High carotenoid corn: agronomic evaluation and interactions with insect pests

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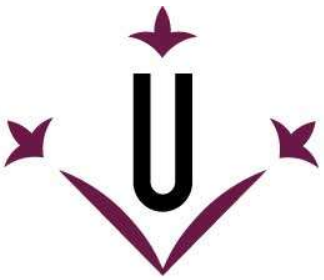

Universitat de Lleida

TESI DOCTORAL

High Carotenoid Corn: Agronomic Evaluation And Interactions With Insect Pests

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Summary

My research project focused on the molecular, biochemical and agronomical characterization of a high carotenoid transgenic corn line, Carolight^{R} under different environmental conditions. Carolight^{R} was transformed with *Zmpsy1* and *PacrtI*, two carotenogenic genes, the expression of which resulted in a massive accumulation of carotenoids in the endosperm. It is important to evaluate the performance of a novel plant variety (transgenic or otherwise) under field conditions in order to determine the impact of the multiple biotic and abiotic stresses to which the plant will be exposed, on its performance characteristics. I determined that the total carotenoid content and composition in the endosperm at maturity were very similar in greenhouse- and field-grown plants. No differences were found in endogenous carotenogenic or transgene expression levels, throughout endosperm development, between greenhouse- and field-grown plants. The overall agronomic performance and characteristics such as yield components of Carolight^{R} and its near isogenic line, M37W, were indistinguishable. In follow up experiments I introgressed the *Bt cry1Ac* insecticidal transgene (named 4Bt) into Carolight^{R} . The new line, 4BtxHC, was toxic to larvae of the corn borer *Ostrinia nubilalis*. However, the insecticidal activity of 4BtxHC grains was lowered compared to the original 4Bt line by 17%. This difference was statistically significant and I generated experimental evidence which suggests that the introduced carotenogenic pathway influences the response of insects to the Bt toxin in the presence of high carotenoid levels. Furthermore larvae fed on leaves from Carolight^{R} (with no Bt gene introgressed) or its near isogenic line exhibited different feeding behavior. I measured a higher degree of feeding when M37W leaves rather than Carolight^{R} leaves were used. I attribute this to a knock-on effect of carotenoid metabolism in the seeds which affects the leaves and, more specifically, how attractive they are to insect pests. Further studies need to be carried out to confirm this hypothesis. Finally I explored the intellectual property landscape relevant to the production and commercialization of Carolight^{R} highlighting relevant patents which would require particular attention in future commercialization activities.

Resumen

Mi proyecto de investigación está centrado en la caracterización molecular, bioquímica y agronómica, de una línea transgénica de maíz con elevado contenido en carotenoides, denominada, Carolight^{R} bajo diferentes condiciones ambientales. La línea Carolight^{R} fue transformada con *Zmpsy1* y *PacrtI*, dos genes carotenogénicos, la expresión de los cuales resultó en una acumulación masiva de carotenoides en el endospermo. Es importante evaluar el comportamiento de una nueva variedad de planta (transgénica o no) en condiciones de campo con el fin de determinar el impacto de múltiples estreses bióticos y abióticos a los que estara sometida la planta. Con los análisis que he realizado, he determinado que tanto el contenido de carotenoides totales y como su composición en el endospermo en madurez fisiológica fueron muy similares entre las plantas cultivadas en el invernadero y las cultivadas en el campo. No encontré diferencias significativas entre los niveles de expresión de los genes carotenogenicos endogenos o en la expresión de los transgenes durante todo el desarrollo del endospermo, al comparar la plantas cultivadas en invernadero con las cultivadas en el campo. El rendimiento y sus componentes (biomasa total, número y peso de los granos)de Carolight^{R} y su línea isogénica, M37W, fueron similares. En experimentos posteriores, realice la introgresión de los transgenes *Btcry1Ac* (llamado 4Bt) con actividad insecticida a Carolight^{R}. La línea obtenida 4BtxHC era toxica a las larvas del taladro *Ostrinia nubilalis*. No obstante la actividad insecticida en las semillas 4BtxHC era inferior en un 17% a la línea original 4Bt. Esta diferencia fue significativa, por lo que he generado evidencias experimentales en las que sugiero que la ruta carotenogenica introducida influye en la respuesta de los insectos a la toxina Bt cuando esta se encuentra con un elevado contenido en carotenoides. Las larvas alimentadas con hojas de la línea Carolight^{R} (sin el gen Bt introducido) o su línea casi isogénica tuvieron un comportamiento diferente. Medí las diferencias en los niveles de alimentación cuando las hojas control M37W fueron utilizadas en lugar hojas de Carolight^{R}. Se observó un menor consumo en las larvas alimentadas con hojas Carolight^{R}; esta diferencia se attribuye a la activación del metabolismo de los carotenoides en semilla que podría haber

producido un efecto secundario en las hojas, en particular de cuan atractivas resultaron a las larvas. Es necesario realizar más estudios para confirmar esta hipótesis. Finalmente, he analizado la propiedad intelectual relevante a la producción y comercialización de Carolight^{R}, remarcando las patentes que requerirían atención especial para futuras actividades comerciales.

Resum

El meu projecte de recerca està centrat en la caracterització molecular, bioquímica i agronòmica, d'un línea transgènica de panís amb un elevat contingut en carotenoides, denominada, Carolight^{R} en condicions ambientals diferents. La línea Carolight^{R} va ser transformada amb *Zmpsy1* i *PacrtI*, dos gens carotenogènics, l'expressió dels quals resulta en una acumulació massiva de carotenoides en l'endosperma. És important analitzar el comportament d'una varietat nova de planta (sigui transgènica o no) en condicions de camp amb la finalitat d'avaluar l'impacte dels múltiples estressos biòtics i abiòtics als que estarà sotmesa. Amb les anàlisis que he dut a terme, he determinat que tant el contingut en carotenoides totals, com la seva composició en l'endosperma, en l'etapa de maduresa, van ser molt similars entre las plantes cultivades a l'hiverncle i les cultivades al camp. No vaig trobar diferencies significatives entre els nivells d'expressió dels gens carotenogènics endògens o en l'expressió dels transgens durant tot el desenvolupament de l'endosperma, en comparar la plantes cultivades en l'hivernacle amb les cultivades en el camp. El rendiment agronòmic general i les característiques específiques dels components del rendiment de Carolight^{R} i la seva línia isogénica, M37W, van ser indistingibles. En experiments posteriors vaig dur a terme la introgressió dels transgens *Btcry1Ac* (anomenat 4Bt) amb activitat insecticida a Carolight^{R}. La línea obtinguda 4BtxHC era tòxica a les larves del barrinador *Ostrinia nubilalis*. No obstant, l' activitat insecticida en les llavors 4BtxHC era inferior en un 17% a la línia original 4Bt. Aquesta diferencia era significativa, i per tant he generat evidencies experimentals en lae que suggereixo que la ruta carotenogènica introduïda influeix en la resposta dels insectes a la toxina Bt quan aquesta es troba amb un elevat contingut en carotenoides. Les larves alimentades amb fulles de la línea Carolight^{R} (sense el gen Bt introduït) o la seva línia gairebé isogènica van tenir un comportament diferent. Vaig mesurar més alimentació quant les fulles control M37W eren utilitzades en lloc de les fulles de Carolight^{R}.Es va observar un menor consum en les larves alimentades amb fulles de la línia Carolight^{R} ; aquesta diferencia s'atribueix a l'activació del metabolisme dels carotenoids en llavors que podria haver

produït un efecte secundari a les fulles, en particular de quan atractives varen ser aquestes per a les larves. Es necessari realitzar més estudis per a confirmar aquesta hipòtesis. Finalment he analitzat la propietat intel·lectual rellevant en la producció i comercialització de Carolight^{R}, tot remarcant les patents que requeririen una atenció especial en el cas de que es consideressin futures activitats comercials.

LIST OF ABBREVIATIONS

ABA	Abscisic Acid
ANOVA	Analysis Of Variance
ARMD	Age-Related Macular Degeneration
BCH	Non-Heme Di-Iron B-Carotene Hydroxylase
Bt	*Bacillus thuringiensis*
cDNA	Complementary DNA
CRTI,	Bacterial Phytoene Desaturase/Isomerase
CRTISO	Carotenoid Isomerase;
Cry1Ac	Crystal Protein Ac coding gene
Cry1Ca	Crystal Protein Ca coding gene
CYP97A and CYP97B	Heme-Containing Cytochrome P450 B-Ring Hydroxylases
CYP97C	Carotene Ɛ-Ring Hydroxylase
DAP	Days After Pollination
DMAPP (or DMADP)	Dimethylallyl Diphosphate
DNA	Deoxyribonucleic Acid
DW	Dry Weight
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Environmental Protection Agency
EcfolE	*Escherichia coli* GTP Cyclohydrolase (GCH1) coding gene
F	Field
FTO	Freedom-To-Operate
FW	Fresh Weight
GM	Genetically Modified
GGDP	Geranyl Geranyl Diphosphate
GGDPS	Geranyl Geranyl Diphosphate Synthase
GH	Greenhouse
GTP	Guanosine-5'-Triphosphate
HC	High Carotenoid
HPLC	High Performance Liquid Chromatography
HR	Herbicide Resistance
IP	Intellectual Property
IPDP (or IPP)	Isopentyl Diphosphate
IPPI	Isopentenyl Diphosphate Isomerase
IR	Radiation Interception
IZE	Immature Zygotic Embryo
L (1-6)	Instar (Insect Nymphal Stages)
LAI	Leaf Area Index
LMICs	Low-And-Middle-Income Countries
LMW	Low Molecular Weight
LUT1	E-Ring Carotene Hydroxylase
LYCB	Lycopene B-Cyclase;
LYCE	Lycopene Ɛ-Cyclase
MTAs	Material Transfer Agreements

MEP	Methylerythritol Phosphate
mRNA	Messenger RNA
NHI	N Harvest Index
NS	Not Significant
NSY	Neoxanthin Synthase
NUtE	N Utilization Efficiency
OECD	Convention On The Organisation For Economic Co-Operation And Development
Osdhar	Rice Dehydroascorbate Reductase
Pa	*Pantoea ananatis*
PCR	Polymerase Chain Reaction
PCT	Patent Cooperation Treaty
PDS	Phytoene Desaturase
PSY	Phytoene Synthase
qRT-PCR	Quantitative PCR
RT-PCR	Reverse transcription PCR
R1	Silking
R6	Maturity
RNA	Ribonucleic Acid
SD	Standard Deviation
SEM	Standard Error Of The Mean
SLA	Specific Leaf Area
SLN	Specific Leaf Nitrogen
TP	Tangible Property
UHPLC	Ultra High Performance Liquid Chromatography
UV	Ultra Violet
V8	Eight Fully Expanded Leaves
VDE	Violaxanthin De-Epoxidase
vip3	Vegetative Insecticidal Protein 3 coding gene
WIPO	World Intellectual Property Organization
WTO	World Trade Organisation
ZDS	Z-Carotene Desaturase
ZEP	Zeaxanthin Epoxidase
Z-ISO	Z-Carotene Isomerase
Zm	*Zea Mays*

Aims And Objectives

AIMS AND OBJECTIVES

The general aim of my project is to characterize the performance of transgenic high carotenoid line Carolight^{R} in different environments. The specific objects are:

- To determine the agronomic performance of Carolight^{R} in the field in comparison to its near-isogenic line M37W **(Chapter 2)**.
- To compare Carolight^{R} endosperm carotenoid composition and transgene/gene expression profile over time in greenhouse- and field-grown plants, to assess the stability of the transgenic trait under different environmental conditions **(Chapter 3)**.
- To test the toxicity of a new high carotenoid line with insecticidal properties against two maize borers (*Ostrinia nubilalis* and *Mythimna unipuncta*) **(Chapter 4)**.
- To compare the feeding behaviour of two maize borers (*Ostrinia nubilalis* and *Mythimna unipuncta*) on Carolight^{R} and M37W leaves and grains **(Chapter 4)**.
- To explore the intellectual property (IP) landscape relative to the production and commercialization of Carolight^{R} **(Chapter 5)**.

Chapter 1

GENERAL INTRODUCTION

1.1 Classification of carotenoids

Carotenoids are natural pigments belonging to a large isoprenoid family of mostly C40 tetraterpenoids. They are synthesized in the plastids of plants and in other photosynthetic organisms such as algae, bacteria and fungi (Zhu et al. 2010) and are responsible for bright red, yellow and orange hues in many fruits and vegetables (Britton et al., 2004). They comprise over 800 molecules which are divided into two groups: hydrocarbon carotenes such as β-carotene, α-carotene and lycopene and xanthophylls, which contain oxygenated substituents, such as lutein, zeaxanthin and violaxanthin (Zaripheh & Erdman, 2002). They are essential components of photosynthetic membranes and have a fundamental role in photosynthesis as accessory pigments. Furthermore, they are involved in the xanthophyll cycle preventing photo-oxidative damage and they can also prevent the oxidative disruption of the photosynthetic process (Havaux and Niyogi, 1999¸Bartley and Scolnik, 1995; Bassi et al., 1993).

1.2 Natural sources of carotenoids

Humans and animals need to introduce carotenoids with the diet through consumption of fresh fruits and vegetables. A variety of yellow-orange or dark green coloured fruits such as mangoes, papaya, carrots, sweet potatoes, spinach, kale and pumpkin, typically, are good sources of β-carotene (Farré et al., 2010; Bai et al., 2011).

Bright red colour of watermelons, tomatoes, guavas and grapefruit is due to lycopene (Bramley, 2000). Lutein and zeaxanthin are the most common xanthophylls in green leafy vegetables (spinach, kale, broccoli, peas and lettuce) and egg yolks (Perry et al., 2009).

Zeaxanthin is the pigment that gives corn, saffron, wolfberries, and many other plants their characteristic yellow color (Sommerburg et al., 1998).

1.3 Carotenoid biosynthesis

The carotenoid biosynthesis pathway starts with the synthesis of the five-carbon isopentyl diphosphate (IPDP) and its allylic isomer, dimethylallyl diphosphate (DMADP); both are derived from the methylerythritol phosphate (MEP) pathway (Eisenreich et al., 2001; Hunter, 2007). Three molecules of IPDP are combined with DMADP by geranyl geranyl diphosphate (GGDP) synthase (GGDPS) to produce GGDP, a 20-carbon molecule, which is the key precursor in the carotenoid biosynthesis pathway. Condensation of two GGDP molecules by phytoene synthase (PSY) is the first committed step in the carotenoid biosynthetic pathway resulting in the production of phytoene, a 40-carbon molecule. This is the main rate-limiting step in the carotenoid biosynthetic pathway (Lu and Li, 2008). Phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) carry out desaturation of phytoene into lycopene. In non-photosynthetic bacteria, the single enzyme CRTI (bacterial-type phytoene desaturase) accomplishes all the above steps. Lycopene is the critical branching point in the pathway (Cazzonelli and Pogson, 2010) because it acts as the substrate for two competing enzymes: lycopene β-cyclase (LYCB), and lycopene ε-cyclase (LYCE) (Cunningham and Gantt, 2011). Both enzymes cyclize the linear backbone to generate terminal α- or β-ionone rings, differing by the 4,5- or 5,6-position of the double bond. The addition of one β-ring by LYCB generates γ-carotene, and the addition of a second β-ring to the free end of γ-carotene by the same enzyme produces β-carotene. This reaction is rapid, so γ-carotene tends not to accumulate. Alternatively, the addition of one ε-ring to lycopene by LYCE generates δ-carotene. This is a poor substrate for LYCE so it is unusual for the second ε-cyclization to

take place, but it is a good substrate for LYCB, which adds a β-ring to the free end to produce α-carotene. α and β-Carotene are hydroxylated to produce lutein and zeaxanthin, respectively. These hydroxylation reactions are catalyzed by the β-ring carotene hydroxylase and the ε-ring carotene hydroxylase (LUT1) (Galpaz et al., 2006; Kim and DellaPenna, 2006; Tian et al., 2004). Epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) produces violaxanthin (Zhai et al., 2016). This reaction is reversed by violaxanthin de-epoxidase (VDE) to give rise to the xanthophyll cycle, which helps plants acclimatize to high light stress by stimulating energy dissipation in photosystem II. Violaxanthin is further converted to neoxanthin by neoxanthin synthase (NSY). The formation of neoxanthin represents the last step in the carotenoid biosynthesis pathway (Lu and Li, 2008). Through the xantophylls cycle, with a number of additional steps, zeaxanthin can be converted into abscisic acid, an important plant hormone (Seo and Koshiba, 2002).

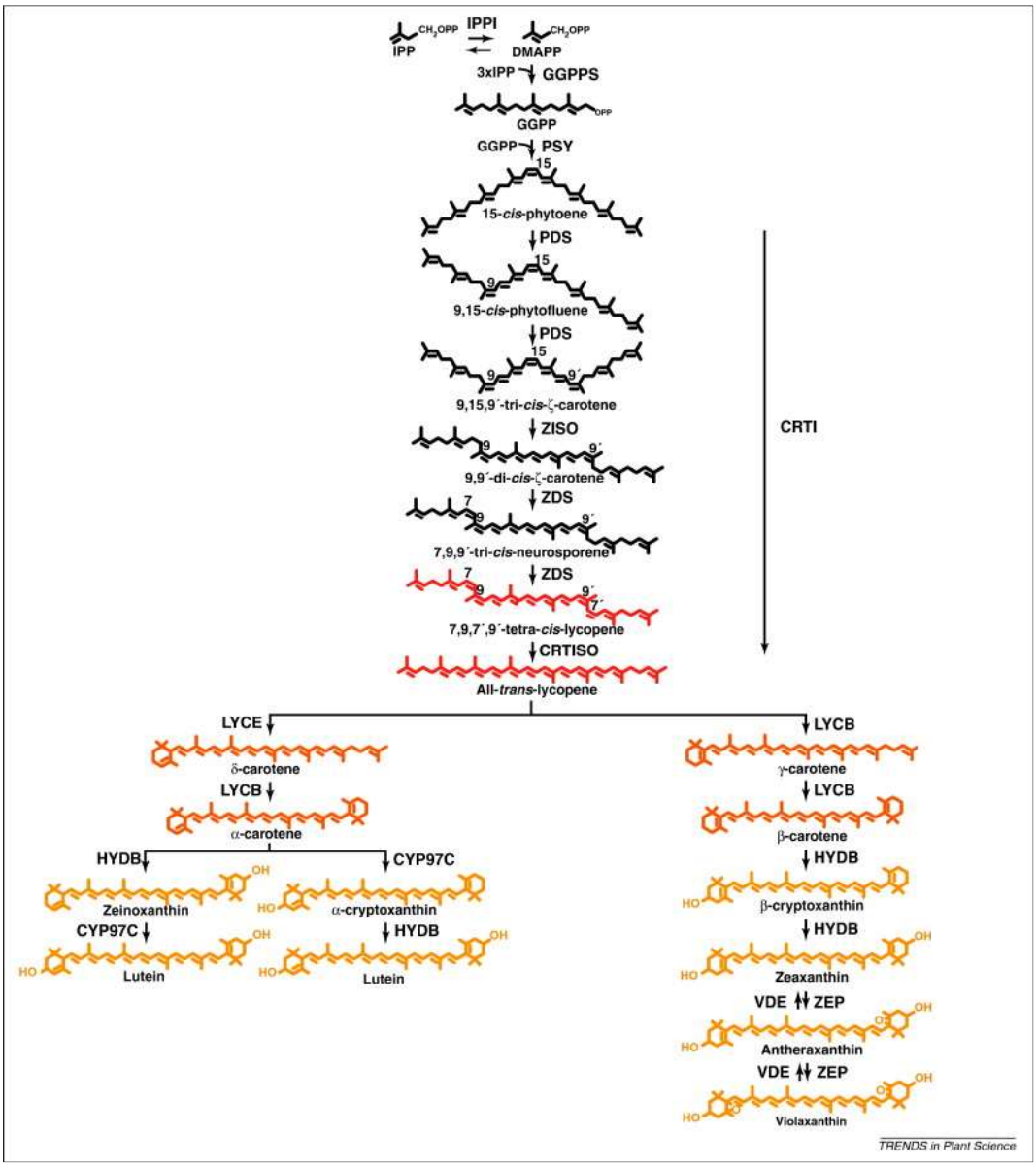

Figure 1: Carotenoid biosynthesis pathway in plants. Figure adapted from Farre' et al. (2011). CRTISO, carotenoid isomerase; CYP97C, carotene ε-ring hydroxylase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; HYDB, β-carotene hydroxylase [non-heme di-iron hydroxylases, β-carotene hydroxylase (BCH) and heme-containing cytochrome P450 β-ring hydroxylases, CYP97A and CYP97B]; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; LYCB, lycopene β-

cyclase; LYCE, lycopene ε-cyclase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, ζ-carotene isomerase. CRTI, bacterial phytoene desaturase/isomerase, is also shown (Farre' et al., 2011).

