transcription factor 5	1,10	3,83	1,08E-4	6,83E-3
OS05G0530400	Heat stress transcription factor Spl7	1,09	5,64	1,49E-8	4,96E-6
OS02G0314600	Chloroplast nucleoid DNA binding protein	1,09	4,27	3,97E-5	3,14E-3
OS11G0513900	Conserved hypothetical protein.	1,08	4,22	9,51E-5	6,25E-3
OS11G0227800	NBS-LRR type disease resistance protein	1,08	5,11	1,91E-6	2,86E-4
OS02G0790500	Glycosyl transferase, family 20 domain containing protein.	1,07	7,27	1,43E-4	8,30E-3
OS02G0126400	OsCPK4	1,06	6,92	2,39E-6	3,20E-4
OS06G0143400	Acyl-ACP thioesterase	1,06	5,07	1,34E-7	2,86E-5
OS04G0667600	Heavy metal transport/detoxification protein	1,06	5,98	1,41E-5	1,38E-3
OS02G0594900	Glycosyl transferase, family 17 protein.	1,05	5,49	6,39E-6	7,46E-4
OS01G0833400	Conserved hypothetical protein.	1,05	3,50	1,63E-4	9,17E-3
OS02G0251900	OsSub13 - Subtilisin homologue	1,05	2,29	1,60E-4	9,01E-3
OS09G0486500	Multiple stress-responsive zinc-finger protein ISAP1 (Stress- associated protein 1) (OsISAP1).	1,05	5,56	8,12E-4	3,05E-2
OS01G0189100	Ubiquitin domain containing protein.	1,04	3,32	2,01E-6	2,95E-4
OS03G0761100	Protein phosphatase 2C-like protein	1,03	6,38	3,83E-7	6,82E-5
OS12G0242500	Conserved hypothetical protein.	1,02	3,51	1,14E-3	3,89E-2
OS01G0739500	Conserved hypothetical protein.	1,02	8,91	1,47E-3	4,72E-2
OS04G0584600	OsCPK13	1,02	7,53	2,09E-8	6,40E-6
OS12G0141000	Conserved hypothetical protein.	1,01	2,67	4,22E-4	1,91E-2
OS04G0640900	Lambda repressor-like, DNA-binding domain containing protein	1,01	4,94	2,79E-7	5,27E-5
OS03G0133000	NAC-domain protein	1,01	5,85	2,41E-4	1,25E-2
OS03G0299700	Conserved hypothetical protein.	-9,29	4,20	4,85E-114	4,45E-110
OS12G0481400	NBS-LRR type disease resistance protein	-9,28	1,85	3,88E-35	2,37E-31
OS01G0382450	Unknown protein	-7,35	-0,44	1,75E-4	9,60E-3
OS04G0682600	Integrase, catalytic region domain containing protein.	-6,66	-0,07	7,20E-7	1,24E-4

OS11G0549300	KI domain interacting kinase 1	-5,05	1,47	3,65E-13	6,69E-10
OS10G0397900	Nuclear transcription factor Y subunit	-5,02	0,32	5,42E-9	2,16E-6
OS10G0432200	Retrotransposon protein	-4,92	4,92	4,14E-114	4,45E-110
OS04G0677000	Protein of unknown function DUF1677	-4,54	2,28	3,68E-33	1,69E-29
OS06G0271400	Hypothetical protein.	-4,27	1,37	5,88E-15	1,54E-11
OS09G0508500	OsDRB2 - Double-stranded RNA-binding protein	-3,98	0,59	1,57E-8	5,13E-6
OS01G0873000	Conserved hypothetical protein.	-3,88	1,50	2,04E-11	1,97E-8