1.4 Impact of carotenoid enhancement on general metabolism

A recent comprehensive transcriptomic, proteomic and metabolomic analysis revealed that the endosperm-specific carotenoid biosynthesis in Carolight^{R} correlated with changes in endosperm carbohydrate metabolism, as well as sterol and fatty acid biosynthesis. These changes in core metabolism were probably due to pleiotropic effect (Decourcelle et al., 2015). Genetic engineering of a pathway may have an effect on global metabolism due to the fact that precursors are consumed at the expense of other resident pathways (Sandmann, 2001). For example, a significantly lower value of total fruit weight was measured in one transgenic tomato plant (HighCaro) constitutively expressing the tomato *lycb* gene (Giorio et al., 2006). Lower yield may be attributed to a number of different factors, including perturbation of other pathways induced by transgene expression. In other cases, collateral effects were not negative for plant metabolism. Recent studies reported that carotenoid enhancement was positively correlated with stress tolerance (Goo et al., 2015; Kim et al., 2013) or enhancement of other nutrients such as proteins and oleates (Schmidt et al., 2015). Organ-specific promoters are often used to prevent or reduce effects on general metabolism (reviewed in Peremarti et al., 2010) so that plant phenology or development are not affected. Since an indirect impact of carotenoid enhancement on general metabolism cannot be excluded, it is important to characterize the growth and behaviour of such transgenic plants in comparison to their near-isogenic lines.

1.5 Carolight^{R}

Micronutrients are substances required in small quantities for basic physiological functions. They include minerals, vitamins and phytochemicals that must be absorbed from the diet in amounts generally <100 mg/day. Micronutrient deficiency is a major threat to public health, particularly for children and pregnant women in low-and-middle-income countries (LMICs) as defined by the World Bank (http://data.worldbank.org/about/country-and-lending-groups). This issue can be addressed by the fortification of processed foods such as flour, bread, dairy products, packaged cereals or salt, which is a common practice in OECD countries (Zhu et al., 2007). However, this approach is often unsustainable in LMICs because of inefficient food distribution capacity and networks, and the prevalence of subsistence agriculture in rural populations, where the diet mainly consists of milled cereal grains (Yuan et al., 2011; Perez-Massot et al., 2013). A more sustainable approach is the biofortification of staple crops by genetic engineering (Farre` et al., 2014). For example, several transgenic crops expressing carotenogenic genes have been developed to boost levels of carotenoids, particularly the essential nutrient β-carotene, including Golden Rice II (Ye et al., 2000; Paine et al., 2005), Golden Potato (Ducreux et al., 2005) and Carolight^{R} (Naqvi et al., 2009; Farre' et al., 2011). This variety has been generated by transforming a white endosperm corn variety with two carotenogenic genes: *Zmpsy1* (corn phytoene synthase 1) and *PacrtI* (bacterial phytoene desaturase). The endosperm thus accumulates 169-fold more β-carotene (pro-vitamin A) as well as other nutritionally important carotenoids such as lycopene, lutein and zeaxanthin (Naqvi et al., 2009; Zhu et al., 2008). An improved version of Carolight^{R} will also be resistant to lepidopteran insect pests due to the presence of one or more *Bacillus thuringiensis* (*Bt*) genes.

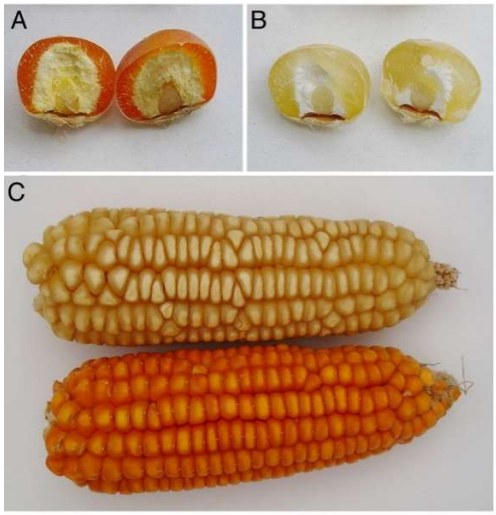

Figure 2: Accumulation of carotenoids in the endosperm of transgenic corn line Carolight^{R} (a) Orange-yellow phenotype of the transgenic endosperm. (b) Normal phenotype of the wt M37W endosperm. (c) Comparison of M37W and transgenic cobs, showing significant increases in the levels of key carotenoids in the transgenic cobs. (Naqvi et al., 2009)

1.6 Carolight^{R}: benefits in human health

The potential health benefits of Carolight^{R} include the alleviation of vitamin A deficiency, which affects more than 20 million pregnant women and 100 million children in LMICs, up to 500,000 of whom go blind and/or suffer growth defects (Bates, 1995; Duester, 2008; Harrison, 2005; HarvestPlus, 2014 http://www.harvestplus.org/content/vitamin; McCullough et al., 1999). Vitamin A cannot be synthesized de novo by humans but can be synthesized from the pro-vitamin A carotenoid β-carotene. The other carotenoids in

Carolight^{R} provide additional health benefits, for example by acting as antioxidants that help to prevent diseases caused by accumulation of reactive oxygen species, for example certain types of cancer, cardiovascular disease and neurodegenerative disorders (Berman et al., 2015; Zhu et al., 2013). More specifically, lutein and zeaxanthin accumulate in the macula of the eye protecting the retina from damaging blue and near-ultraviolet light and thus preventing or delaying age-related macular degeneration (Berman et al., 2015). Several studies suggest that multiple carotenoids provide synergistic benefits (Diplock et al., 1998; Van Poppel, 1996). The deployment of Carolight^{R} in LMICs could therefore address the high level of preventable diseases, particularly those affecting children and pregnant women.

1.7 Introgression of insect tolerance into Carolight^{R}

Since the ultimate goal of Carolight^{R} is to be introduced into agricultural systems in developing countries, it is extremely important to guarantee the best possible performance in the field, including resistance to pest infestations, which often result in devastating yield losses. In developing countries it is estimated that about half of all crop production is lost due to insect damages (Christou et al., 2006). Insects can reduce crop yield not only by directly destroying parts of the plants, but also by acting as vectors for many viral and bacterial diseases. Furthermore, resulting damage may result in fungal infections, which can cause contamination with mycotoxins (Yuan et al., 2011). Mycotoxins, such as aflatoxin and fumonisin, are extremely dangerous for human health since they act as antinutritional factors when present at low doses in food (Wu, 2007), are carcinogenic, and can also act as immune system repressors (Williams et al., 2010). One way to prevent this damage is to continue using huge amounts of exogenously applied pesticides or to introgress insecticidal transgenes

into the plant. Genetically-engineered crops expressing insecticidal transgenes have been used to control pests since 1985. Most of these genes derive from the Gram-positive, soil-dwelling bacterium *Bacillus thuringiensis* which produces insecticidal δ-endotoxins that are highly specific to particular taxonomic groups of insects. Many different crops have been transformed with a variety of *Bt* genes, each of them having different insect targets and different insecticidal efficacy. The use of these crops in agricultural settings has dramatically reduced the need for highly contaminating pesticides and the fuel needed for spraying, thus resulting in an environmental benefit. Their high specificity makes them innocuous to mammals and to non-target beneficial insects (Sanahuja et al., 2011). These reasons have allowed both improvement of economic status of farmers and biodiversity preservation (James, 2010; Brookes and Barfoot, 2010). We introduced the *Bt cry1Ac* gene into Carolight^{R} to test its efficiency against lepidopteran maize pests which are common in the region of our study. However, the use of a single Bt gene could cause insect resistance to occur (Tabashnik et al., 2013). Bt toxins, in fact, bind to a specific receptor on the surface of midgut epithelial cells so that resistance can evolve via receptor mutations that abolish such binding. Many different strategies (including creation of refugia) can be used to prevent the emergence of resistance in target pests. One of the most effective is the pyramiding strategy, which consists of the introduction of different insecticidal genes targeting different receptors in the same insect providing a multi-mechanistic defence. Resistance could only occur in the case that insects acquire simultaneous mutations in different genes, which is very unlikely, thus making pyramiding an effective strategy for durable defence against insect pests (Gatehouse and Gatehouse, 1998). This is the reason why our final goal will be to introduce other two additional *Bt* genes into Carolight^{R}, namely *cry1Ca* and *vip3* (ongoing work).

1.8 Lepidopteran corn pests in Spain

In 2015 Spain grew 92% of all biotech maize in Europe, with 107,749 hectares of Bt maize, being by far the largest adopter between the 5 European countries (Spain, Portugal, Czechia, Slovakia and Romania) which are currently growing transgenic crops (ISAAA Brief 51-2015; http://isaaa.org/resources/publications/briefs/51/executivesummary/default.asp). At present, the only Bt maize hybrids allowed for cultivation in the EU are those based on the event MON810 (Monsanto) expressing the Cry1Ab toxin. Bt maize expressing Cry1Ab toxin is an effective way to control the European corn borer (ECB) *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) which is one of the most important maize pests throughout the USA and Europe (Mason et al., 1996; Comas et al., 2013), and the Mediterranean corn borer (MCB) *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae) which appears to be the most damaging pest of maize in Spain (Castañera, 1986) and other Mediterranean countries (Anglade, 1972; Melamed-Madjar & Tam, 1980). However, available Bt maize cultivars have shown a much lower efficacy for controlling some secondary Spanish maize lepidopteran pests present in maize fields in our region of study. Maize crops are in fact attacked by *Mythimna unipuncta* (Haworth) and *Helicoverpa armigera* (Hübner), two other non-target Lepidopteran species belonging to the Noctuidae family. Few studies are present regarding the effect of Bt maize on these two species (i.e. Eizaguirre et al., 2010; Pilcher et al., 1997; Schaafsma et al., 2007) but it has been reported that at least some individuals can survive in maize crops taking advantage of the absence of corn borers (Eizaguirre et al., 2010). *H. armigera*, is primarily a cotton pest but can feed on crops belonging to very different botanical families; little is known on the damage that this species is able to inflict to maize but it is always present in low number on the ear silks. *M. unipuncta* is normally

feeding on leaves of many gramineous plants such as maize, millet and weeds and is able to consume all the leaves of a maize field when its population is high (Eizaguirre et al., 2010). It is therefore important to study the effect of Bt maize not only in primary pests such as *O. nubilalis* and *S. nonagrioides* but also on non-target species present in the region of the study, with a particular attention to *M. unipincta*, since L5 larvae of this species can migrate from other crops and grassy areas to corn fields during the growing season causing high yield losses (Pilcher et al., 1997). In particular, there are very few studies regarding the efficiency of Cry1Ac expressing maize against the four main corn borers in our region of study. Cry1Ac has been proven effective against *H. armigera* in cotton (Wu et al. 2008), and recently in maize (Chen et al., 2016). Wang et al. (2014) reported that their transgenic maize line transformed with the *cry1Ac* gene provides good resistance to *O. furnacalis* (Guenée) (Lepidoptera: Crambidae) while the first commercial Bt soybean (MON 87701 × MON 89788), expressing Cry1Ac protein has been proven effective against soybean looper *Chrysodeixis includes* (Walker) (Lepidoptera: Noctuidae) (Yano et al., 2015).

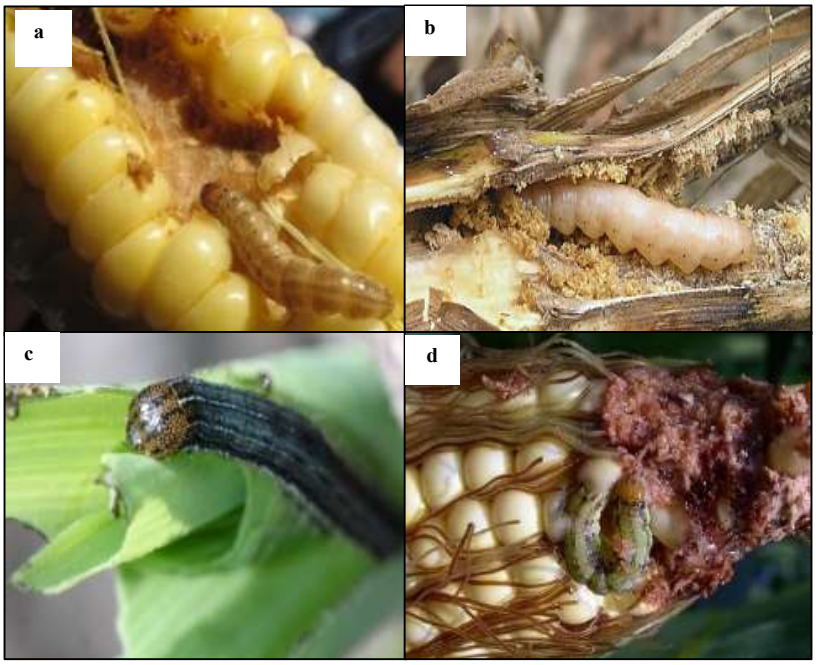

Figure 3: Common Lepidopteran species attacking maize in Spain. The stem borers a) *O. nubilalis* and b) *S. nonagrioides*; c) the leaf feeder *M. unipuncta*; d) the earworm *H. armigera*.

1.9 References

- Anglade, P. (1972). Les sesamia. Entomologie Appliqué à l'Agriculture. II, Lépidoptères (ed. by Balachowsky, A. S.), pp. 1389– 1400. Masson et Cie, Paris, France.
- Bai, C., Twyman, R.M., Farre, G., Sanahuja, G., Christou, P., Capell, T., et al. (2011). A golden era pro-vitamin A enhancement in diverse crops. *Vitr Cell Dev Biol - Plant*. **47**:205-221.
- Bartley, G.E. & Scolnik P.A. (1995). Plant carotenoids: pigments for photoprotection, visual attraction, and human health. *Plant Cell*.**7**:1027-1038.
- Bassi, R. Pineau, B., Dainese, P., Marquardt, J. (1993). Carotenoid-binding proteins of photosystem II. *Eur J Biochem*. **212**:297-303.
- Bates, C.J. (1995). Fat-soluble vitamins: vitamin A. *Lancet*. **345**:31-34.
- Berman, J., Zorrilla-López, U., Farré, G., Zhu, C., Sandmann, G., Twyman, R.M., et al. (2015). Nutritionally important carotenoids as consumer products. *Phytochem. Rev.* **5**:727-743 (2015).
- Bramley, P.M. (2000). Is lycopene beneficial to human health? *Phytochemistry*. **54**:233-236.
- Britton, G., Liaaen-Jenson, S. & Pfander, H. (2004). Carotenoids Handbook. *Photosynthetica* **42**:186-186.
- Brookes, G. & Barfoot, P. (2010). GM crops: global socio-economic and environmental impacts 1996-2008. *PG Economics Ltd*, Dorchester, UK.

- Castañera, P. (1986). Plagas del Maíz. IV. Jornadas Técnicas Sobre El Maíz, Lérida. Plagas, pp. 1–24. Ministerio de Agricultura Pesca y Alimentación, Madrid, Spain.
- Cazzonelli, C.I. & Pogson, B.J. (2010). Source to sink: regulation of carotenoid biosynthesis in plants. *Trends Plant Sci.* **15**, 266-274.
- Christou, P., Capell, T., Kohli, A., Gatehouse, J.A., Gatehouse, A.M. (2006). Recent developments and future prospects in insect pest control in transgenic crops. *Trends Plant Sci*. 11:302-308.
- Comas, J., Lumbierres, B., Pons, X., Albajes, R. (2013). Ex-ante determination of the capacity of field tests to detect effects of genetically modified corn on non-target arthropods. *J Econ Entomol*. **106**:1659-1668.
- Cunningham, F.X., Pogson, B., Sun, Z., McDonald, K.A., DellaPenna, D., Gantt, E. (1996). Functional analysis of the beta and epsilon lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell.* **8**:1613-1626.
- Decourcelle, M., Perez-Fons, L., Baulande, S., Steiger, S., Couvelard, L., Hem, S., et al. (2015). Combined transcript, proteome, and metabolite analysis of transgenic maize seeds engineered for enhanced carotenoid synthesis reveals pleotropic effects in core metabolism. *J Exp Bot*. **66**:3141-50.
- Diplock, A.T. Charleux, J.L., Crozier-Willi, G., Kok, F.J., Rice-Evans, C., Roberfroid, M., Stahl, W., Viña-Ribes, J. (1998). Functional food science and defence against reactive oxidative species. *Br. J. Nutr.* **80**:77-112.

- Ducreux, L.J., Morris, W.L., Taylor, M.A., Millam, S. (2005) Agrobacterium-mediated transformation of *Solanum phureja*. *Plant Cell Rep.* **24**:10-14.
- Duester, G. (2008). Retinoic acid synthesis and signaling during early organogenesis. *Cell*. **134**:921-931.
- Eisenreich, W., Rohdich, F. & Bacher, A.(2001) Deoxyxylulose phosphate pathway to terpenoids. *Trends Plant Sci.* **6**:78-84.
- Eizaguirre, M., Madeira, F., López, C., & Romeis, J. (2010). Effects of Bt maize on non-target lepidopteran pests. *IOBC/wprs Bulletin*. **52**:49-55.
- Farré, G., Sanahuja, G., Naqvi, S., Bai, C., Capell, T., Zhu, C., et al. (2010). Travel advice on the road to carotenoids in plants. *Plant Sci*. **179**:28-48.
- Farré, G., Bai, C., Twyman, R.M., Capell, T., Christou, P., Zhu, C. (2011). C Nutritious crops producing multiple carotenoids - a metabolic balancing act. *Trends Plant Sci*. **16**:532-540.
- Farre, G., Blancquaert, D., Capell, T., Van Der Straeten, D., Christou, P., Zhu, C. (2014). Engineering complex metabolic pathways in plants. *Annu. Rev. Plant Biol.* **65**:187-223.
- Galpaz, N., Ronen, G., Khalfa, Z., Zamir, D., Hirschberg, J. (2006). A chromoplastspecific carotenoid biosynthesis pathway is revealed by cloning of the tomato white-flower locus. *Plant Cell.* **18**:1947-1960.
- Gatehouse, A.M.R. & Gatehouse J.A. (1998). Identifying proteins with insecticidal activity: use of encoding genes to produce insect-resistant transgenic crops. *Pestic. Sci*. **52**:165-175.

- Giorio G., Stiglian A.L. & D'Ambrosio C. (2006). Agronomic performance and transcriptional analysis of carotenoid biosynthesis in fruits of transgenic HighCaro and control tomato lines under field conditions. *Transgenic Res*. **16**:15-28.

- Goo, Y.M., Han, E.H., Jeong, J.C., Kwak, S.S., Yu, J., Kim, Y.H., et al. (2015). Overexpression of the sweet potato *IbOr* gene results in the increased accumulation of carotenoid and confers tolerance to environmental stresses in transgenic potato. *C R Biol*. **338**:12-20.

- Havaux, M. & Niyogi, K.K. (1999). The violaxanthin cycle protects plants from photo-oxidative damage by more than one mechanism. *Proc Natl Acad Sci U S A*. **96**: 8762-8767.

- Houghton, L.A., Gray, A.R., Rose, M.C., Miller, J.C., Hurthouse, N.A., Gregory, J.F. (2011). Long-term effect of low-dose folic acid intake: potential effect of mandatory fortification on the prevention of neural tube defects. *Am. J. Clin. Nutr.* **94**:136-141.

- Hunter, W.N. (2007). The non-mevalonate pathway of isoprenoid precursor biosynthesis. *J Biol Chem.* **282**:21573-21577.

- James, C. (2010). Global status of commercialized biotech/GM crops: 2009. *ISAAA Brief* 41-2009. ISAAA, Ithaca, NY.

- Kim, J. & DellaPenna, D. (2006). Defining the primary route for lutein synthesis in plants: the role of Arabidopsis carotenoid beta-ring hydroxylase CYP97A3. *Proc Natl Acad Sci USA.* **103**:3474-3479.

- Kim, S.H., Ahn, Y.O., Ahn, M.J., Jeong, J.C., Lee, H.S., Kwak, S.S. (2013). Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and

salt stress tolerance in transgenic sweetpotato cultures. *Plant Physiol Biochem*. **70**:445-454.

- Lu, S. & Li, L.(2008). Carotenoid metabolism: biosynthesis, regulation, and beyond. *J Integr Plant Biol.* **50**, 778-785.
- McCullough, F.S., Northrop-Clewes, C.A. & Thurnham, D.I. (1999). The effect of vitamin A on epithelial integrity. *Proc. Nutr. Soc.* **58**:289-293.
- Melamed-Madjar, V. & Tam, S. (1980). A field survey of changes in the composition of corn borer populations in Israel. *Phytoparasitica*. **8**: 201–204.
- Naqvi, S., Zhu, C., Farre, G., Ramessar, K., Bassie, L., Breitenbach, J., et al. (2009). Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proc. Natl. Acad. Sci. USA*. **106**:7762-7767.
- Paine, J.A, Shipton, C., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., et al. (2005). Improving the nutritional value of golden rice through increased pro-vitamin A content. *Nat. Biotechnol.* **23**:482-487.
- Peremarti, A., Twyman, R.M., Gómez-Galera, S., Naqvi, S., Farré, G., Sabalza, M., et al. (2010). Promoter diversity in multigene transformation. *Plant Mol Biol*. **73**:363-378.
- Pérez-Massot, Banakar, R., Gómez-Galera, S., Zorrila-López, U., Sanahuja, G., Arjó, G et al. (2013). The contribution of transgenic plants to better health through improved nutrition: opportunities and constraints. *Genes Nutr.* **8**:29-41.

- Perry, A., Rasmussen, H., & Johnson, E. J. (2009). Xanthophyll (lutein, zeaxanthin) content in fruits, vegetables and corn and egg products. *J Food Compost Anal*. **22**:9-15.

- Pilcher, C.D., Rice, M.E., Obrycki, J.J., Lewis, L.C. (1997). Field and laboratory evalua-tions of transgenic Bacillus thuringiensis corn on secondary lepidopteran pests (Lepidoptera: Noctuidae). *J. Econ. Entomol*. **90**:669-678.

- Sadras, V. O. & Richards R. A. (2014). Improvement of crop yield in dry environments: benchmarks, levels of organisation and the role of nitrogen. *J Exp Bot.* **65**:1981.

- Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., Christou, P. (2011). *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnol. J*. **9**:283-300.

- Sandmann, G. (2001) Genetic manipulation of carotenoid biosynthesis: strategies, problems and achievements. *Trends Plant Sci*. **6**:14-17.

- Schaafsma, A. W., Holmes, M. L., Whistlecraft, J., Dudley, S. A. (2007). Effectiveness of three Bt corn events against feeding damage by the true armyworm (*Pseudaletia unipuncta* Haworth). *Can. J. Plant Sci.* **87**:599–603

- Schmidt, M., Parrott, W., Hildebrand, D.F., Berg, R.H., Cooksey, A., Pendarvis, K., et al. (2015). Transgenic soya bean seeds accumulating β-carotene exhibit the collateral enhancements of oleate and protein content traits. *Plant Biotechnol J.* **13**:590-600.

- Seo, M. & Koshiba, T. (2002). Complex regulation of ABA biosynthesis in plants. *Trends Plant Sci*. **7**:41-48.
- Sommerburg, O., Keunen, J., Bird, A., van Kuijk, F.J. (1998). Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes. *Br J Ophthalmol*. **82**:907-910.
- Tian, L., Musetti, V., Kim, J., Magallanes-Lundback, M., DellaPenna, D. (2004). The Arabidopsis LUT1 locus encodes a member of the cytochrome p450 family that is required for carotenoid epsilon-ring hydroxylation activity. *Proc Natl Acad Sci USA*. **101**:402-407.
- Tabashnik, B.E., Brévault, T. & Carrière, Y. (2013). Insect resistance to Bt crops: lessons from the first billion acres. *Nat Biotechnol*. **31**:510-21.
- Van Poppel, G. (1996). Epidemiological evidence for beta-carotene in prevention of cancer and cardiovascular disease. *Eur. J. Clin. Nutr.* 50:57-61.
- Yuan, D., Bassie, L., Sabalza, M., Miralpeix, B., Dashevskaya, S., Farre, G., et al. (2011). The potential impact of plant biotechnology on the Millennium Development Goals. *Plant Cell Rep*. **30**:249-265.
- Williams, J.H., Grubb, J.A., Davis, J.W., Wang, J.S., Jolly, P.E., Ankrah, N.A. et al. (2010). HIV and hepatocellular and esophageal carcinomas related to consumption of mycotoxin prone foods in sub-Saharan Africa. *Am. J. Clin. Nutr*. **92**:154-160.
- Wimsatt, W.C. (1994). The ontology of complex systems: levels of organization, perspectives and causal thickets. *Can J Philos.* **20**:207-274.
- Wu, F. (2007). Bt corn and impact on mycotoxins. *CAB Rev*. **2**:1-8.

- Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P., Potrykus, I. (2000). Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science.* **287**:303-305.
- Yuan, D., Bassie, L., Sabalza, M., Miralpeix, B., Dashevskaya, S., Farre, G. (2011). The potential impact of plant biotechnology on the Millennium Development Goals. *Plant Cell Rep.* **30**:249-265.
- Zaripheh, S. & Erdman J.W. Jr. (2002). Factors that influence the bioavailablity of xanthophylls. *J Nutr.* **132**:531-534.
- Zhai, S., Xia, X., He, Z. (2016). Carotenoids in Staple Cereals: Metabolism, Regulation, and Genetic Manipulation. *Front Plant Sci*. **7**:1197.
- Zhu, C., Naqvi, S., Gomez-Galera, S., Pelacho, A.M., Capell, T., Christou, P. (2007). Transgenic strategies for the nutritional enhancement of plants. *Trends Plant Sci*. **12**:548-55.
- Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P., Capell, T. (2008). Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proc. Natl. Acad. Sci.USA*. **105**:18232-18237.
- Zhu, C., Bai, C., Sanahuja, G., Yuan, D., Farré, G., Naqvi, S., et al. (2010). The regulation of carotenoid pigmentation in flowers. *Arch. Biochem. Biophys*. 132-141.
- Zhu, C., Sanahuja, G., Yuan, D., Farre, G., Arjó, G., Berman, J. (2013). Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies. *Plant Biotechnol. J.* **11**:129-141.

Chapter 2

INTRODUCING A CAROTENOGENIC MINI-PATHWAY IN WHITE CORN DOES NOT AFFECT DEVELOPMENT AND AGRONOMIC PERFORMANCE

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Chapter 2:

A carotenogenic mini-pathway introduced into white corn does not affect development or agronomic performance

2.1 Abstract

High carotenoid maize may be useful to overcome vitamin deficiencies in developing countries. However, it is possible that transgene introduction in a plant may alter its physiology and there could be a competition in terms of resources between the production of novel heterologous molecules with other essential processes thus making the plant less productive in terms of biomass/yield. A transgenic crop needs to be at least as productive in terms of yield as the wild-type crops normally used by farmers to be adopted into agricultural systems. We compared field performance of a transgenic high carotenoid corn (Carolight^{R}) versus its wild-type near isogenic line. Our results show that the insertion of a transgenic cassette for high carotenoid accumulation did not affect crop general performance including grain yield and its main components compared to wild type M37W.

2.2 Introduction

Several transgenic crops with high carotenoid levels have been generated by overexpressing carotenogenic genes, including Golden Rice II (Paine et al. 2005) and high-carotenoid Carolight^{R} corn (Zhu et al., 2008), both of which overexpress corn *psy1* and *Pantoea ananatis crt*I. Golden Rice II produces 37 µg g^{-1} dry weight (DW) of total carotenoids (26-fold more than the near isogenic wild-type variety) and Carolight^{R} corn produces 84 µg g^{-1} DW of total carotenoids (156-fold more than the near isogenic wild-type variety) (Paine et al. 2005; Zhu et al., 2008). Such biofortified crops provide an excellent and sustainable strategy to combat pro-vitamin A deficiency, which is prevalent in many developing countries (Farré et al., 2011). Transgenic crops have increased the productivity and sustainability of agriculture, not only in industrialized countries but also in developing countries that have adopted them successfully (Farré et al., 2011; Sanahuja et al., 2013; Zhu et al., 2013). As part of the development process, novel plant varieties including genetically engineered crops are tested under field conditions to determine whether their performance meets the standards set by commercial developers. In cases of crops engineered for metabolic or development traits, the resources needed to produce novel heterologous molecules may compete with other processes, resulting in a trade-off between improved nutritional value and yield or agronomic performance. Both conventional breeding and genetic engineering may therefore affect physiological traits such as yield or biomass production in a positive (Subedi and Ma, 2007), negative (Elmore et al., 2001a, 2001b) or neutral manner (Marra et al., 2004). A recent comprehensive transcriptomic, proteomic and metabolomic analysis revealed that the endosperm-specific carotenoid biosynthesis in Carolight^{R} correlated with changes in

endosperm carbohydrate metabolism, as well as sterol and fatty acid biosynthesis (Decourcelle et al., 2015).

Transgene introduction may affect plant physiology and phenotype, as shown by different studies comparing transgenic (Bt) and non-transgenic (near-isogenic line) maize hybrids (Ma and Subedi, 2005; Subedi and Ma, 2007; Laserna et al., 2012; Shi et al., 2013). These effects were not only related to the ability of Bt maize to control pest and therefore reduce the impact of biotic stress, but probably also to epistatic (Falconer, 1981), pleiotropic (Ge et al., 2004; Decourcelle et al., 2015) or positional effect of transgene insertion (Feldmann et al., 1989). For example, Subedi and Ma (2007) not only reported higher grain yield for *Bt* hybrids than their non-transgenic isogenic line (in the absence of pests) but they also detected significant differences in time to flowering (*Bt* hybrids took 2–3 additional days to reach silking) between Bt hybrids and their near isogenic line (Ma and Subedi, 2005). The same authors reported greater vegetative growth at the beginning of the early reproductive period for *Bt* maize hybrids compared to their non-transgenic counterparts even if similar leaf N concentration were measured (Subedi and Ma, 2007). Regarding herbicide resistance (HR, plants expressing a phosphinotricin acetyl transferase transgene), despite properties that result in indirect yield benefits, some farmers observed a yield drop when using HR varieties (Raymer and Grey, 2003). Evidence for a reduced yield was found when comparing five HR varieties with five non-HR varieties in four locations in Nebraska, attributed to the presence of the HR gene (Elmore et al., 2001a, 2001b). In contrast, a 2002 national survey of soybean producers indicated no statistical difference in yield between non-transgenic soybean and HR soybean (Marra et al., 2004). In general, it has been reported that the impact of transgenic traits *Bt* and phosphinotricin acetyl transferase, both single or stacked traits) on mean yield,

compared with conventional hybrids, ranges from -765.6 Kg/Ha to +407.9 Kg/Ha (Shi et al., 2013).The HC maize used in this study has *Zmpsy1* and *PacrtI* transgenes driven by endosperm-specific promoters (LMW glutenin promoter and barley D hordein promoter, respectively). Nevertheless, possible side effects both in the endosperm and in other organs, resulting from the substantial increase in carotenoid biosynthesis, cannot be predicted and must be evaluated. Therefore, we tested the hypothesis that Carolight^{R} may differ in agronomic performance from its non-transgenic near isogenic counterpart. The key objective of our study was to determine whether the accumulation of carotenoids in Carolight^{R} affects the rates of photosynthesis, biomass accumulation and partitioning into different organs in experimental field trials. In order to broaden the range of environments and to test the consistency of adaptation and performance between Carolight^{R} and its near isogenic counterpart, we compared both genotypes in field plots under different soil nitrogen (N) availability regimes and contrasting source-sink relationships during grain filing. N availability is known to influence photosynthesis and biomass accumulation (Gastal and Lamaire, 2002) and modifying the source-sink relationship may alter grain weight and N concentration in the grain to differing degrees in different crops (Borrás et al., 2004; Seebauer et al., 2010).

2.3 Materials and methods

2.3.1 Treatments

We used two corn (*Zea mays*) near-isogenic genotypes: the wild-type white endosperm variety M37W and the high-carotenoid transgenic line Carolight^{R}, expressing corn *psy1* and *Pantoea annatis crt*I in a M37W genetic background. M37W seeds were obtained from CSIR (Pretoria, South Africa). Seeds were planted in the experimental fields of the School of Agronomy, University of Lleida, Spain (41° 37' 50" N, 0° 35' 27" E, 180m mean sea level). Two different fertilization regimes were applied: N_0 = 0 kg ha^{-1} and N_{200} = 200 kg ha^{-1}. The N was applied as urea at the V_6 stage (six fully expanded leaves). Source-sink relationships were manipulated at silking (R_1 stage) by removing most of the leaves, and tests were carried out using plants that were distributed uniformly at the same density and were visually identical in terms of size, leaf number and developmental stage. Most of the leaf laminae were removed by cutting the leaves on the collar, between the lamina and sheath. Only the two leaves adjacent to the ear were left on the plant (those immediately below and above). The effect of all treatments and their interactions were tested for statistical significance by the analysis of variance (ANOVA).

2.3.2 Experimental design

In Study 1, seeds were sown in the field on 5 May 2013 and treatments consisted of a factorial combination of the two maize genotypes and two N treatments (N_0 and N_{200}) with subplots consisting of source-sink treatments. Plots were randomized with four replicates, each consisting of six rows, 70 cm apart and 6.47 m in length. Each plot was fully irrigated. Pests,

diseases and weeds were controlled or avoided by spraying recommended insecticides, fungicides and herbicides at the doses recommended by their manufacturers whenever necessary.

2.3.3 Sampling and analysis of plants

Three developmental stages were chosen for biomass sampling: V_8 (eight fully expanded leaves), R_1 (silking) and R_6 (maturity) (Ritchie et al., 1993). Plants were cut at ground level, and the stems (including leaf sheaths), leaf laminae, ears and grains (at maturity) were separated in the laboratory. The ears were divided into basal, central and apical sections, and grains in each section were separated from the cob and counted. The area of green leaf tissue in all samples was determined at stages V_8 and R_1 using a Li-3100C area meter (Li-COR Biosciences, Lincoln, NE, USA). All plant materials were oven dried for 72 h at 65ºC after processing and weighed to determine the dry biomass of each fraction.

After weighing, all plant materials were ground in analytical mills, and the N concentration was determined using the Kjeldahl method. The specific leaf area (SLA) was calculated by dividing the leaf area per plant by leaf mass, and the specific leaf nitrogen (SLN) content was calculated as the ratio of leaf nitrogen content to leaf area. The leaf area index (LAI) was calculated as the total green area of leaf tissue per unit ground area. N utilization efficiency (NUtE) was calculated as the ratio between yield and N uptake at physiological maturity. The N harvest index (NHI) was calculated as the ratio between N content in the grains and aboveground biomass at maturity.

Photosynthetic rates and related traits were determined for individual leaves every 2 weeks from V_8 until grain filling was complete, using a LCi portable photosynthesis system (ADC BioScientific, Great Amwell, UK) which measures net CO_2 assimilation (net photosynthetic rate). Measurements were taken using the last fully expanded leaf between mid-morning and noon on cloudless days by holding the photosynthetic chamber perpendicular to the direction of incident solar radiation. The leaf chlorophyll concentration from V_8 to R_6 was estimated *in situ* using a SPAD-520 portable chlorophyll meter (Minolta, Tokyo, Japan) on the same leaves used to measure gas exchange. The duration of photosynthesis was calculated by integrating values across the growth cycle and measuring the area underneath the curve (Abeledo et al., 2014).

2.4 Results

2.4.1 Leaf traits and their relationship with photosynthetic rate

As expected, the chlorophyll content of leaves (i) declined during plant growth from 43.8±2.7 SPAD units at 70 DAS to 26.1±2.0 SPAD units at 141 DAS and (ii) was slightly higher under the N_{200} treatment than the N_0 treatment **(Fig. 1)**. No consistent differences in chlorophyll level ranges were observed between the wild-type and transgenic corn lines **(Fig. 1)**.

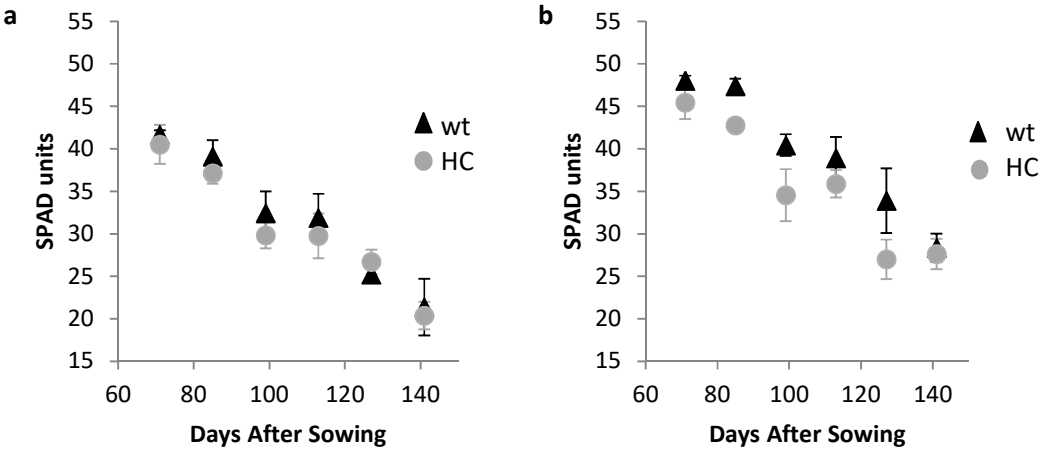


Figure 1: Chlorophyll content in the last fully expanded leaf during development under (a) N_0 and (b) N_{200} treatments (values are means ± SD, n = 4) for wild-type M37W (wt, triangles) and Carolight^R (HC, circles). N_0 = 0 kg ha^{-1} of N; N_{200} = 200 kg ha^{-1} of N.