OS06G0549900	FAD linked oxidase, N-terminal domain containing protein.	-3,66	1,01	6,59E-8	1,55E-5
OS12G0249900	Protein kinase-like domain containing protein.	-3,49	0,55	1,34E-4	7,90E-3
OS06G0570900	bHLH protein	-3,36	0,47	1,19E-4	7,27E-3
OS09G0425200	Fibroin heavy chain (Fib-H) (H-fibroin)-like protein	-3,35	1,63	6,12E-13	1,02E-9
OS11G0641500	OsLAC19 - Laccase	-3,17	4,28	1,63E-10	1,15E-7
OS01G0872900	Transposon protein, putative, Pong sub-class	-3,10	0,42	1,32E-6	2,00E-4
OS04G0122200	Protein kinase	-2,91	2,44	2,17E-6	3,09E-4
OS08G0140300	AADC1 --- Tryptophan decarboxylase	-2,87	3,40	3,05E-12	3,74E-9
OS10G0409400	BURP domain-containing protein 16	-2,87	3,96	6,22E-9	2,38E-6
OS03G0215400	MADS1	-2,78	0,79	1,32E-6	2,00E-4
OS09G0554101	Conserved hypothetical protein	-2,70	0,57	7,68E-6	8,64E-4
OS12G0258700	OsLAC24 - Laccase	-2,70	1,38	6,61E-6	7,67E-4
OS03G0307300	OsNAS1 - Nicotianamine synthase 1	-2,39	2,63	3,07E-4	1,49E-2
OS07G0429700	Thionin-like peptide	-2,27	3,63	2,80E-4	1,40E-2
OS02G0823200	Conserved hypothetical protein.	-2,15	2,01	9,22E-10	4,98E-7
OS01G0106300	Isoflavone reductase homolog IRL	-2,13	1,27	2,67E-8	7,90E-6
OS03G0288000	OsMT1-1b - Metallothionein-like protein 1B	-2,13	1,53	2,38E-6	3,20E-4

Table Annex II.2. Genes differentially expressed in *MIR7695-Ac* plants in comparision to wild-type (Azygous), in infection conditions.

Locus	Gene Description	logFC	logCPM	PValue	FDR
		10,65	3,88	1,33E-7	3,35E-5
OS05G0103800	Conserved hypothetical protein.	5,04	3,32	1,82E-8	4,97E-6
OS12G0113600	Embryogenesis transmembrane protein	3,98	0,98	1,19E-4	8,71E-3
OS01G0310100	Phospholipase D	3,27	7,70	5,10E-5	4,64E-3
OS11G0119900	Hypothetical protein	3,11	2,55	1,41E-4	9,86E-3
OS01G0310800	OsWAK4 (OsWAK-RLCK)	2,46	6,50	2,45E-5	2,62E-3
OS01G0237750	Conserved hypothetical protein.	2,35	0,48	3,62E-4	1,92E-2
OS01G0310500	Conserved hypothetical protein.	2,18	8,26	8,88E-5	7,17E-3
OS04G0121300	OsSub35 - Putative Subtilisin homologue	2,18	2,31	4,24E-5	4,01E-3
OS06G0185400	Conserved hypothetical protein	2,16	1,02	6,10E-4	2,69E-2
OS02G0571900	CYP76M6	2,15	3,23	1,82E-6	3,04E-4
OS08G0100300	Non-protein coding transcript, uncharacterized transcript.	2,15	1,89	2,35E-6	3,71E-4
OS01G0106400	Isoflavone reductase-like protein	1,89	4,08	3,99E-10	1,70E-7
OS12G0629700	OsPR5 - Thaumatin-like protein	1,87	1,13	1,81E-7	4,25E-5
OS01G0189100	Ubiquitin domain containing protein.	1,86	3,32	1,54E-16	2,82E-13
OS06G0552900	Phosphatidylethanolamine-binding protein - Similar to Flowering Locus T (FT)	1,85	3,81	4,48E-5	4,16E-3
OS10G0178500	Cytokinin-O-glucosyltransferase 3	1,82	1,86	1,02E-4	7,89E-3
OS05G0368000	Conserved hypothetical protein	1,76	1,47	4,28E-4	2,14E-2
OS12G0215950	LRR protein	1,69	2,65	1,63E-7	3,98E-5
OS03G0594100	Cytochrome P450	1,69	1,37	2,02E-5	2,24E-3
OS01G0108500	Conserved hypothetical protein.	1,68	2,07	2,02E-4	1,27E-2
OS10G0538200	Eukaryotic aspartyl protease	1,64	3,91	5,78E-4	2,63E-2
OS02G0699000	nitrate transporter NRT1	1,64	2,88	1,28E-4	9,12E-3
OS04G0301500	OsRERJ1 - JA-responsive bHLH protein	1,62	1,33	9,91E-4	3,82E-2
OS06G0493100	Planthopper-inducible protein BpHi008A	1,62	6,14	2,19E-16	3,35E-13
OS06G0313440	SAM dependent carboxyl methyltransferase family protein.	1,59	2,33	4,46E-4	2,21E-2
OS07G0417200	Fatty acid desaturase	1,59	2,92	5,92E-4	2,65E-2
OS01G0130200	NPR1 interactor-like	1,54	2,23	1,04E-4	8,01E-3
OS01G0326300	Peroxidase component PR-2 and/or 4	1,50	4,36	1,51E-9	5,44E-7
OS01G0326100	Peroxidase component PR-2 and/or 4	1,50	2,05	6,53E-4	2,83E-2
OS12G0221600	RALFL46 - Rapid ALkalinization Factor RALF family protein	1,50	1,86	1,28E-6	2,21E-4
OS09G0417600	OsWRKY76	1,48	4,85	7,31E-7	1,38E-4
OS03G0235000	Prx38 - Peroxidase prxRPN	1,47	2,26	7,06E-5	5,95E-3
OS04G0581100	Isopenicillin N synthase family protein.	1,47	3,48	1,21E-6	2,15E-4
OS02G0571800	OsKLS6 - Ent-isokaur-15-ene synthase	1,42	1,29	1,58E-4	1,07E-2
OS04G0150300	Conserved hypothetical protein	1,42	0,87	1,23E-3	4,43E-2
OS11G0475200	Non-protein coding transcript	1,41	0,97	2,62E-4	1,55E-2
OS06G0521500	Peroxidase Class III	1,40	1,90	4,60E-4	2,26E-2

OS02G0582600	Heavy metal transport/detoxification protein domain containing protein.	1,37	3,36	5,42E-6	7,71E-4
OS01G0508500	Conserved hypothetical protein.	1,36	4,15	1,91E-4	1,23E-2
OS07G0187900	Nodulin	1,36	2,55	2,45E-5	2,62E-3
OS10G0537800	Eukaryotic aspartyl protease	1,36	5,08	4,20E-4	2,11E-2
OS01G0307686	Embryonic abundant protein-like	1,33	1,30	1,76E-4	1,16E-2
OS10G0320100	Flavonoid 3'-hydroxylase	1,32	2,13	2,43E-5	2,62E-3
OS05G0567600	hhH-GPD superfamily base excision DNA repair protein	1,32	2,30	7,12E-5	5,95E-3
OS04G0209300	ABC transporter family protei	1,30	5,24	3,48E-6	5,32E-4
OS02G0517900	Conserved hypothetical protein.	1,30	4,16	5,35E-10	2,18E-7
OS01G0339500	No apical meristem (NAM) protein	1,29	3,21	1,23E-5	1,51E-3
OS03G0122300	Flavone synthase (DOX)	1,29	3,51	3,10E-7	6,54E-5
OS09G0417800	OsWRKY62 (SA pathway)	1,26	4,39	5,00E-4	2,38E-2