A similar decrease during growth was observed for the photosynthetic rate, with the mean value of both genotypes and treatments starting from 27.0±0.9 mmol CO_2 m^{-2} s^{-1} at V_8 and slowly declining to 13.7±0.4 mmol CO_2 m^{-2} s^{-1} at maturity, but soil N availability did not affect the leaf photosynthetic rate. Both genotypes exhibited similar photosynthetic rates across treatments and throughout development. Consequently, the photosynthetic rate averaged across time and N levels was almost identical for both genotypes **(Fig. 2a)**, and the duration of photosynthesis (an integral of photosynthesis over time) did not significantly differ between the genotypes under either of the N treatments **(Fig. 2b).**

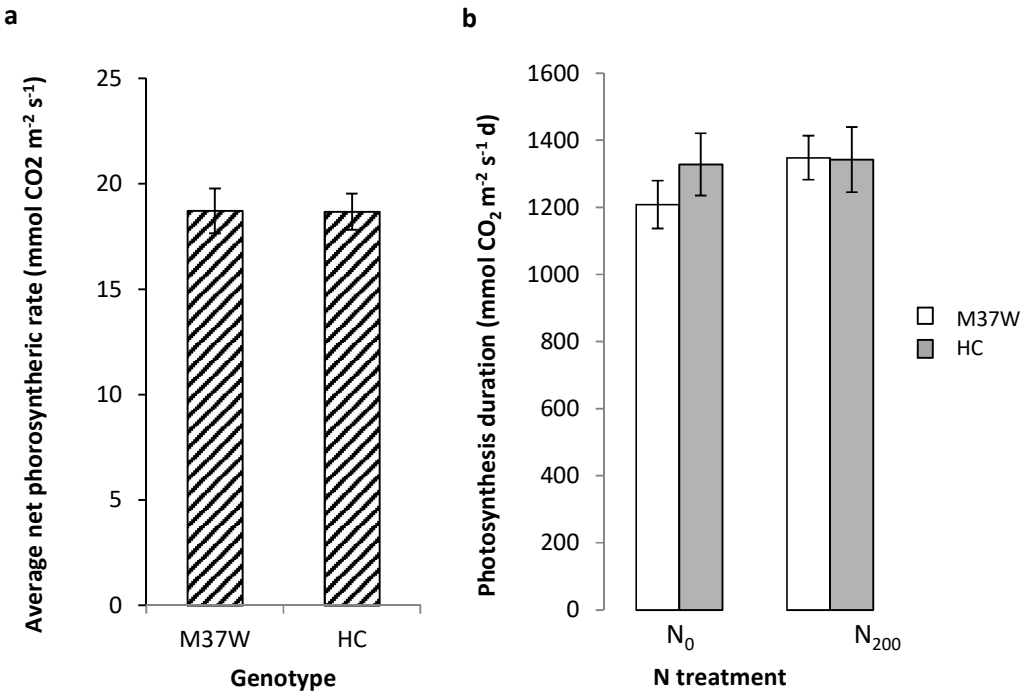
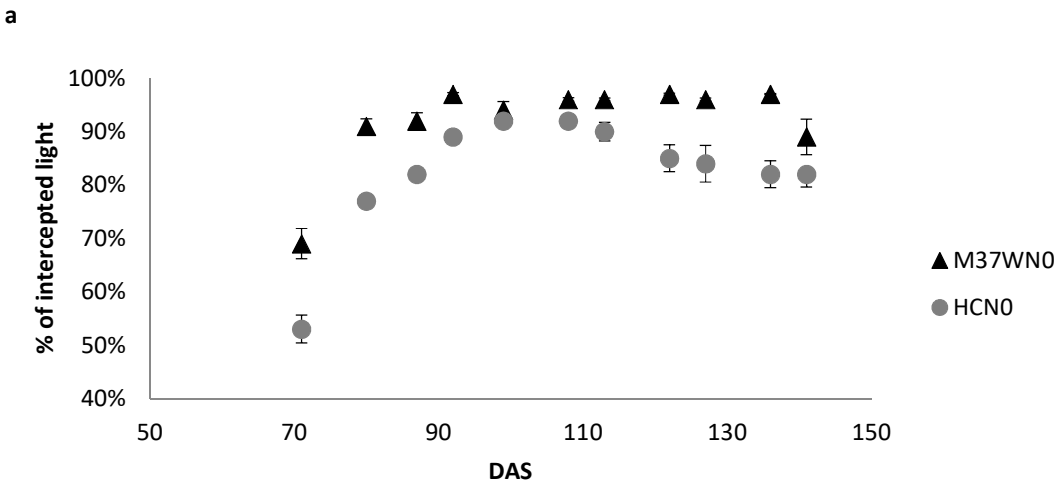


Figure 2: (a) Photosynthetic rate averaged from V_8 (eight fully expanded leaves) to R_6 (maturity) and for N levels for the two genotypes (M37W and Carolight^R). (b) Duration of photosynthesis (integral of area under the curve of photosynthetic rate vs time) from V_8 to

R_6 for the two genotypes under N_0 (0 kg ha^{-1} of N) and N_{200} (200 kg ha^{-1} of N) treatments. Data presented as means ± SD (n = 4).

Intercepted light showed significant differences at N_0 treatment: HC genotype had an average 10 ± 6 % reduction in radiation interception compared to M37W during the entire plant growth cycle **(Fig. 3a)**. At N_{200} no significant differences between genotypes were found **(Fig. 3b)**.



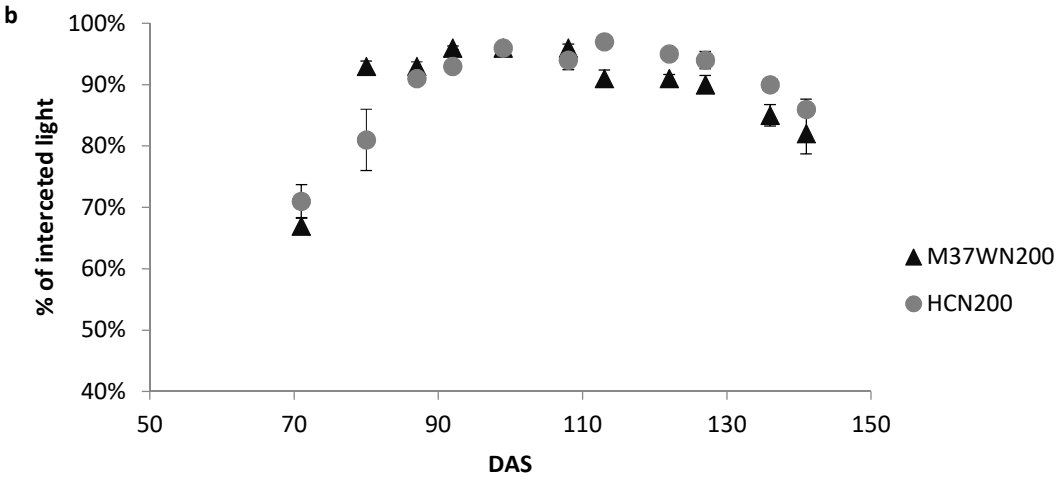


Figure 3: Percentage of intercepted light for M37W and HC during development under (a) N_0 and (b) N_{200} treatments (values are means ± SD), n = 4 N_0 = 0 kg ha^{-1} of N; N_{200} = 200 kg ha^{-1} of N.

LAI values were significantly higher in Carolight^R than wild-type at V_8 under the N_0 treatment (1.5±0.1 for M37W *vs* 2.4±0.3 for Carolight^R) but there were no genotype-specific differences at either developmental stage under the N_{200} treatment **(Table 1)**. The SLN was similar in both genotypes at V_8 but was significantly higher under the N_{200} treatment: the means for the two genotypes were 2.3±0.1 g m^{-2} and 3.9±0.3 g m^{-2} under the N_0 and N_{200} treatments, respectively. At silking, there were no statistically significant differences between the genotypes or treatments **(Table 1).**

Stage of development	N treatment	Genotype	LAI	SLA ($cm^2\ g^{-1}$)	SLN (mg N cm^{-2})
V_8	N_0	**M37W**	1.5±0.1	208.3±7.4	2.2±0.4
		Carolight^R	2.4±0.3	216.9±7.1	2.5±0.6
	N_{200}	**M37W**	2.7±0.3	201.6±3.2	4.2±0.4
		Carolight^R	2.5±0.1	201.5±6.9	3.7±0.7
R_1	N_0	**M37W**	4.5±0.2	160.4±9.4	2.2±0.1
		Carolight^R	4.6±0.4	151.7±8.5	2.8±0.4
	N_{200}	**M37W**	4.6±0.3	148.8±7.4	3.2±0.3
		Carolight^R	5.6±1.2	157.1±3.1	2.3±0.4

Table 1: Leaf traits of wild-type and Carolight^R grown in the field (values are means ± SD, n = 4) with no fertilizer (N_0) or fertilized with 200 kg ha^{-1} of N (N_{200}) at V_8 and R_1. LAI = leaf area index, SLA = specific leaf area, and SLN = specific leaf nitrogen.

2.4.2 Biomass accumulation

Biomass increased from V_8 to R_1 and subsequently to R_6, but surprisingly response to N fertilization was negligible **(Fig. 4)**. There were no statistically significant differences in total biomass accumulation between the two genotypes **(Fig. 4)**, and similar values were measured when analysing the biomass partition between stems, leaves and cobs in both genotypes under the N_0 and N_{200} treatments.

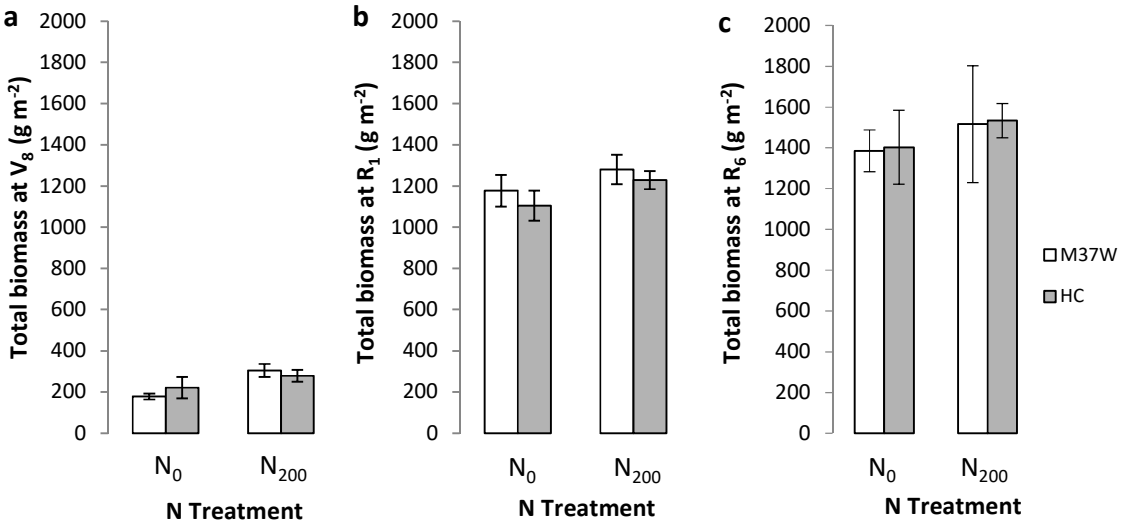


Figure 4: Biomass accumulation at (a) V8, (b) R1 and (c) R6. Values are means ± SD, n = 4. N_0 = 0 kg ha^{-1} of N (urea); N_{200} = 200 kg ha^{-1} of N (urea).

2.4.3 Grain yield and its components

Grain yield was similar in both genotypes under both N treatments **(Fig. 5a)**. The grain yield was more closely related to grain number (R^2=0.85, P<0.05) than to average grain weight (R^2=0.34, NS). There was no significant difference in grain number between genotypes, but there was a tendency towards a higher grain number in Carolight^R than wild-type corn: 1,895±247 vs 1,656±126 grains m^{-2} under the N_0 treatment and 1,807±378 vs 1,554±72 grains m^{-2} under the N_{200} treatment **(Fig. 5b)**. There was no significant difference between the genotypes in terms of average grain weight, and no consistent trends either **(Fig. 5c)**.

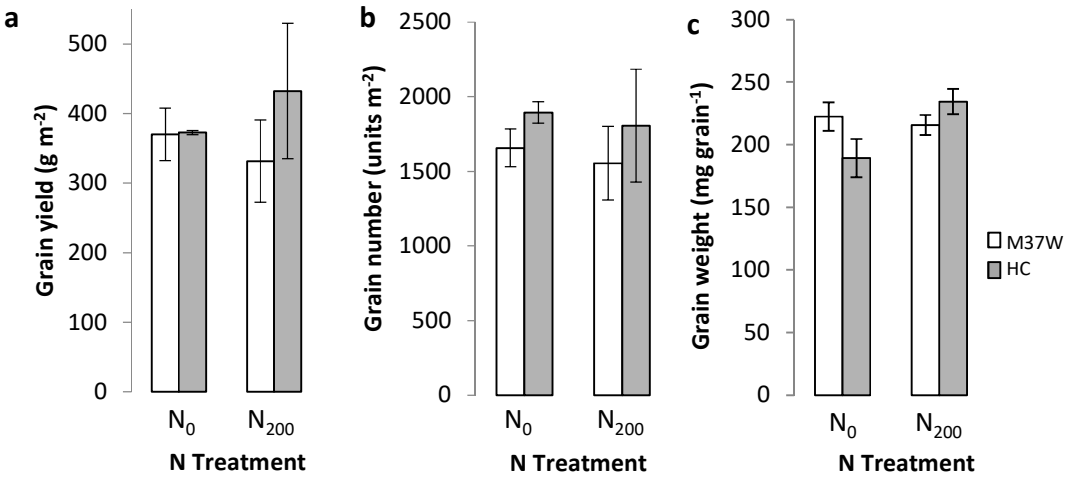


Figure 5: (a) Grain yield, (b) grain number and (c) average weight of an individual grain for the two genotypes (M37W and Carolight^{R}). Values are means ± SD, n = 4. N_0 = 0 kg ha^{-1} of N (urea); N_{200} = 200 kg ha^{-1} of N (urea).

2.4.4 Nitrogen content and economy

Nitrogen uptake was similar for the Carolight^{R} and M37W plants under both N treatments **(Table 2)**. NHI and NUtE, which were reduced by fertilization, were similar for both genotypes, and the similar levels of N partitioning and utilization efficiency were consistent regardless of the N treatment **(Table 2)**.

N treatment	Genotypes	NHI (%)	NUtE (g_{grain} g_N^{-1})	N Uptake (g_N m^{-2})
N_0	**M37W**	54.6±1.1	29.0±0.9	12.7±1.0
	Carolight^R	56.0±5.1	29.7±2.7	12.7±2.21
N_{200}	**M37W**	38.7.±3.1	20.4±0.9	16.4±0.87
	Carolight^R	45.1±8.7	24.3±4.8	17.3±1.0

Table 2: Nitrogen harvest index (NHI), nitrogen utilization efficiency (NUtE) and total nitrogen uptake at maturity for wild-type M37W and transgenic Carolight^R corn under two N treatments (values are means ± SD, n = 4). N_0 = 0 kg ha^{-1} of N (urea); N_{200} = 200 kg ha^{-1} of N (urea).

2.4.5 Source/sink relationship

At maturity, the average grain weight was consistently higher in the intact undefoliated plants compared to plants that had been defoliated with the exception of two leaves adjacent to the ear **(Fig. 6 and 7)**. The average response to defoliation was similar in both genotypes: the grain weight was reduced by 31±4% in M37W and by 28±5% in Carolight^R **(Fig. 6 and 7)**. Interestingly, in both genotypes, the grains from the apical part of the cob (the smallest grains) were the most affected: the weight of apical grains was reduced by 41±9% due to defoliation whereas the weight of the central and basal grains was reduced by only 26±8% **(Fig. 6 inset)**.

In intact undefoliated plants, the percentage of N in the grains was similar in both genotypes and consistently lower (1.87±0.01%) than in the defoliated plants (2.05±0.01%) **(Fig. 8)**. This

may have occurred because defoliation also reduced the grain number by ~25% in both genotypes.

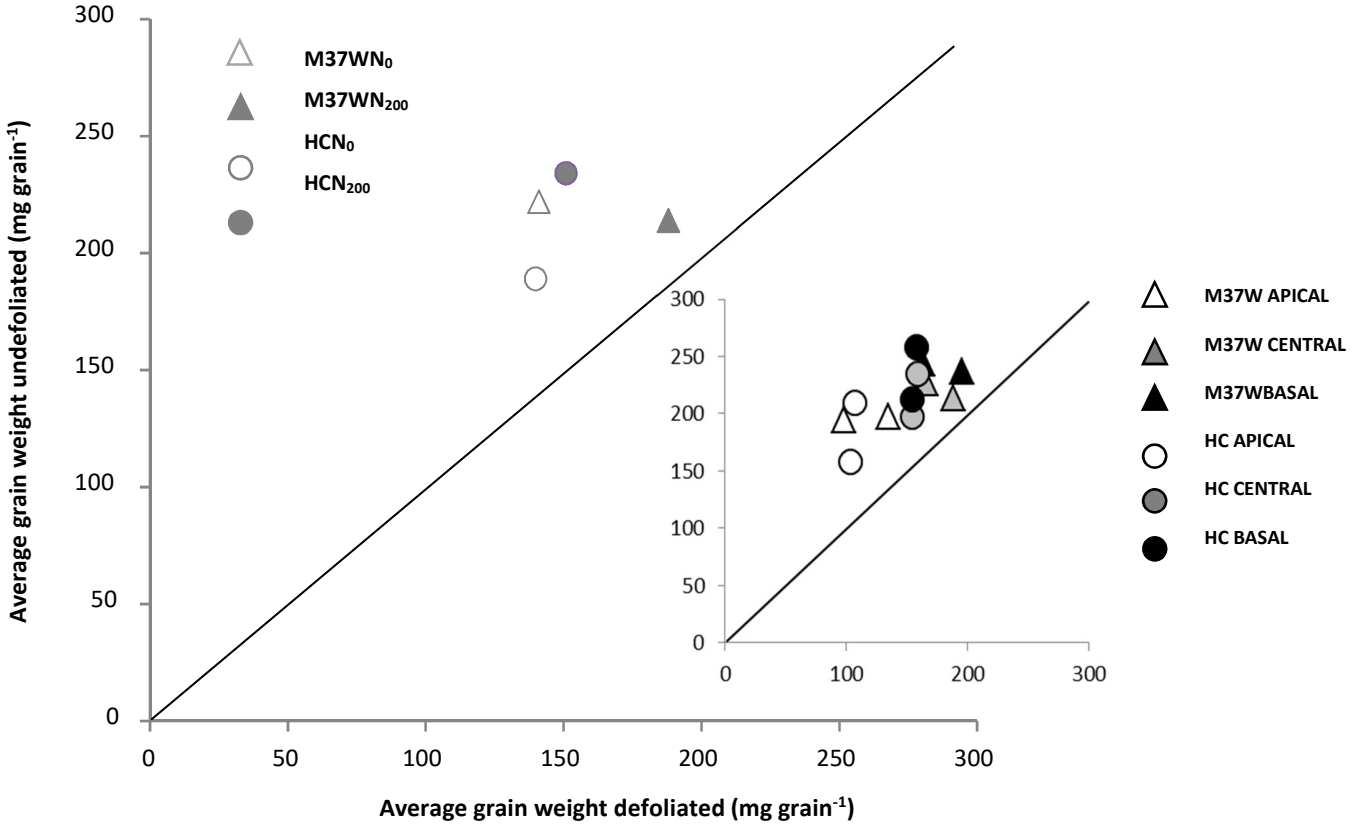
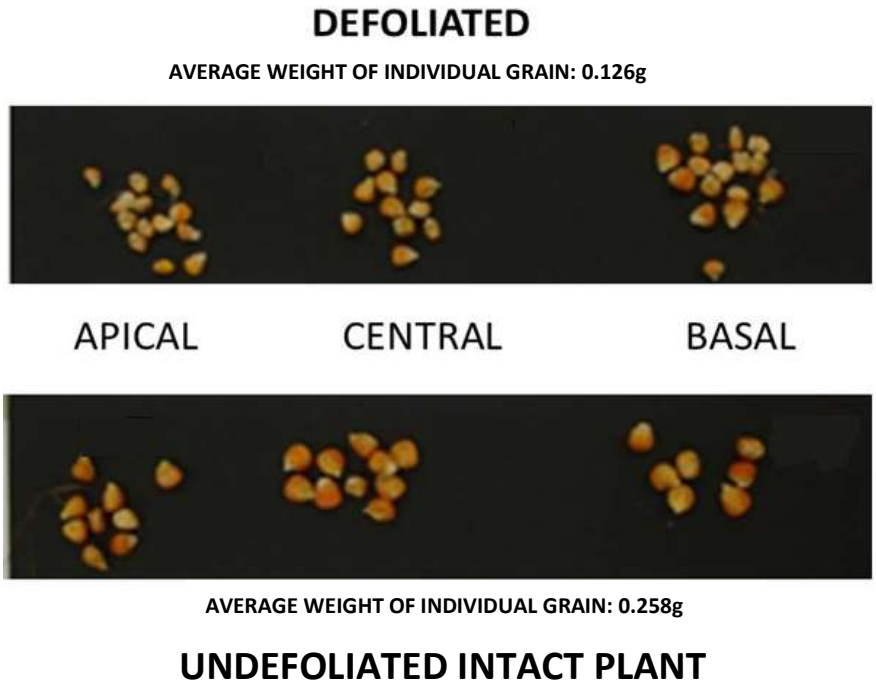


Figure 6: Average grain weight in intact undefoliated plants plotted against the corresponding values in defoliated plants for M37W (triangles) and Carolight^{R} (circles) grown under N_0 (open symbols) and N_{200} treatments (closed symbols). The y = x line represents the 1:1 ratio. N_0 = 0 kg ha^{-1} of N; N_{200} = 200 kg ha^{-1} of N. **Inset**: grain weight in intact undefoliated plants plotted against the corresponding values in defoliated M37W and Carolight^{R} for the basal, central and apical grains.

a



b

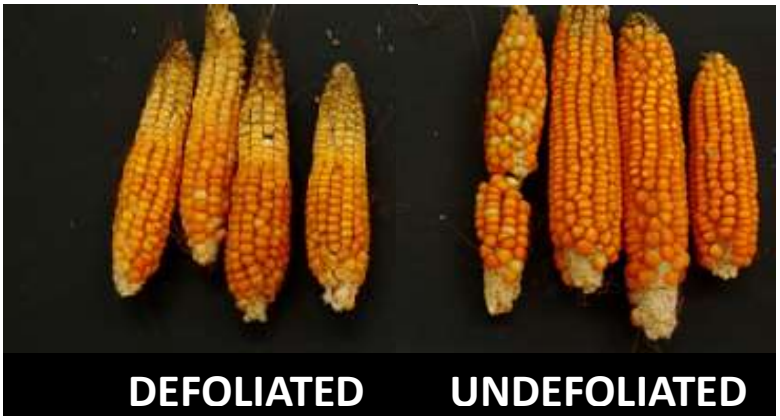


Figure 7: Phenotype of HC a) grains and b) cobs from defoliated plants and undefoliated intact ones. Average grain weight (in grams) of an individual grain is shown.