OS12G0555500	OsPBZ1	1,26	9,06	1,13E-5	1,41E-3
OS01G0962700	Prx20 - Peroxidase	1,26	5,72	9,44E-11	4,68E-8
OS05G0390800	VQ domain containing protein	1,25	1,93	1,24E-4	8,98E-3
OS10G0580900	Conserved hypothetical protein	1,24	4,42	8,45E-5	6,88E-3
OS07G0605400	EGG APPARATUS-1 protein	1,24	4,57	2,54E-5	2,70E-3
OS06G0314600	OsJMT1 (MeJa) SAM dependent carboxyl methyltransferase family protein.	1,22	1,17	5,79E-4	2,63E-2
OS12G0555300	Similar to PBZ1	1,19	4,06	7,24E-4	3,07E-2
OS03G0285700	APx01 (APX1) - Ascorbate peroxidase	1,18	6,79	7,07E-12	4,42E-9
OS06G0314501	Conserved hypothetical protein	1,18	1,85	9,73E-5	7,61E-3
OS04G0127200	OsSub37 - Putative Subtilisin homologue	1,18	7,23	5,85E-4	2,64E-2
OS04G0122200	Protein kinase	1,18	2,44	1,32E-4	9,32E-3
OS06G0293500	Embryogenesis transmembrane protein-like	1,18	3,06	4,70E-4	2,28E-2
OS10G0109300	Peroxidase precursor	1,17	2,85	5,86E-5	5,17E-3
OS03G0181100	Os-JAZ10	1,16	3,99	5,38E-4	2,49E-2
OS10G0468500	Leucine Rich Repeat family protein	1,16	2,13	1,47E-5	1,76E-3
OS02G0274100	Peroxisomal fatty acid beta-oxidation multifunctional protein (MFP)	1,16	6,95	4,16E-12	2,72E-9
OS07G0217600	Cytochrome P450	1,15	6,01	1,19E-8	3,52E-6
OS11G0117400	OsWRKY100	1,13	3,79	1,11E-3	4,13E-2
OS04G0688100	OsCPX1 - Cationic Peroxidase	1,12	7,10	1,75E-4	1,16E-2
OS09G0375000	Enolase	1,10	1,79	8,51E-4	3,43E-2
OS07G0129200	OsPR1a	1,10	4,44	1,74E-8	4,84E-6
OS03G0335200	OsWRKY44	1,10	4,26	6,50E-4	2,83E-2
OS04G0175600	0-methyltransferase	1,08	4,52	7,13E-5	5,95E-3
OS01G0723600	Ribose-phosphate pyrophosphokinase 3	1,08	4,31	1,39E-9	5,20E-7
OS11G0601700	bHLH protein	1,07	2,73	6,31E-4	2,77E-2
OS02G0575700	Loricrin-like protein (CPuORF)	1,07	4,41	2,01E-6	3,30E-4
OS02G0151300	Endoglucanase	1,07	2,74	1,12E-5	1,41E-3
OS04G0542000	HAT dimerisation domain containing protein	1,06	2,19	8,24E-4	3,35E-2
OS07G0663700	Momilactone A synthase-like	1,06	5,71	3,55E-4	1,90E-2
OS03G0314500	WD40-like Beta Propeller Repeat family protein	1,06	3,30	9,45E-4	3,71E-2
OS04G0179200	OsMAS1 - Momilactone A synthase	1,06	3,29	1,09E-3	4,10E-2
OS02G0542400	OsGTγ-1 (TF)	1,05	1,94	7,57E-4	3,16E-2

OS01G0834900	Hypothetical protein	1,04	2,85	6,76E-4	2,91E-2
OS09G0388400	Hydrolase, HAD superfamily, Cof family	1,04	1,75	1,08E-3	4,09E-2
OS04G0316200	Membrane binding protein (MBP)	1,03	3,02	4,69E-4	2,28E-2
OS06G0591200	Conserved hypothetical protein.	1,02	4,75	1,83E-7	4,26E-5
OS05G0455400	bHLH protein	1,02	3,36	3,07E-4	1,72E-2