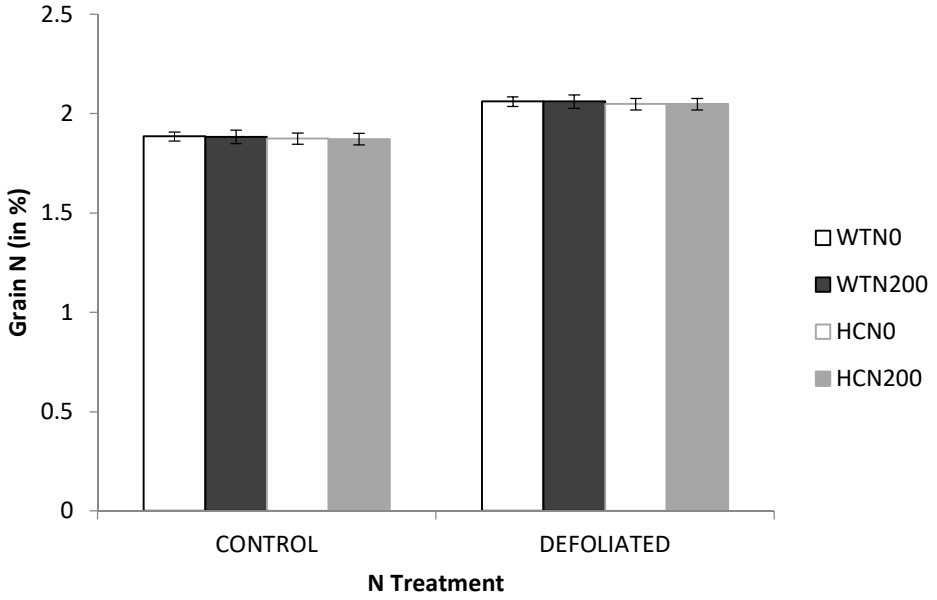


Figure 8: Percentage of N in the grains of M37W and HC under the two N treatments. Values are means ± SD, n = 4 N_0 = 0 kg ha^{-1} of N; N_{200} = 200 kg ha^{-1} of N.

2.5 Discussion

2.5.1 Differences in LAI between genotypes do not lead to differences in photosynthesis or biomass accumulation

During early growth (V_8) without fertilizer treatment (N_0), the LAI of Carolight^R plants was significantly higher than that of M37W plants, but by silking (R_1) no significant differences were detected between the genotypes. However, HC genotype had lower radiation interception (IR) compared to M37W during the entire plant growth cycle at N_0. The early differences in LAI or IR between genotypes were probably not substantial enough to cause statistically significant differences in photosynthetic rate or duration **(Fig. 2)**. Furthermore, there were no differences between the two genotypes in terms of plant biomass at the three developmental stages we investigated **(Fig. 4)**. These results indicate that the plant biomass, as well as height and other morphological characteristics were similar in the two genotypes.

2.5.2 Nitrogen economy is similar between genotypes

N fertilization affects crop radiation interception, as N is involved in leaf area development and senescence (Eik and Hanway, 1965; Lemcoff and Loomis, 1986). Nitrogen is a key element of the chlorophyll molecule and chloroplasts contain 70–80% of the cell N (Makino and Osmond, 1991). Photosynthesis requires correct amount of all the necessary proteins for all photosynthetic steps, such as formation of the light-harvesting chlorophyll-protein complexes of the photosystem II antenna (Bungard et al., 1997). When N fertilizer was provided (N_{200}), most of the measured traits remained at similar levels **(Table 2, Figs. 1, 2, 3 and 4)** or increased **(Table 1, Fig. 5)** compared to the N_0 treatment. The response to N

availability may vary under different agronomic environments depending on the genotype and environmental conditions. It has been demonstrated how both crop growth and grain yield of maize crops vary widely in response to N availability; N deficiency can severely affect leaf area index, light interception, biomass production, and grain yield (Uhart and Andrade, 1995). Maize grain yield is determined by crop growth and its allocation to kernels at harvest (Cirilo et al., 2009). Crop growth depends on the amount of intercepted radiation (IR) and the plant efficiency to convert it in aboveground biomass, commonly referred to as radiation use efficiency (RUE). The ability of a crop to intercept and use photosynthetically active radiation depends on canopy size and canopy architecture (Boote and Loomis, 1991; Maddonni et al., 2001a) which differ among maize genotypes with concomitant effects on light attenuation by the canopy (Maddonni and Otegui, 1996). Furthermore, maize genotypes differ in plant height, leaf number, individual leaf area, vertical leaf angle, and leaf area density distribution along the main stem (Maddonni and Otegui, 1996; Maddonni et al., 2001b). The response of both our genotypes to N fertilization was generally minimal. However, our experiments were designed to determine whether the transgenic and non-transgenic plants responded differently to changes in the N supply. The response to N was generally similar in both genotypes, indicating that the introduced transgenes and their expression do not influence the manner in which corn plants respond to N availability.

2.5.3 Response to defoliation treatment is similar between genotypes

Changing the source-sink relationship by defoliation at silking reduced the grain weight by ~30% in both genotypes, but no significant differences were found between them (**Fig. 6 and 7**). The response was strongest in the apical grains (in the apical third of the ear) which are

always smaller than those of the basal and central thirds because growth starts 4–5 days later than the basal grains and there is a greater likelihood of abortion (Tollenaar and Daynard, 1978). Environmental stress and defoliation result in sterility and/or reduced size of apical kernels without a proportional reduction in the weight and fertility of basal kernels (Daynard and Duncan, 1969; Schoper et al., 1982; Jones and Simmons, 1983). For this reason, the apical grain weight reduction we observed both in Carolight^{R} and M37W plants was likely due to the great severity of source-limitation for grain growth. Importantly, the additional metabolic requirements of Carolight^{R} endosperm did not result in a higher sensitivity to source limitation compared to the wild-type, suggesting that sensitivity to abiotic stress (impairing the balance of resources between grain growth and assimilate availability) does not increase by the introgression of carotenogenic transgenes. The reduction in grain size and number in response to defoliation at silking (before grain set) agrees with earlier reports of significant decreases in grain yield caused by defoliation at this developmental stage (Borrás et al, 2004). Grain number is mainly determined during the critical 30-day period bracketing silking (Andrade et al., 2002), which is characterized by the growth of the juvenile ear, containing the female florets (Otegui & Bonhomme, 2008). The abortion process then affects a proportion of the pollinated florets. Grain weight potential is largely determined during the same period (Gambín et al., 2006) but final grain weight is realised during the effective period of grain filling (Borrás et al, 2004). The relative N content in the grains was similar in both genotypes, and increased significantly (~10%) in both varieties following defoliation. N provision to growing grains can be restricted at source by limiting either its availability from post-anthesis assimilation or its translocation from leaves (Simpson et al., 1983). Grain growth in cereals therefore tends to be sink-limited whereas N accumulation in grains is usually source limited (Dreccer et al., 1997). Defoliation is therefore expected to result in a

lower proportional incorporation of N (Borrás et al, 2004). However, defoliation also reduced the grain number in our study, and this may explain the higher rate of N incorporation we observed in both genotypes.

2.5.4 Conclusions

In conclusion, I determined experimentally that a transgenic maize plant (Carolight^{R}) with increased carotenoid production is no different in terms of phenology and agronomic performance from its wild-type counterpart. Minor differences in some parameters, such as LAI or IR, were measured in the early stages of plant life cycle, but they did not impact yield or development. This demonstrates that the introduction of an exogenous carotenogenic pathway into M37W did not have any detectable negative pleiotropic effect, probably also due to the use of an endosperm specific promoter. GM crops with increased nutritional value can be, if properly designed, as agronomically efficient as their near isogenic lines, without performance/yield penalties.

2.6 References

- Abeledo, L. G., Savin, R. & Slafer, G. A. (2014). Leaf Photosynthesis During Grain Filling Under Mediterranean Environments: Are Barley or Traditional Wheat More Efficient Than Modern Wheats? *J Agro Crop Sci.* **200**:172-182.

- Andrade, F.H., Echarte, L., Rizzalli, R., Della Maggiora, A., Casanovas, M. (2002). Kernel number prediction in maize under nitrogen or water stress. *Crop Sci*. **42**:1173–1179.

- Boote, K.J. & Loomis, R.S. (1991) . The prediction of canopy assimilation. K.J. Boote, R.S. Loomis (Eds.), Modeling Crop Photosynthesis—From Biochemistry to Canopy, CSSA Spec. Publ. 19. CSSA and ASA, Madison, WI pp. 109–140.

- Borrás, L., Slafer, G.A. & Otegui, M.E. (2004). Seed dry weight response to source–sink manipulations in wheat, maize and soybean: a quantitative reappraisal. *Field Crop Res*. **86**: 131–146.

- Bungard, R.A., McNeil, D., & Morton, J.D. (1997). Effects of nitrogen on the photosynthetic apparatus of Clematis vitalba grown at several irradiances. Aust J Plant Physiol. 24: 205–214.

- Cirilo, A., Dardanelli, J., Balzarini, M., Andrade, F.H., Cantarero, M., Luque, S., Pedrol, H.M. (2009). Morphophysiological traits associated with maize crop adaptations to environments differing in nitrogen availability. *Field Crop Res*. **113**:116-124.

- Daynard, T. B. & Duncan, W. G. (1969). The black layer and grain maturity in corn. *Crop Sci*. **9**:473-476.

- Decourcelle, M., Perez-Fons, L., Baulande, S., Steiger, S., Couvelard, L., Hem, S., et al. (2015). Combined transcript, proteome, and metabolite analysis of transgenic maize seeds engineered for enhanced carotenoid synthesis reveals pleotropic effects in core metabolism. *J Exp Bot*. **66**:3141-3150.

- Dreccer, M.F., Grashoff, C. & Rabbinge, R. (1997). Source-sink ratio in barley (*Hordeum vulgare* L.) during grain filling: effects on senescence and grain protein concentration. *Field Crops Res.* **49**:269-277.

- Eik, K. & Hanway, J.J. (1965). Some factors affecting development and longevity of leaves of com. *Agron J*. **57**: 7-12.

- Elmore, R.W., Roeth, F.W., Klein, R.N., Knezevic, S.Z., Martin, A., Nelson, L.A. Shapiro, Charles, A. (2001a). Glyphosate-resistant soybean cultivar response to glyphosate. *Agron J.* **93**:404–407.

- Elmore, R.W., Roeth, F.W., Nelson, L.A., Shapiro, C.A., Klein, R.N., Knezevic, S.Z., Martin, A. (2001b). Glyphosate-resistant soybean cultivar yields compared with sister lines. *Agron J.* **93**:408-412.

- Falconer, D.S. (1981). Introduction to Quantitative Genetics (2nd ed) (Longman Inc.).

- Farré, G., Bai, C., Twyman, R.M., Capell, T., Christou, P., Zhu, C. (2011). Nutritious crops producing multiple carotenoids – a metabolic balancing act. *Trends Plant Sci*. **16:**532-540.

- Feldmann, K.A., Marks, M.D., Christianson, M.L., Quatrano, R.S. (1989). A dwarf mutant of Arabidopsis generated by T-DNA insertion mutagenesis. *Science* **243**:1351–1354.

- Gambín, B.L., Borrás, L. & Otegui, M.E. (2006). Source-sink relations and kernel weight differences in maize temperate hybrids. *Field Crops Res*. **95**:316-326.

- Gastal, F. & Lemaire, G. (2002). N uptake and distribution in crops: an agronomical and ecophysiological perspective. *J Exp Bot.* **53**:789-799.

- Ge, L., Chen, H., Jiang, J.F., Zhao, Y., Xu, M.L., Xu, Y, Y., Tan, K.H., Xu, Z.H., Chong. K. (2004). Overexpression of OsRAA1 causes pleiotropic phenotypes in transgenic rice plants, including altered leaf, flower, and root development and root response to gravity. *Plant Physiol*. **135**:1502–1513.

- Laserna, M.P., Maddonni, G.A. & Lopez, C.G. (2012). Phenotypic variations between non-transgenic and transgenic maize hybrids. *Field Crop Res*. **134**:175-184.

- Lemcoff, J.H. & Loomis, R.S. (1986). Nitrogen influences on yield determination in maize. *Crop Sci*., **26**: 1017-1022.

- Ma, B.L. & Subedi, K.D. (2005). Development, yield, grain moisture and nitrogen uptake of Bt corn hybrids and their conventional near-isolines. *Field Crops Res***.** **93**:199–211.

- Maddonni, G.A. & Otegui, M.E. (1996). Leaf area, light interception, and crop development in maize. *Field Crops Res.* **48**:81–87.

- Maddonni, G.A., Otegui, M.E. & Cirilo, A.G. (2001a). Plant population density, row spacing and hybrid effects on maize canopy architecture and light attenuation. Field Crops Res., **71**:183–193.

- Maddonni, G.A., Chelle, M., Drouet, J.-L., Andrieu, B. (2001b). Light interception of contrasting azimuth canopies under square and rectangular plant spatial distributions: simulations and crop measurements. Field Crops Res. 70:1–13.

- Makino, A. & Osmond, B. (1991). Effects of nitrogen nutrition partitioning between chloroplasts and mitochondria in pea and wheat. *Plant Physiology*. **96**: 355–362.

- Marra, M., Piggott, N.E. & Carlson, G.A. (2004). The net benefits, including convenience, of Roundup Ready® soybeans: Results from a National Survey. (NSF Center for IPM Technical Bulletin).

- Otegui, M.E. & Bonhomme, R. (1998). Grain yield components in maize: I. Ear growth and kernel set. *Field Crops Res*. **56**:247–256.

- Paine, J., Shipton, C., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., et al. (2005). Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat Biotechnol***. 23**:482-487.

- Raymer, P.L. & Grey, T.L. (2003). Challenges in comparing transgenic and nontransgenic soybean cultivars. *Crop Sci*. **43**:1584-1589.

- Ritchie, S.W., Hanway, J.J. & Benson, G.O. (1993). How a Corn Plant Develops. CES 21. Iowa State Univ., Ames, IA.

- Sanahuja, G. et al. (2013). A question of balance - achieving appropriate nutrient levels in biofortified staple crops. *Nutr Res Rev.* **26**: 235-245.

- Schoper, J. B., Johnson, R, R. & Lambert, R. J. (1982). Maize yield response to increased assimilate supply. *Crop Sci*. **22**:1184-1189.

- Seebauer, J. R., Singletary, G. W., Krumpelman, P. M., Ruffo, M. L., Below, F. E. (2010). Relationship of source and sink in determining kernel composition of maize. *J Exp Bot*. **61**: 511-519.

- Shi, G., Chavas, J.P. & Lauer, J. (2013). Commercialized transgenic traits, maize productivity and yield risk. *Nat. Biotechnol*. **31**:111-114.

- Simpson, R.J., Lambers, H. & Dalling, M.J. (1983). Nitrogen redistribution during grain growth in wheat (*Triticum aestivum* L) IV Development of a quantitative model of the translocation of nitrogen to the grain. *Plant Physiol*. **71**:7-14.

- Subedi, K.D. & Ma, B.L. (2007). Dry matter and nitrogen partitioning patterns in Bt and non-Bt near-isoline maize hybrids. *Crop Sci*. **47**:1186-1192.

- Tollenaar, M. & Daynard, T. B. (1978). Kernel growth and development at two positions of the ear of maize (Zea mays). *Can. J. Plant Sci*. **58**:189-197.

- Uhart, S.A. Andrade, F.H. (1995). Nitrogen deficiency in maize. I. Effects on crop growth, development, dry matter partitioning, and kernel set. Crop Sci. 35:1376–1383.

- Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P., Capell, T. (2008). Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proc Natl Acad Sci U S A*. **105**:18232–18237.

- Zhu, C., Sanahuja, G., Yuan, D., Farre, G., Arjó, G., Berman, J. (2013). Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies. *Plant Biotechnol. J.* **11**:129-141.

Chapter 3

CAROTENOID ACCUMULATION IN HIGH CAROTENOID CORN ENDOSPERM IS SIMILAR UNDER GREENHOUSE AND FIELD CONDITIONS

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Chapter 3:

Carotenoid accumulation in High Carotenoid corn endosperm is similar under greenhouse and field conditions

3.1 Abstract

Performance of a plant under laboratory and/or greenhouse conditions might be very different from that in a more complex environment such as in the field. A major contributor to this behaviour is the multiple stresses (biotic or abiotic) that the plant is exposed to in the field, together with the variability of several factors (e.g. temperature, humidity, light intensity). Carolight^{R} is an effective vehicle to address vitamin A deficiency in developing countries, but this would be possible only if functionality of the transgenic trait is uniform under different conditions. We evaluated Carolight^{R} plants under greenhouse and field conditions and analysed carotenoid content and accumulation in developing endosperm over time. Additionally, we determined endogenous carotenogenic gene and transgene transcript accumulation during endosperm development. We demonstrate that Carolight^{R} behaves similarly under both environments.

3.2 Introduction

Carotenoids are essential pigments found mainly in plants and microbes (Zhu et al., 2010). They consist of eight isoprenoid units joined end to end to form a C_{40} hydrocarbon skeleton that includes a chromophore and linear or cyclic end groups. Plant carotenoids accumulate in plastids and play fundamental roles in photosynthesis as accessory pigments and in the xanthophyll cycle by preventing photo-oxidative damage (Havaux at al., 1999). They also facilitate plant interactions with the biotic environment by conferring characteristic colors to fruits and flowers, making them attractive to insects and thus favoring pollination and seed dispersal (Zhu et al., 2010). The degradation of carotenoids, such as 9'-cis-neoxanthin and 9-cis-violaxanthin, also provides precursors for the synthesis of abscisic acid (ABA) (Li et al., 1990; Lindgren et al., 2003; Parry et al., 1990). From a nutritional perspective, carotenoids such as β-carotene have pro-vitamin A activity and are therefore essential for animals and humans that cannot synthesize retinoids *de novo*. Carotenoids such as lycopene provide general health-promoting antioxidant activity whereas lutein and zeaxanthin protect the retinal cells of the macula against phototoxic damage, thus preventing age-related macular degeneration (ARMD) (Omoni et al., 2005).

Carolight^{R} is an effective vehicle to alleviate vitamin A deficiency in developing countries, it can also provide additional health benefits due to its antioxidants content which might help to prevent diseases caused by accumulation of reactive oxygen species, including certain cancers, cardiovascular disease and neurodegenerative disorders (Berman et al., 2015; Zhu et al., 2013). This would be possible only if its performance is stable and predictable under different environments. Performance of novel crops (genetically engineered or otherwise) under laboratory and/or greenhouse conditions may differ considerably compared to the field,

where several factors can vary simultaneously (e.g. temperature, humidity, light intensity) together with other biotic and abiotic stresses. In this study we compared the performance of the introduced transgenic trait in a controlled greenhouse environment (GH) and in the field (F).

3.3 Materials and methods

3.3.1 Plant material

We used two corn (*Zea mays*) near-isogenic genotypes: the wild-type white endosperm variety M37W and the high-carotenoid transgenic line Carolight^{R} (T12 generation seeds) expressing corn *psy1* and *Pantoea annatis crtI* in a M37W genetic background. M37W seeds were obtained from CSIR (Pretoria, South Africa). Seeds were planted in the experimental fields of the School of Agronomy, University of Lleida, Spain (41° 37' 50" N, 0° 35' 27" E, 180 m mean sea level) or in pots maintained in the greenhouse in May 2013. In the greenhouse, for the first 50 days, the day/night temperature was 28/20°C with a 10-h photoperiod and 60–90% relative humidity, and thereafter a day/night temperature of 21/18°C day with a 16-h photoperiod was used. Seeds were harvested at 15, 20, 25, 30, 40, and 60 days after sowing (DAS), and the dissected endosperm was frozen in liquid nitrogen and stored at –80°C.

3.3.2 Total RNA isolation and mRNA analysis

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and DNA was removed with DNase I (RNase-free DNase Set, Qiagen). Total RNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Vernon Hills, IL, USA), and 2 µg total RNA was used as template for first strand cDNA synthesis with Ominiscript reverse transcriptase (Qiagen) in a 20-µl total reaction volume, following the manufacturer's recommendations.

3.3.3 Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was used to analyze *Zmbch1*, *Zmbch2*, *Zmcrtiso, Zmcyp97a*, *Zmcyp97b*, *Zmcyp97c*, *Zmlycb*, *Zmlyce*, *Zmpds*, *Zmzds*, *Pacrti* and *Zmpsy1* gene expression in a BioRad CFX96 system with 25-µl reaction mixtures containing 10 ng cDNA, 1x iQ SYBR Green Supermix (BioRad, Hercules, CA, USA) and 0.2 µM forward and reverse primers (Farré et al., 2013; Naqvi et al., 2011). Relative expression levels were calculated on the basis of serial dilutions of cDNA (125–0.2 ng) which were used to generate standard curves for each gene. PCR was carried out in triplicate using 96-well optical reaction plates. The reaction conditions comprised an initial heating step at 95°C for 5 min followed by 44 cycles of 95°C for 10 s, 58°C for 35 s and 72°C for 15s. Specificity was confirmed by product melt curve analysis over the temperature range 50–90°C with fluorescence acquired after every 0.5°C increase and the fluorescence threshold value and gene expression data were calculated using BioRad CFX96™ software. Values represent the mean of 10 biological replicates ± SEM. Amplification efficiencies were compared by plotting ΔCt values of different primer combinations in serial dilutions against the log of starting template concentrations using CFX96 software.

3.3.4 Carotenoid extraction from maize endosperm

Maize endosperm was excised by removing the seed coat and embryo. Samples were freeze-dried before extraction and were ground to a fine powder. Carotenoids in 50-100 mg samples were extracted in 15 ml methanol:ethyl acetate (6:4 v/v) at 58°C for 20 min. The mixture was filtered and transferred to a separation funnel before 15 ml of hexane:diethyl ether (9:1 v/v) were added and agitated gently for 1 min. Fifteen ml of saturated NaCl solution was added,

the aqueous phase was removed, and the organic phase was washed twice with water. The samples were dried under N_2 at 37°C, flushed with argon and stored at –80°C.

3.3.5 UHPLC-MS

The extracts were dissolved in 210–600 µl injection solvent, which was 3 parts acetonitrile/methanol (7:3 v/v) to 2 parts acetone. UHPLC analysis was carried out at SCT-DATCEM (Servei Científic-Tècnic DATCEM, University of Lleida), using an Acquity Ultra Performance LC system linked to a PDA 2996 detector (Waters Corp., Milford, MA, USA). Mass detection was achieved using an Acquity TQD tandem quadrupole MS equipped with a Z spray electrospray interface (Waters). MassLynx software v4.1 (Waters) was used to control the instruments and also for data acquisition and processing. UHPLC separations were carried out on an Acquity UPLC C18 BEH 130 Å, 1.7 µm, 2.1 × 150 mm reversed-phase column (Waters). The mobile phase consisted of acetonitrile/methanol 7:3 v/v (solvent A) and 100% water (solvent B). Carotenoids were quantified using a PDA detector and identified as previously described (Rivera et al., 2013) based on the order of elution from the column, ultraviolet and visible spectra, the spectral fine structure (Britton et al., 2004), and mass fragments reported in the literature (Rivera et al., 2011) and determined by comparison with the following authentic standards: β-carotene, lutein, β-cryptoxanthin and astaxanthin (Sigma-Aldrich, St Louis, MO, USA), zeaxanthin (Fluka, Buchs SG, Switzerland), phytoene and antheraxanthin (Carotenature, Lupsingen, Switzerland). MS analysis was carried out by atmospheric pressure chemical ionization (APCI) as previously described (Rivera et al., 2011).

3.4 Results

3.4.1 Expression of *Zmpsy1* and *PacrtI* in greenhouse and field-grown plants

Zmpsy1 and *PacrtI* expression levels in the endosperm of plants grown in the field and in the greenhouse were measured by quantitative real-time RT-PCR at four time points, corresponding to four different endosperm developmental stages during grain filling (15, 20, 25 and 30 DAP). Both transgenes were expressed in both environments, and in both cases *Pacrt*I mRNA accumulated at three-fold lower levels than *Zmpsy1* mRNA at all time points **(Fig. 1a)**. The expression profile of *Pacrt*I was similar under field and greenhouse conditions **(Fig. 2a)**. There was a 2-fold increase in *Zmpsy1* mRNA levels in young endosperm (15 DAP) for plants growing in the greenhouse compared to plants in the field, but the expression levels in the two environments converged during seed development **(Fig. 2b)**.

3.4.2 Endogenous carotenogenic gene expression

We measured the accumulation of transcripts representing 10 endogenous carotenogenic genes in the developing endosperm of transgenic plants in both environments: β-carotene hydroxylase 1 (*Zmbch1*), β-carotene hydroxylase 2 (*Zmbch2*), carotene isomerase (*Zmcrtiso*), carotene ε-hydroxylase (*Zmcyp97a*, *Zmcyp97b*, *Zmcyp97c*), lycopene β-cyclase (*Zmlycb*), lycopene ε-cyclase (*Zmlyce*), phytoene desaturase (*Zmpds*) and ζ-carotene desaturase (*Zmzds*). No significant differences in the expression levels of these endogenous carotenogenic genes were observed between the two environments, and the abundance of each transcript did not change substantially over time **(Fig 2 c-l)**. *Zmbch2* mRNA was more than 1000-fold more abundant than *Zmbch1* mRNA **(Fig. 1b)**, as previously reported (Li et

al., 2010). *Zmcyp97A* and *Zmcyp97B* mRNA accumulated to high levels in both environments and were more than 100-fold more abundant than *Zmcyp97C* mRNA **(Fig. 1c)**. *Zmlycb* mRNA was more abundant than *Zmlyce* mRNA **(Fig. 1d)**.

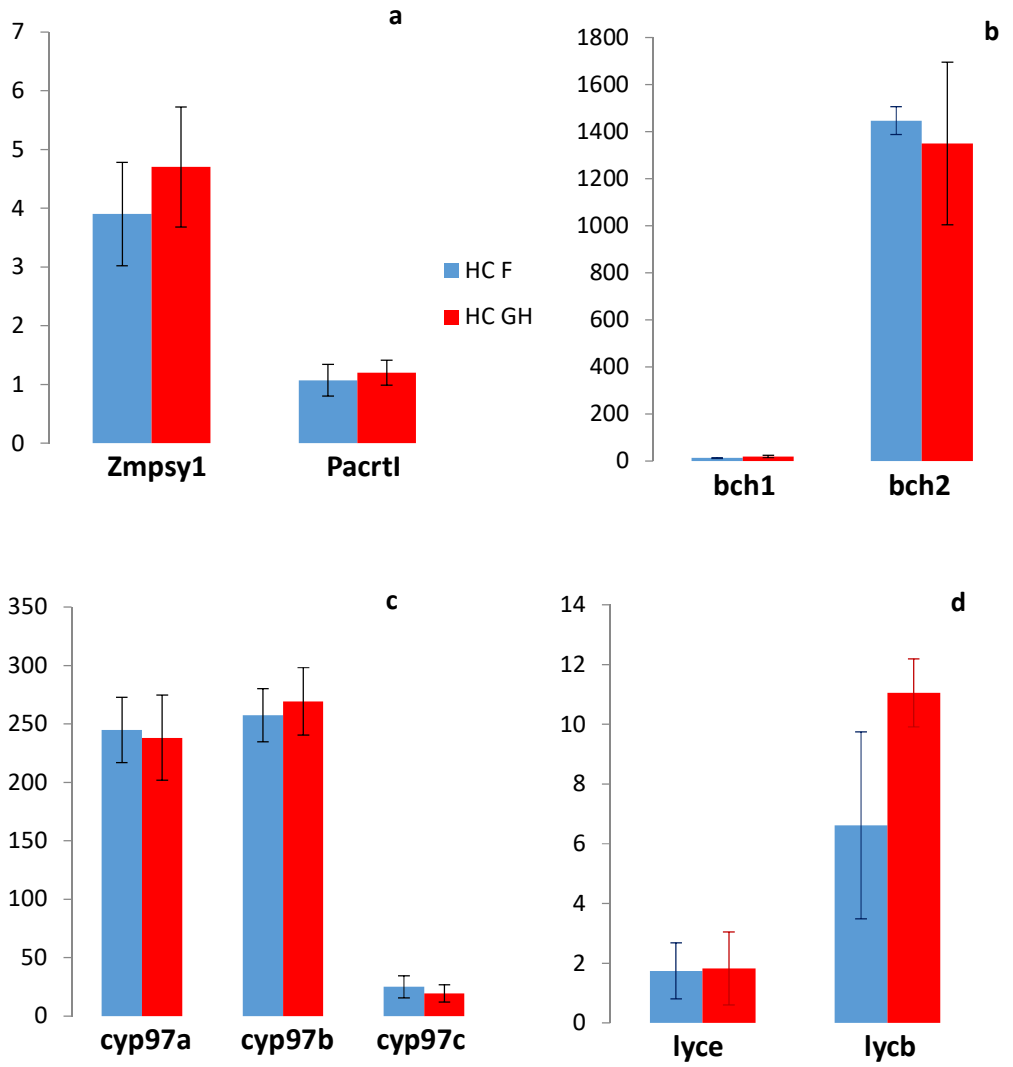


Figure 1: Average gene transcript accumulation in developing endosperm samples (from 15 to 30 DAP) of HC F and HC GH plants **a)** *Zmpsy1* and *PacrtI,* **b)** *Zmbch1* and *Zmbch2* **c)** *Zmcyp97a*, *Zmcyp97b*, *Zmcyp97c* and **d)** *Zmlyce* and *Zmlycb.*

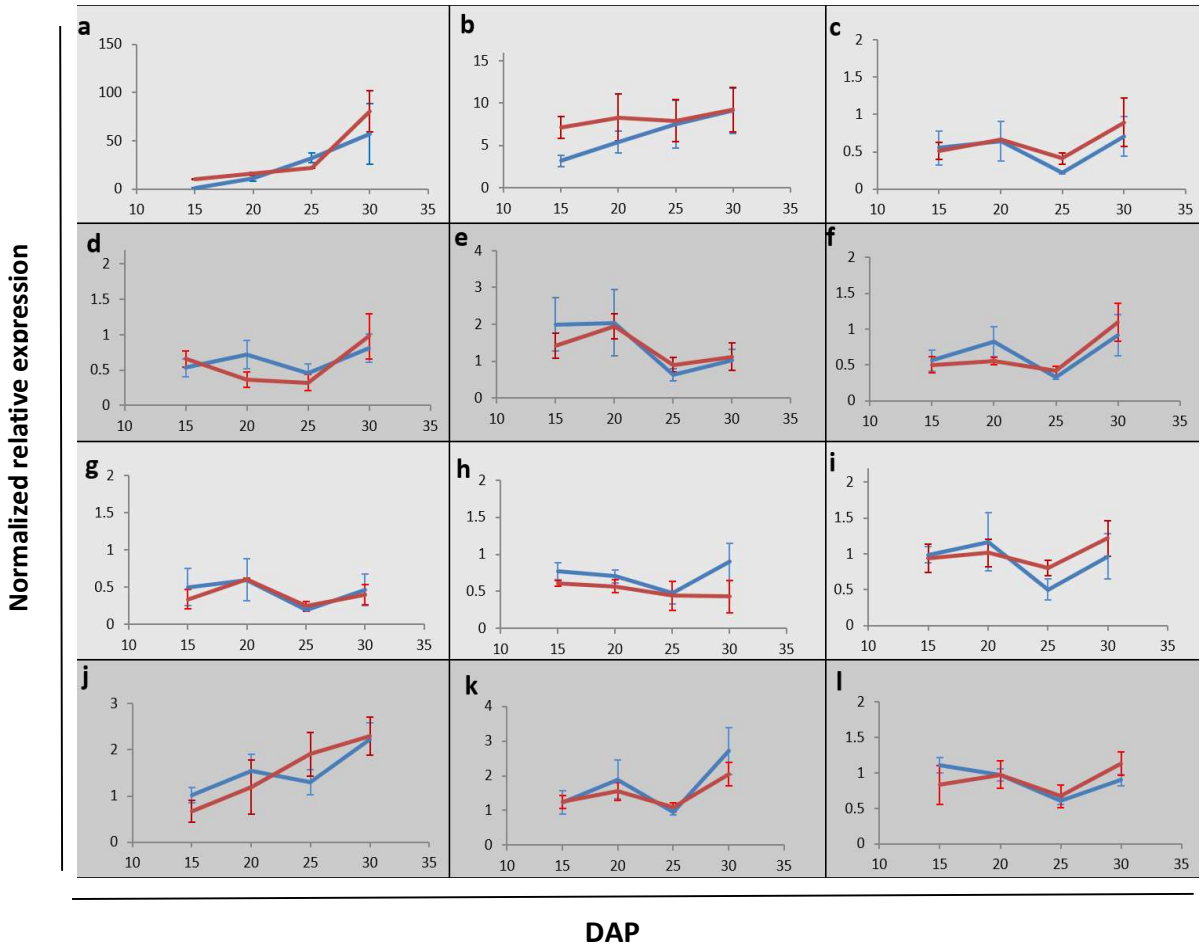


Figure 2: Quantitative real-time RT-PCR analysis of endogenous carotenogenic genes and transgenes in HC plants grown in the field (HC-F) or in the greenhouse (HC-GH). Data represent relative mRNA levels in the immature maize endosperm of HC-F and HC-GH plants at four developmental stages (15, 20, 25 and 30 DAP) normalized against *actin* mRNA and presented as the mean of 10 biological replicates. **(a)** *Pacrti*, phytoene desaturase; **(b)** *Zmpsy1,* phytoene synthase. **(c)** *Zmbch1*, β-carotene hydroxylase 1; **(d)** *Zmbch2*, β-carotene hydroxylase 2; **(e)** *Zmcrtiso,* carotene isomerase; **(f)** *Zmcyp97a*, carotene ε-hydroxylase; **(g)** *Zmcyp97b*; **(h)** *Zmcyp97c*; **(i)** *Zmlycb*, lycopene β-cyclase; **(j)** *Zmlyce*,

lycopene ε-cyclase; **(k)** *Zmpds*, phytoene desaturase; **(l)** *Zmzds*, ζ-carotene desaturase. *Zm, Zea mays; Pa, Pantoea ananatis.*

3.4.3 Carotenoid accumulation during endosperm development

We measured the carotenoid content and composition in the endosperm of the plants in both environments at three developmental stages (25, 40 and 60 DAP). The prevalent carotenoids were phytoene, zeaxanthin, lutein, β-carotene, α-cryptoxanthin, β-cryptoxanthin and antheraxanthin. The maximum total carotenoid accumulation occurred at ~40 DAP in both environments, and was 113±7.5 µg g^{-1} DW for plants in the greenhouse compared to 99±2.7 µg g^{-1} DW for plants in the field. However, no significant differences in the total carotenoid content were observed in the mature kernels at 60 DAP. The total carotenoid content was 96.8±3.7 µg g^{-1} DW for plants in the greenhouse compared to 88.7±6.5 µg g^{-1} DW for plants grown in the field **(Fig. 3)**.