OS01G0291500	OsPMT - p-coumarate monolignol transferase	1,01	3,41	1,97E-4	1,27E-2
OS06G0670300	MYB transcription factor	1,01	3,12	9,19E-6	1,19E-3
OS02G0718100	Non protein coding transcript	1,00	3,32	9,26E-4	3,66E-2
OS04G0615200	Adhesive/proline-rich protein	1,00	2,23	5,71E-4	2,61E-2
OS07G0526400	CHS - Chalcone synthase 1	1,00	2,74	1,59E-4	1,08E-2
OS03G0299700	Conserved hypothetical protein.	-9,34	4,20	1,90E-117	1,74E-113
OS06G0271400	Hypothetical protein.	-8,92	1,37	1,56E-27	5,72E-24
OS04G0682600	Integrase, catalytic region domain containing protein.	-7,38	-0,07	1,63E-9	5,76E-7
OS12G0481400	NBS-LRR type disease resistance protein	-6,86	1,85	1,17E-31	5,39E-28
OS04G0677000	Protein of unknown function DUF1677	-6,39	2,28	2,74E-37	1,68E-33
OS10G0432200	Retrotransposon protein	-5,67	4,92	6,29E-131	1,15E-126
OS01G0872900	Transposon protein, putative, Pong sub-class	-5,37	0,42	7,20E-11	3,78E-8
OS09G0554101	Conserved hypothetical protein	-4,91	0,57	5,95E-13	4,75E-10
OS10G0397900	Nuclear transcription factor Y subunit	-4,53	0,32	7,74E-10	3,09E-7
OS07G0429700	Thionin-like peptide	-4,49	3,63	9,03E-11	4,60E-8
OS03G0307200	OsNAS2 - Nicotianamine synthase 2	-4,07	3,65	7,49E-14	7,23E-11
OS09G0425200	Fibroin heavy chain (Fib-H) (H-fibroin)-like protein	-4,05	1,63	6,30E-18	1,28E-14
OS07G0642800	Proline rich protein	-3,64	0,47	4,10E-7	8,26E-5
OS11G0151500	ENA1 - Major facilitator superfamily protein	-3,64	0,81	1,51E-8	4,33E-6
OS01G0873000	Conserved hypothetical protein.	-3,55	1,50	1,16E-9	4,44E-7
OS02G0823200	Conserved hypothetical protein.	-3,19	2,01	3,91E-19	8,98E-16
OS03G0307300	OsNAS1 - Nicotianamine synthase 1	-3,12	2,63	1,05E-5	1,34E-3
OS03G0293700	Hypothetical protein	-3,05	3,36	1,95E-12	1,49E-9
OS01G0356900	Leucine-rich repeat/extensin 1	-3,05	2,33	2,28E-12	1,67E-9
OS12G0282000	OsIRNLPl (Fe-regulated TF)	-2,81	7,30	3,40E-13	2,83E-10
OS09G0508500	OsDRB2 - Double-stranded RNA-binding protein	-2,75	0,59	5,47E-5	4,94E-3
OS10G0472900	3-ketoacyl-CoA synthase family protein	-2,71	1,63	4,04E-9	1,24E-6
OS06G0320200	Glycoside hydrolase, family 1 protein.	-2,63	0,67	4,05E-6	5,94E-4
OS10G0475100	Oxidoreductase	-2,57	4,54	1,27E-26	3,89E-23
OS04G0405500	Conserved hypothetical protein	-2,34	1,37	1,10E-5	1,40E-3
OS01G0800900	Conserved hypothetical protein.	-2,27	1,13	6,96E-7	1,33E-4
OS03G0215400	MADS1	-2,26	0,79	5,59E-5	4,98E-3
OS11G0549300	KI domain interacting kinase 1	-2,23	1,47	1,94E-7	4,46E-5

OS12G0249900	Protein kinase-like domain containing protein.	-2,22	0,55	2,94E-4	1,67E-2