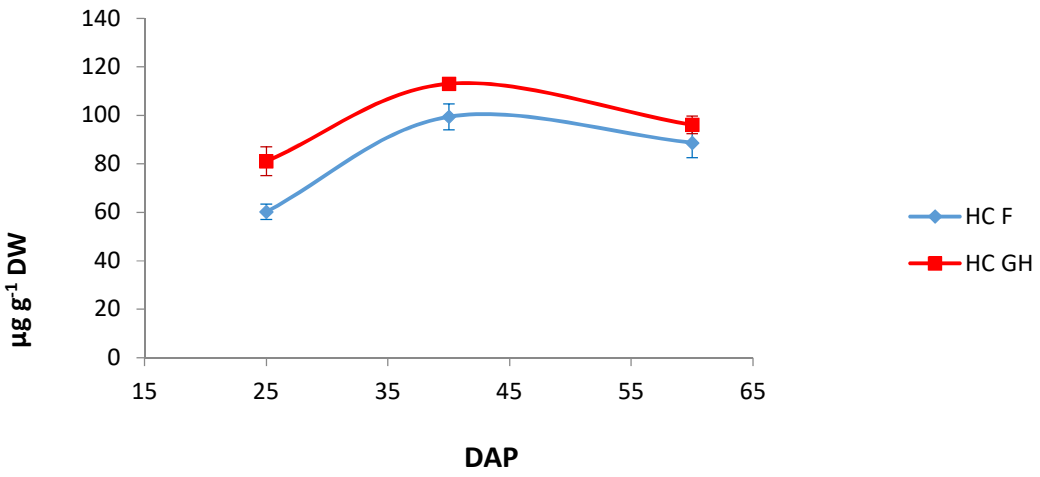


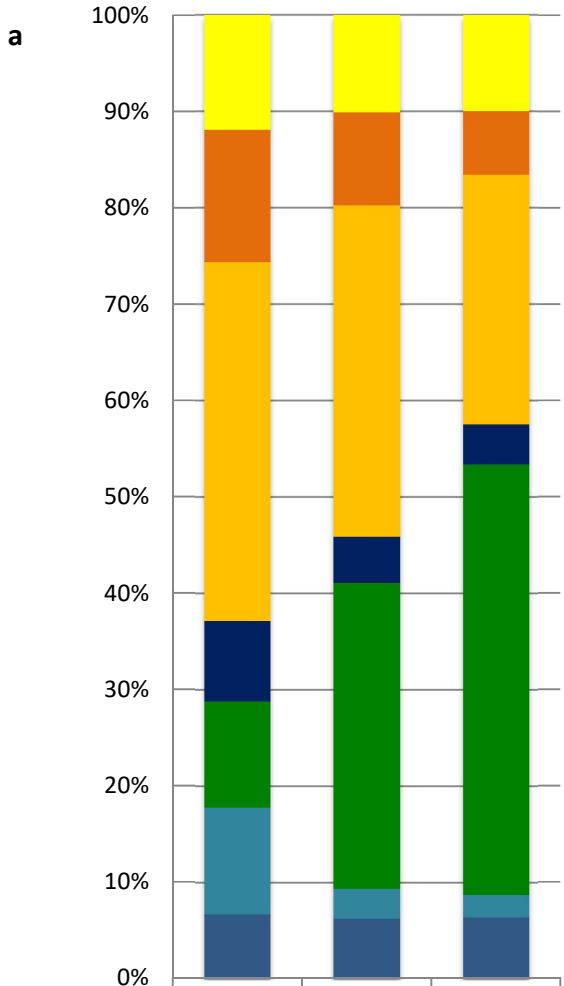
Figure 3: Total carotenoid content of HC-F and HC-GH endosperm at 25, 40 and 60 DAP.

M37W plants under the same conditions accumulated only minimal amounts of lutein, zeaxanthin and antheraxanthin in both environments, consistent with earlier reports (Farré et al., 2013).

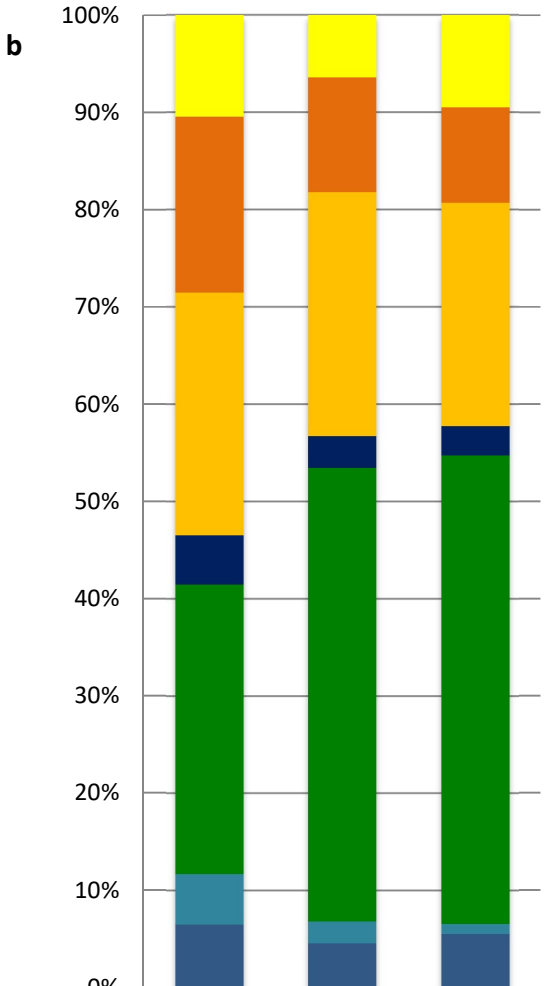
The levels of each carotenoid varied during endosperm development, with significant differences in their relative proportions when comparing plants in the greenhouse and in the field. However, these differences became less pronounced during further development and plants in both environments contained similar levels of each carotenoid at maturity **(Fig. 4** and **Table 1)**.

25 DAPs	HC F	HC GH	M37W F	M37W GH
ZEAXANTHIN	22.41±7.36	20.21± 10.91	0.31±0.57	0.66±0.07
BETACAROTENE	8.26±1.25	14.65±1.85		
LUTEIN	7.17±0.60	8.48±0.53	0.33±0.53	0.46±0.28
ANTERAXANTHIN	6.68±4.03	4.17±1.61	0.56±0.75	0.61±0.34
BETACRYPTOXANTHIN	5.03±1.21	4.12±0.95		
PHYTOENE	6.62±0.63	24.15±3.06		
ALPHACRYPTOXANTHIN	4.03±1.45	5.29±1.19		
TOTAL	60.20±3.09	81.06±5.88	1.20±0.17	1.73±0.15
40 DAPS				
ZEAXANTHIN	34.13±17.64	28.34±8.69	0.86±0.08	1.19±0.62
BETACAROTENE	9.60±1.21	13.27±0.95		
LUTEIN	10.06±1.90	7.30±0.73	0.61±0.18	0.59±0.31
ANTERAXANTHIN	3.13±0.08	2.55±0.51	0.23±0.08	0.30±0.21
BETACRYPTOXANTHIN	4.77±0.25	3.72±0.97		
PHYTOENE	31.53±8.92	52.68±2.76		
ALPHACRYPTOXANTHIN	6.19±0.59	5.18±1.26		
TOTAL	99.41±2.71	113.04±7.05	1.70±0.04	2.08±0.23
60 DAPS				
ZEAXANTHIN	22.99±13.55	22.04±10.54	0.68±0.15	1.24±0.76
BETACAROTENE	5.85±1.69	9.45±0.43		
LUTEIN	8.86±0.37	9.13±0.00	0.56±0.29	0.60±0.44
ANTERAXANTHIN	2.08±0.42	1.01±0.02	0.16±0.16	0.03±0.03
BETACRYPTOXANTHIN	3.65±0.45	2.85±0.16		
PHYTOENE	39.63±0.88	46.28±2.60		
ALPHACRYPTOXANTHIN	5.67±0.87	5.30±0.59		
TOTAL	88.71±6.25	96.07±3.67	1.4±0.13	1.87±0.39

Table 1: Content (in μg g^{-1} DW) of individual carotenoids in HC and M37W plants grown in field (F) or greenhouse (GH) conditions.



	25	40	60
lutein	12%	10%	10%
β-carotene	14%	10%	7%
zeaxanthin	37%	34%	26%
β-cryptoxanthin	8%	5%	4%
phytoene	11%	32%	45%
antheraxanthin	11%	3%	2%
α-cryptoxanthin	7%	6%	6%



	25	40	60
lutein	10%	6%	10%
β-carotene	18%	12%	10%
zeaxanthin	25%	25%	23%
β-cryptoxanthin	5%	3%	3%
phytoene	30%	47%	48%
antheraxanthin	5%	2%	1%
α-cryptoxanthin	7%	5%	6%

Figure 4: **Carotenoid content and composition in the endosperm of HC plants grown in the field (HC-F) or in the greenhouse (HC-GH).** (a) Individual carotenoids (%) in 25, 40 and 60 DAP endosperm of transgenic HC-F plants, (b) As above for HC-GH plants.

This was confirmed by measuring the distribution of carotenoids representing the β and ε branches of the carotenoid pathway **(Fig. 5)**. Young endosperm tissue (25 DAP) accumulated a higher proportion of β branch carotenoids in the field (70% of total carotenoids) than the greenhouse (53% of total carotenoids) **(Fig. 5)** perhaps reflecting the higher rate of conversion of β-carotene into downstream products such as zeaxanthin (37% for field plants *vs* 25% for greenhouse plants), antheraxanthin (11% *vs* 5%) and β-cryptoxanthin (8% *vs* 5%) **(Fig. 4** and **Table 1).** Indeed, higher proportions of these carotenoids were detected in field-grown plants compared to their counterparts in the greenhouse, with corresponding lower amounts of β-carotene **(Fig. 4** and **5**, **Table 1)**. We measured a gradual decline in the accumulation of β branch carotenoids in the endosperm of field-grown plants over time, compensated by a concomitant increase in the levels of early carotenoids up to lycopene. This resulted in a similar carotenoid profile in mature endosperm (60 DAP) in both environments. The proportion of β-branch carotenoids was higher than the proportion of ε-branch carotenoids at all time points. The proportion of total carotenoids in the ε-branch (α-cryptoxanthin and lutein) remained constant during seed development **(Fig. 5)**.

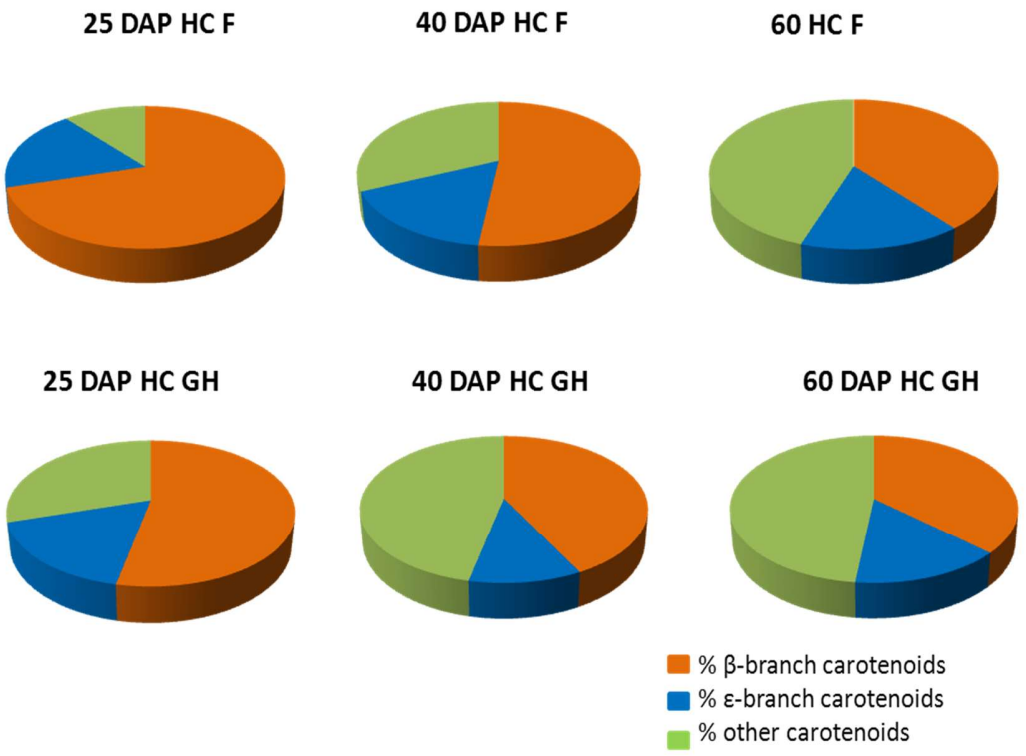


Figure 5: Distribution of β and ε branch carotenoids in HC-F and HC-GH endosperm at 25, 40 and 60 DAP.

Phytoene was the most abundant carotenoid in the endosperm of plants grown in both environments. The levels of phytoene increased during endosperm maturation under both conditions, representing ~50% of total carotenoids at maturity **(Fig. 4** and **Table 1).** Plants in the greenhouse accumulated more β-carotene (~14.6±1.85 μg g^{-1} DW, equivalent to 10% of total carotenoids) than field-grown plants (~9.60±1.21 μg g^{-1} DW, equivalent to 7% of total carotenoids) **(Fig. 4** and **Table 1).**

3.5 Discussion

Total carotenoid content at maturity was similar in greenhouse- and field-grown plants and reached the maximum of ~106±7 µg g^{-1} DW at 40 DAP, which is significantly higher than the maximum of 86±0.65 µg g^{-1} DW previously reported for a T1 Carolight^{R} line (Farré et al., 2013). The capacity for carotenoid accumulation in Carolight^{R} corn therefore appears to increase by nearly 20% between generations T1 and T12. A 7–20-fold increase in the accumulation of a recombinant protein expressed in transgenic corn endosperm was reported between generations T1 and T6 (Hood et al., 2012). A further 2–4-fold increase can be achieved by self-pollination (Hood et al., 2012) and an increase of 30–40% can be achieved by including a dedifferentiation-regeneration cycle compared to plants restricted to the sexual reproduction cycle (Ramessar et al., 2008).

I observed a 25% higher total carotenoid content in the young endosperm of greenhouse plants compared to field plants, but this gap had closed by maturity (60 DAP) with plants in both environments accumulating ~95 µg g^{-1} DW total carotenoids in the endosperm. Studies on the effects of cultivation practices and conditions (such as field vs greenhouse) are limited in cereals, and have been reported mainly in other crops such as tomato (Ehret et al., 2013; Brandt et al., 2003) and pepper (Lee et al., 2013: Russo and Howard, 2002; Keyhaninejad et al., 2012). A greenhouse study in tomato found that β-carotene, lycopene and lutein levels were negatively affected by light intensity (Ehret et al., 2013). Brandt et al. (2003) detected higher lycopene levels in tomato fruits grown in a greenhouse compared to those grown in the field. Thirteen greenhouse-grown varieties of *Capsicum annuum* and *C. chinense* produced more carotenoids than field-grown plants, reflecting the 20% higher light intensity in the field compared to the greenhouse (Lee et al., 2005). Cultivar-specific responses were

measured among red pepper cultivars but the majority produced more carotenoids in the greenhouse than the field (Russo et al., 2002). The carotenoid content of three other *C. annuum* cultivars has also been shown to decline by up to three-fold in the field compared to the greenhouse (Keyhaninejad et al., 2012). Light intensity can vary substantially in a field or in a greenhouse, thus possibly affecting carotenogenic gene expression and/or carotenoid production. Corn is cultivated in fields during summer, when the light intensity can reach extreme values. In our region, the temperature can reach 40°C in August, which is when Carolight^{R} reaches its reproductive stage, whereas the temperature of the greenhouse did not exceed 28°C. This may explain the relatively low levels of carotenoid production in the Carolight^{R} plants growing in the field compared to the greenhouse during the first period of endosperm development, whereas the lower field temperatures later in development allowed the field-grown plants to catch up with their greenhouse-dwelling counterparts.

When I analyzed the carotenoid profile, the proportions of the major carotenoids found in the endosperm (phytoene, zeaxanthin, lutein, β-carotene, α-cryptoxanthin, β-cryptoxanthin and antheraxanthin) varied during development between the plants in the greenhouse and field, although again these differences had disappeared by maturity and the profiles in mature kernels were similar regardless of where the plants were cultivated. The young endosperm tissue of field plants contained ~20% higher levels of β-branch carotenoids than greenhouse plants, but this reflected the specific accumulation of more zeaxanthin, β-cryptoxanthin and antheraxanthin and not β-carotene. Indeed, β-carotene was only ~3% more abundant in the greenhouse plants, consistent with the negative response of β-carotene to light intensity in tomato (Ehret et al. 2013). Higher temperatures and increased exposure to solar radiation were shown to reduce β-carotene accumulation in the fruits of the same tomato cultivar

(Rosales et al., 2006). These data suggest that the high temperature and light intensity in the field during summer growth may negatively affect the β-carotene content of Carolight^{R} corn. The proportion of ε-branch carotenoids remained constant in both environments during development (25–60 DAP) which suggests that differences in β-branch carotenoids are compensated by changes in the levels of phytoene, the precursor of downstream metabolites in the pathway. In both environments, the phytoene content increased over time, from young to mature endosperm, consistent with a gradual decline in the level of β-branch carotenoids. Phytoene was the most abundant endosperm carotenoid measured in both greenhouse and field plants, representing ~50% of the total carotenoids at maturity. This gradual accumulation probably reflects a metabolic bottleneck at the level of *Pacrt*I (phytoene desaturase), which converts phytoene into lycopene. This finding is important because it suggests that the performance of *Pacrt*I might not be optimal, and this could be remedied in subsequent Carolight^{R} products, particularly with the rapid development of genome editing technologies (Bortesi et al., 2016; Zhu et al., 2016).

The transient differences in β-branch carotenoids described above are likely to reflect the abundance and/or activity of the enzymes acting downstream of phytoene and they also suggest an additional level of control of transgene expression by the environment. Carotenoid accumulation in seeds, fruits and flowers correlates with the abundance of transcripts representing key carotenogenic genes (Zhu et al., 2010; Fraser et al., 1994). Therefore, quantitative real-time RT-PCR was used to determine whether the bottleneck could be explained by the low abundance of *Pacrt*I mRNA, and whether the transient differences in β-branch carotenoids also reflected differences at the mRNA level. In both environments and at all time points, *Pacrt*I mRNA accumulated in the endosperm at significantly lower levels

(~3-fold lower) than *Zmpsy1* mRNA. We also found that greenhouse plants accumulated twice as much *Zmpsy1* mRNA in the young endosperm (15 DAP) than field plants which partially explains the transient differences in carotenoid profiles we observed. This is consistent with the significant difference in the total carotenoid content of the endosperm of greenhouse and field plants at 25 DAP, suggesting that *Zmpsy1* mRNA accumulates at higher levels in the endosperm of greenhouse plants at ~15 DAP thus increasing the flux in the downstream carotenoid pathway. *Zmpsy1* mRNA levels then converged in the greenhouse and field plants between 20 and 30 DAP. Similarly, a correlation between *psy1* expression and total carotenoid levels was specifically observed at 20 DAP (Li et al., 2008a). The differential expression of *Zmpsy1* mRNA alone while other endogenous or exogenous carotenogenic transcripts were expressed at consistent levels suggests that *Zmpsy1* mRNA and/or its promoter is peculiarly sensitive to the different environmental conditions in the greenhouse and field. These environments are characterized by differences in light quality and temperature, as well as biotic and abiotic stresses, and this could influence carotenogenic gene/transgene expression or carotenoid accumulation. Light regulates genes and gene products related to photosynthesis, including carotenoids (Pizarro and Stange, 2009). The rice genes *Ospsy1* and *Ospsy2* contain *cis*-acting elements involved in light regulation (Dibari et al., 2012) as are the three equivalent corn genes, *Zmpsy1-3* (Gallagher et al., 2004; Li et al., 2008b). It is unclear whether the three *psy* genes in corn have overlapping functions in the modulation of carotenoid synthesis in different tissues and in response to developmental and/or stress signals. In corn leaves, carotenogenesis may require both phytochrome-dependent and phytochrome-independent photoregulation of *psy2* as well as non-photoregulation of *psy1* (Li et al., 2008a). Non-photoregulated PSY1 is the main enzyme responsible for carotenoid synthesis in corn endosperm (Faqiang et al., 2009) whereas PSY3

mediates carotenoid synthesis in the roots induced by abiotic stress (Li et al., 2008b). PSY1 is required for heat stress-induced carotenoid biosynthesis in corn to protect plastid membranes in photosynthetic tissues (Davison, 2002; Havaux et al., 2007). However, although there is a correlation between abiotic stress and *Zmpsy2* mRNA levels in the leaves and *Zmpsy3* mRNA levels in the roots, *Zmpsy1* mRNA levels do not appear to respond to abiotic stress at all. Importantly, these studies were performed under laboratory conditions and there are no studies thus far reporting the response of *Zmpsy1* to the combined stresses that may be encountered under field conditions. Interestingly, *Zmpsy1* transgene in Carolight^{R} was controlled using the wheat low-molecular-weight glutenin promoter, which is not known to be influenced by light or abiotic stress. This study therefore provides the first evidence that environmental factors can influence either this promoter or the *Zmpsy1* gene.