OS06G0294950	Conserved hypothetical protein	-2,17	2,64	3,20E-12	2,26E-9
OS03G0667300	OsIRT2 - Fe2+transporter	-2,17	0,79	1,87E-5	2,11E-3
OS09G0572000	ERF (ethylene response factor)/AP2 transcription factor family	-2,15	0,51	6,89E-6	9,36E-4
OS08G0112700	MADS-box transcription factor	-2,05	2,96	7,36E-15	9,01E-12
OS09G0451400	1-aminocyclopropane-1-carboxylate oxidase 1	-1,85	4,53	1,38E-21	3,61E-18
OS11G0154900	BZIP transcription factor	-1,84	0,97	2,12E-4	1,32E-2
OS03G0718800	LTPL118 - Protease inhibitor/seed storage/LTP family protein precursor	-1,79	1,33	5,36E-4	2,49E-2
OS05G0551000	OsHRZ2	-1,78	7,88	6,00E-14	6,11E-11
OS07G0273301	FAS1	-1,75	1,65	3,65E-6	5,49E-4
OS12G0236100	Conserved hypothetical protein	-1,72	10,76	3,74E-7	7,63E-5
OS04G0231800	Four-helical cytokine	-1,70	3,51	3,61E-10	1,58E-7
OS01G0871600	OsIROPT1	-1,67	4,82	1,45E-15	2,05E-12
OS12G0236225	Conserved protein	-1,66	8,50	2,16E-8	5,82E-6
OS11G0417800	Non-protein coding transcript	-1,61	3,12	1,31E-11	7,50E-9
OS02G0579000	OsNAC1 (induced by Fw def)	-1,61	4,92	1,95E-16	3,25E-13
OS03G0283200	XIG -IN2-1 protein	-1,58	3,37	1,25E-6	2,19E-4
OS11G0125900	Nucleoside phosphatase GDA1/CD39 family protein	-1,56	4,02	1,31E-13	1,20E-10
OS04G0231200	Non-protein coding transcript, uncharacterized transcript.	-1,52	2,94	1,77E-7	4,25E-5
OS04G0578600	OsFRO2 - Ferric reductase	-1,50	6,45	1,02E-10	4,94E-8
OS07G0480800	Xyloglucan endo-transglycosylase	-1,50	0,93	3,17E-4	1,76E-2
OS03G0322900	OsLEA17	-1,49	3,65	1,38E-6	2,37E-4
		-1,47	2,52	1,25E-4	8,99E-3
OS09G0130300	Conserved hypothetical protein	-1,46	5,75	3,69E-9	1,15E-6
OS01G0511000	Similar to LOB domain protein 40.	-1,45	1,34	8,29E-4	3,37E-2
OS10G0555600	OsEXPB6 - Expansin-B6	-1,41	1,34	1,75E-4	1,16E-2
OS05G0519300	DUF506 domain containing protein	-1,39	4,22	2,19E-14	2,52E-11
OS10G0465700	Beta-amylase PCT-BMYI	-1,39	4,76	1,15E-7	2,93E-5
OS01G0952800	OsIRO2 (bHLH protein)	-1,39	10,05	2,11E-15	2,77E-12
OS07G0622700	Hydrolase-like	-1,34	3,17	1,04E-9	4,05E-7
OS12G0236200	Non-protein coding transcript, unclassifiable transcript	-1,33	3,85	1,98E-6	3,27E-4
OS03G0371350	Cytochrome P450	-1,33	4,28	2,41E-10	1,08E-7
OS03G0604566	Conserved hypothetical protein	-1,32	2,66	6,27E-6	8,65E-4
OS06G0498800	osMFT1 MFT-Like1(Mother of FT and TFL1 gene) - Phosphatidylethanolamine-binding protein	-1,30	1,85	2,14E-4	1,33E-2
OS05G0244500	Glycoside hydrolase	-1,30	4,48	4,37E-10	1,82E-7
OS07G0258400	OsNramp1 (Integral membrane protein)	-1,29	9,72	3,63E-12	2,46E-9
OS07G0142100	Conserved hypothetical protein.	-1,27	10,55	2,06E-6	3,31E-4
OS05G0169200	WD40-like domain containing protein	-1,27	1,75	2,01E-4	1,27E-2

OS03G0725200	Conserved hypothetical protein	-1,25	5,96	1,35E-11	7,50E-9
OS03G0371400	Cytochrome P450	-1,24	3,28	2,93E-6	4,60E-4
OS10G0406900	Polygalacturonase isoenzyme 1 beta subunit	-1,23	2,06	5,99E-4	2,67E-2
OS10G0195250	Expressed protein	-1,18	8,57	2,46E-9	8,19E-7
OS01G0608101	Hypothetical protein	-1,17	7,96	3,17E-9	1,02E-6
OS05G0369900	Conserved hypothetical protein.	-1,15	3,20	4,85E-7	9,67E-5
OS09G0451000	1-aminocyclopropane-1-carboxylate oxidase (ACO1)	-1,14	6,38	1,89E-13	1,65E-10
OS03G0857600	Receptor protein kinase	-1,14	2,79	4,74E-5	4,35E-3
OS03G0371300	Cytochrome P450	-1,14	4,80	3,47E-11	1,87E-8
OS03G0126700	Similar to Barley stem rust resistance protein.	-1,14	2,90	1,43E-4	9,91E-3
OS04G0252850	Histone H1	-1,13	2,25	1,06E-4	8,04E-3
OS05G0467400	DUF1618 domain containing protein	-1,12	1,87	3,43E-4	1,86E-2
OS03G0119300	Subtilisin homologue	-1,12	2,82	2,72E-5	2,81E-3
OS06G0133400	Conserved hypothetical protein	-1,11	5,45	2,01E-7	4,49E-5
OS06G0473000	Poor homologous synapsis 1 protein	-1,09	4,50	2,64E-9	8,64E-7
OS02G0594166	Conserved hypothetical protein.	-1,09	7,80	1,73E-8	4,84E-6
OS09G0455300	Similar to INDEHISCENT protein.	-1,08	3,66	6,76E-4	2,91E-2
OS02G0833200	Non-protein coding transcript, uncharacterized transcript.	-1,08	2,90	3,71E-5	3,56E-3
OS07G0142200	Retrotransposon protein	-1,06	4,69	8,14E-4	3,35E-2
OS01G0689451	OsHRZ1 - RING finger protein	-1,05	9,02	1,27E-10	5,81E-8
OS01G0647200	Non-protein coding transcript, unclassifiable transcript.	-1,05	10,93	1,31E-8	3,82E-6
OS06G0268050	Hypothetical protein	-1,04	2,28	2,44E-4	1,48E-2
OS04G0420700	Hypothetical protein	-1,04	2,73	2,19E-5	2,39E-3
OS11G0262600	Conserved hypothetical protein - expressed under carbonate stress	-1,04	7,51	5,18E-7	1,01E-4
OS12G0623600	Cysteine oxidase 2	-1,03	4,66	1,06E-11	6,30E-9
OS05G0592300	Protein of unknown function DUF1637 family protein.	-1,02	6,00	7,23E-12	4,42E-9
OS12G0283300	Conserved hypothetical protein - expressed under carbonate stress	-1,02	2,44	2,00E-4	1,27E-2
OS06G0594400	Kelch repeat-containing F-box-like	-1,01	4,97	3,24E-4	1,79E-2
OS03G0608700	Transposon protein, putative, CACTA, En/Spm subclass	-1,00	5,32	1,13E-10	5,31E-8
OS01G0520800	Conserved hypothetical protein.	-1,00	1,86	1,20E-3	4,36E-2