Although we did not observe any major differences between greenhouse and field plants in the expression of most endogenous carotenogenic genes during endosperm development (15–30 DAP), *Zmlycb* mRNA was four times more abundant than *Zmlyce* mRNA in both environments, which is important because the corresponding enzymes compete to divert flux into the β-branch and ε-branch (Harjes et al., 2008). This expression profile could therefore explain the higher content of β-branch carotenoids (~30%) at maturity compared to ε-branch carotenoids (~10%) in both greenhouse and field plants. However, the differential expression of hydroxylase genes may also influence the carotenoid profile. Two classes of enzymes catalyze the hydroxylation of α and β ionone rings in higher plants: CYP97-type heme-containing cytochrome P450 hydroxylases (Tian et al., 2004; Kim & Dellapenna, 2006) and ferredoxin-dependent β-carotene hydroxylase (BCH)-type non-heme di-iron hydroxylases (Sun et al., 1996; Tian & Dellapenna, 2001; Tian et al., 2003). BCH-type enzymes typically

show limited activity towards the ε-ring of α-carotene but significant activity towards the β-ring, although *Zmbch1* also promotes the accumulation of α-carotene (Zhou et al., 2012). CYP97A is responsible for catalyzing the hydroxylation of the β-ring of α-carotene whereas CYP97C is mainly active against the ε-ring (Kim & DellaPenna, 2006; Chang et al., 2015). PuCHY1, a CYP97B enzyme produced by the red alga *Porphyra umbilicalis*, is also responsible for β-ring hydroxylation (Yang et al, 2014). I found that *Zmbch2* mRNA accumulated to levels 100-fold greater than *Zmbch1* mRNA and that *Zmcyp97a* and *Zmcyp97b* were expressed at levels at least 10-fold higher than *Zmcyp97c* in the endosperm of both the greenhouse and field plants. The higher content of β-branch carotenoids in the Carolight^{R} endosperm in both environments can therefore be explained by these specific gene expression profiles.

In conclusion, minor differences in endosperm carotenoid composition were measured in the early stages of development, but mature endosperm from field- and greenhouse-grown plants were no different in terms of their carotenoid content and composition. Thus, Carolight^{R} behaves in a similar manner in a controlled environment and under field conditions, an essential requirement to support further product development.

3.6 References

- Alba, R., Cordonnier-Pratt, M.M., Pratt, L.H. (2000). Fruit-localized phytochromes regulate lycopene accumulation independently of ethylene production in tomatoes. *Plant Physiol*. **123**:363– 370.
- Berman, J., Zorrilla-López, U., Farré, G., Zhu, C., Sandmann, G., Twyman, R.M., Capell, T., Christou, P. (2015). Nutritionally important carotenoids as consumer products. *Phytochem. Rev.* **5**:727–743.
- Brandt, S., Lugasi, A., Barna, É., Hóvári, J., Pék, Z., Helyes, L. (2003). Effects of growing methods and conditions on the lycopene content of tomato fruits. *Acta Alimentaria*. **32**:269–278.
- Britton, G., Liaaen-Jenson, S. & Pfander, H. (2004). Carotenoids Handbook. *Photosynthetica* **42**:186-186.
- Chang, S., Berman, J., Sheng, Y., Wang, Y., Capell, T., Shi, L., Ni, X., Sandmann. G., Christou, P., Zhu, C. (2015). Cloning and Functional Characterization of the Maize (Zea mays L.) Carotenoid Epsilon Hydroxylase Gene. *PLoS One*. 10:e0128758.
- Ehret, D.L., Usher, K., Helmer, T., Block, G., Steinke, D., Frey, B., Kuang, T., Diarra, M. (2013). Tomato fruit antioxidants in relation to salinity and greenhouse climate. *J Agric Food Chem*. **61:**1138-1145.
- Farre', G., Maiam Rivera, S., Alves, R., Vilaprinyo, E., Sorribas, A., Canela, R., Naqvi, S., Sandmann, G., Capell, T., Zhu, C., Christou, P. (2013). Targeted transcriptomics and metabolic profiling reveals temporal bottlenecks in the maize

carotenoid pathway that can be addressed by multigene engineering. *Plant J*. **75**:441-455.

- Faqiang Li, Oren Tzfadia, Eleanore T. Wurtzel. (2009). The phytoene synthase gene family in the Grasses. *Plant Signaling & Behavior*. **4**:208-211.
- Fraser, P.D., Truesdale, M.R., Bird, C.R., Schuch, W., Bramley, P.M. (1994). Carotenoid biosynthesis during tomato fruit development. *Plant Physiol*. **105**:405–413.
- Harjes, C.E., Rocheford, T.R., Bai, L., Brutnell, T.P., Kandianis, C.B., Sowinski, S.G., Stapleton, A.E., Vallabhaneni, R., Williams, M., Wurtzel, E.T., Yan, J., Buckler, E.S. (2008). Natural genetic variation in lycopene ε-cyclase tapped for maize biofortification. *Science*. **319**:330–333.
- Havaux, M. and Niyogi, K.K. (1999). The violaxanthin cycle protects plants from photo-oxidative damage by more than one mechanism. *Proc Natl Acad Sci U S A*. **96**: 8762-8767.
- Hood, E.E., Devaiah, S.P., Fake, G., Egelkrout, E., Teoh, K.T., Requesens, D.V., Hayden, C., Hood, K.R., Pappu, K.M., Carroll, J., Howard, J.A. (2012). Manipulating corn germplasm to increase recombinant protein accumulation. *Plant Biotechnol J.* **10**: 20-30.
- Keyhaninejad, N., Richins, R.D. & O'Connel, M.A. (2012). Carotenoid Content in Field-grown versus Greenhouse-grown Peppers: Different Responses in Leaf and Fruit. *Hortscience*. **47**:852–855.

- Kim, J. & DellaPenna, D. (2006). Defining the primary route for lutein synthesis in plants: the role of Arabidopsis carotenoid β-ring hydroxylase CYP97A3. *Proc Natl Acad Sci USA*. **103**:3474–3479.
- Lee, J.J., Crosby, K.M., Pike, L.M., Yoo, K.S., Leskovar, D.I. (2005). Impact of genetic and environmental variation on development of flavonoids and carotenoids in pepper (Capsicum spp.) *Sci. Hort*. **106**:341–352.
- Li, Y. & Walton, C. (1990). Violaxanthin Is an Abscisic Acid Precursor in Water-Stressed Dark-Grown Bean Leaves. *Plant Physiol*. **92**:551-559.
- Li, F., Vallabhaneni, R., Yu, J., Rocheford, T., Wurtzel, E.T. (2008a). The Maize Phytoene Synthase Gene Family: Overlapping Roles for Carotenogenesis in Endosperm, Photomorphogenesis, and Thermal Stress Tolerance. *Plant Physiology*. **147**:1334-1346.
- Li, F., Vallabhaneni, R. & Wurtzel, E. T. (2008b). PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and regulator of abiotic stress-induced root carotenogenesis. *Plant Physiol*. **146**:1333-1345.
- Li, Q., Farre', G., Naqvi, S., Breitenbach, J., Sanahuja, G., Bai, C., Sandmann, G., Capell, T., Christou, P., Zhu, C. (2010). Cloning and functional characterization of the maize carotenoid isomerase and β-carotene hydroxylase genes and their regulation during endosperm maturation. *Transgenic Res*.**19**:1053-1068.
- Lindgren, L.O., Stålberg, K.G., Höglund, A.S. (2003). Seed-specific overexpression of an endogenous Arabidopsis phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. *Plant Physiol*. **132**:779-785.

- Naqvi, S., Zhu, C., Farre', G., Sandmann, G., Capell, T., Christou, P. (2011). Synergistic metabolism in hybrid corn reveals bottlenecks in the carotenoid pathway and leads to the accumulation of extraordinary levels of the nutritionally important carotenoid zeaxanthin. *Plant Biotechnol J*. **9**:384-393.
- Omoni ,O.A. & Aluko, E.R. (2005). The anti-carcinogenic and anti-atherogenic effects of lycopene: a review. *Trends Food Sci Technol*. **16**:344-350.
- Parry, A.D., Babiano, M.J. & Horgan, R. (1990). The role of cis-carotenoids in abscisic acid biosynthesis. *Planta*. **182**:118-28.
- Pizarro, L. & Stange, C. (2009). Light-dependent regulation of carotenoid biosynthesis in plants. *Cien. Inv. Agr*. **36**:143–162.
- Ramessar, K. Rademacher, T., Sack, M., Stadlmann, J., Platis, D., Stiegler, G., Labrou, N., Altmann, F., Ma, J., Stöger, E., Capell, T., Christou, P. (2008). Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. *Proc Natl Acad Sci U S A*. **105**:3727-3732.
- Rivera, S., Vilaró, F. & Canela, R. Determination of carotenoids by liquid chromatography/mass spectrometry: Effect of several dopants. *Anal. Bioanal. Chem*. **400**:1339-1346 (2011).
- Rivera, S.M., Vilaró, F., Zhu, C., Bai, C., Farré, G., Christou, P., Canela-Garayoa, R. (2013). Fast quantitative method for the analysis of carotenoids in transgenic maize. *J. Agric. Food Chem*. **61**:5279-5285.
- Rosales, M. A., Ruiz, J. M., Hernández, J., Soriano, T., Castilla, N. and Romero, L. (2006). Antioxidant content and ascorbate metabolism in cherry tomato exocarp in relation to temperature and solar radiation. *J. Sci. Food Agric*. **86**:1545–1551.

- Russo, V.M. & Howard, L.R. (2002). Carotenoids in pungent and non-pungent peppers at various developmental stages grown in the field and glasshouse. *J. Sci. Food Agr.* **82**:615–624.
- Schofield, A. & Paliyath, G. (2005). Modulation of carotenoid biosynthesis during tomato fruit ripening through phytochrome regulation of phytoene synthase activity. *Plant Physiol. Biochem*. **43**:1052–1060.
- Simkin, A.J., Zhu, C., Kuntz, M., Sandmann, G. (2003). Light–dark regulation of carotenoid biosynthesis in pepper (Capsicum annuum) leaves. J. Plant Physiol. 160:439–443.
- Sun, Z., Gantt, E. & Cunningham, F.X. (1996). Cloning and functional analysis of the β-carotene hydroxylase of Arabidopsis thaliana. *J Biol Chem*. **271**:24349–24352.
- Tian, L. & Dellapenna, D. (2001). Characterization of a second carotenoid β-hydroxylase gene from Arabidopsis and its relationship to the LUT1 locus. *Plant Mol Biol*. **47**:379–388.
- Tian, L., Magallanes-Lundback, M., Musetti, V., DellaPenna, D. (2003). Functional analysis of β- and ε-ring carotenoid hydroxylases in Arabidopsis. *Plant Cell*. **15**:1320–1332.
- Tian, L., Musetti, V., Kim, J., Magallanes-Lundback, M., DellaPenna, D. (2004). The Arabidopsis LUT1 locus encodes a member of the cytochrome p450 family that is required for carotenoid ε-ring hydroxylation activity. *Proc Natl Acad Sci U S A*. **101**:402–407.

- Yang, L.E, Huang, X.Q., Hang, Y., Deng, Y.Y., Lu, Q.Q., Lu, S. (2014). The P450-type carotene hydroxylase PuCHY1 from *Porphyra* suggests the evolution of carotenoid metabolism in red algae. *J Integr Plant Biol*. **56**:902-915.
- Zhou, Y., Zhou, Y., Han, Y., Li, Z., Fu, Y., Fu, Z., Xu, S., Li, J., Yan, J., Yang, X. (2012) . ZmcrtRB3 encodes a carotenoid hydroxylase that affects the accumulation of α-carotene in maize kernel. *J Integr Plant Biol*. **54**:260-269.
- Zhu, C., Bai, C., Sanahuja, G., Yuan, D., Farré, G., Naqvi, S., Shi, L., Capell, T., Christou, P. (2010). The regulation of carotenoid pigmentation in flowers. *Arch. Biochem. Biophys*. **504**:132-141.
- Zhu, C., Sanahuja, G., Yuan, D., Farré, G., Arjó, G., Berman, J., Zorrilla-López, U., Banakar, R., Bai, C., Pérez-Massot, E., Bassie, L., Capell, T., Christou, P. (2013). Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies. *Plant Biotechnol. J.* **11**:129-141.

Chapter 4

EFFICACY OF *Bt cry1Ac* GENE AGAINST LEPIDOPTERAN INSECT PESTS IN A HIGH CAROTENOID MAIZE LINE.

Manuscript in preparation

Chapter 4:

Efficacy of Bt *cry1Ac* gene against lepidopteran insect pests in a high carotenoid maize line.

4.1 Abstract

The effectiveness of *Bt cry1Ac* gene in a maize line fortified with carotenoids (4BtxHC) was evaluated against *O. nubilalis* and *M. unipuncta*, two lepidopteran pests. Leaves and grains of 4BtxHC were toxic to larvae of the corn borer *O. nubilalis*. However, the insecticidal activity of 4BtxHC grains was lowered compared to the original 4Bt line by ca: 17%. We suggest that the accumulation of high levels of carotenoids influences the response of insects to the *Bt* toxin. Furthermore larvae of the two lepidoptera fed on leaves from Carolight^{R} (HC, with no *Bt* gene) or its near isogenic line (M37W) exhibited different feeding behaviour. Larvae displayed a higher degree of feeding on M37W rather than on Carolight^{R} leaves. I attribute this to a knock-on effect of carotenoid metabolism in the seeds which affects the leaves and, more specifically, how attractive they are to insect pests. I conclude that antagonistic effects between stacked traits in a transgenic plant are not to exclude and should be carefully studied.

4.2 Introduction

Bacillus thuringiensis (*Bt*) is a Gram-positive bacterium which was first described by Berliner (Berliner, 1911) when he isolated a *Bacillus* species from caterpillars of *Ephestia kuehniella* Zeller, the Mediterranean flour moth, and named it after the German province of Thuringia where the infected moth was found. Direct contact between the spores / crystals and healthy caterpillars had no effect, but when the spores and crystals were coated onto leaves and consumed, the caterpillars stopped feeding and died. The potential of *Bt* as an insecticide was recognized in 1927, and subsequent large-scale aerial spraying of corn fields with the bacteria proved to be effective against the European corn borer (*Ostrinia nubilalis*) (Husz, 1930). This eventually led to the development of Sporeine, a commercial *Bt* insecticide, which was used for the first time in 1938 (Lambert and Peferoen, 1992). The insecticidal activity of *B. thuringiensis* is due to the crystalline protein inclusions formed in the course of sporulation (Angus, 1956). These proteinaceous inclusions, called Cry proteins or δ-endotoxins, are composed of crystal proteins which are highly toxic to a wide variety of important insect pests. Each Cry toxin has a highly specific activity against insect species of the orders lepidoptera, diptera, coleoptera, hymenoptera as well as nematodes (Schnepf et al., 1998). Their high specificity for target pests makes them a valuable alternative to chemical pesticides for integrated pest management in agriculture. *Bt* toxins need to be activated in the insect gut, and it was soon discovered that the critical factors for this activation were an alkaline environment and the presence of specific proteases, which cleaved the innocuous pro-toxin into its active form. Once activated by proteolysis, the toxins were shown to bind to receptors in the brush border membrane of midgut epithelial cells, thus causing pores to open, disrupting the movement of solutes across the gut epithelium and

promoting the influx of water. The toxins were shown to be orally lethal to caterpillars in pure form, and the pro-toxins could be converted into active toxins in vitro using specific proteases under alkaline conditions. The requirements for alkaline conditions, specific proteases and specific receptors are the reason why *Bt* toxins are harmless to mammals (which have an acidic gut and lack the corresponding receptors) and why each toxin has a narrow host range (Sanahuja et al., 2011).

The discovery that genes coding for these crystal proteins are localized on transmissible plasmids (Gonzalez et al., 1982), allowed the cloning of *cry* genes from many *Bt* subspecies and subsequent development of *Bt* transgenic plants. In 1995, the US Environmental Protection Agency (EPA) approved the first registration of *Bt* potato, corn and cotton crops, and the deployment of the latter two crops has increased year on year, with an immense positive impact on agriculture and the environment. The deployment of *Bt* crops has reduced the use of pesticides and fossil fuels required for spraying, reduced CO_2 emissions by limiting the need for plowing, and conserved soil and moisture by encouraging no-till agriculture (Sanahuja et al., 2011). Insect susceptibility to Bt toxins can be influenced by a variety of different factors, many of which are still unknown or not completely understood. Nutrition is one of these factors, but has recently started to attract attention (Deans et al., 2016). Very little, for example, is known about how carotenoid enhancement, particularly in association with *Bt* toxins, may affect herbivore development and behaviour. Carotenoids are responsible for visual pigments and body color in insects and crustaceans, have strong anti-oxidant activity and are insect hormone precursors but their transition along food webs has been poorly investigated (Heath et al., 2013). The oxidative cleavage of carotenoids produces volatile apocarotenoids which are important compounds in herbivore-plant communication since they can be either strongly attractive or repellent for insects (Caceres et al., 2016). For

example, Cruz and Eizaguirre (2015) reported that Carolight^{R} was found to be less attractive for the corn stem borer *Sesamia nonagrioides* (Lef) to lay eggs on. Therefore, it is important to study how a *Bt* plant with enhanced carotenoid production behaves in terms of controlling lepidopteran corn pests most commonly present in the region of study.

The aims of my experiments were (i) to determine the toxicity of a transgenic corn line (4Bt) expressing the *Btcry1Ac* gene on different lepidopteran corn pests (Study 1), (ii) to determine the toxicity of a second event (4BtxHC) obtained by crossing 4Bt with the high carotenoid line Carolight^{R} (Zhu et al., 2008) (Study 2), and (iii) to determine if there are differences in feeding behaviour between insects fed on Carolight^{R} and its near-isogenic line, M37W.

In study 2, in particular, we wished to test the hypothesis that the accumulation of high levels of carotenoids might interfere with the effectiveness of Cry1Ac and therefore alter toxicity towards insect pests.

In study 1, L2 larvae of four different corn borer species (lepidoptera family) were used: *O. nubilalis* (Hübner) and *S. nonagrioides* as *Bt*-target species and *Mythimna unipuncta* (Haworth) and *Helicoverpa armígera* (Hübner) as secondary corn pests (non-target). The latter pests have been designated as non-target pests since Bt crops have not been designed to specifically control them; nonetheless they can be susceptible to Bt toxins. *M. unipuncta* was studied both at L2 and L5 stages since it is the most mobile species, capable of moving from one field to the other.

In study 2 I focused on the most susceptible (*O.nubilalis*) and the least susceptible (*M. unipuncta*) species.

4.3 Material and methods

4.3.1 Plant material

Corn (*Zea mays*) M37W and transgenic lines 4Bt, expressing *Bacillus thuringiensis cry1Ac*, and MON810 (DKC6667YG, Dekalb, containing MON810 event), expressing *Btcry1Ab*, were germinated in the greenhouse. They were grown at 28/20ºC day/night temperature with a 10 h photoperiod and 60-90 % relative humidity for the first 50 days, followed by maintenance at 21/18ºC day/night temperature with a 16-h photoperiod, thereafter. The South African elite white corn inbred M37W seeds were obtained from CSIR (Pretoria, South Africa). Heterozygous 4Bt plant seeds were available in the laboratory (Sanahuja, G., PhD thesis, 2013). An elite event with the highest Cry1Ac protein accumulation was self-pollinated to obtain the 4Bt homozygous line.

By crossing 4Bt homozygous plants with Carolight^{R}, expressing corn *psy1* and *Pantoea annatis crt*I in a M37W genetic background (Zhu et al. 2008), a new event was obtained: 4BtxHC. A homozygous 4BtxHC line was obtained through self-pollination.

4.3.2 Quantification of Cry1Ac toxin

The content of Cry1Ac protein in plant material was determined before the start of the experiments using the EnviroLogix Cry1Ab/Cry1Ac ELISA kit (EnviroLogix Inc., Portland, Maine, USA). For calibration, Cry1Ac standards at 8.0, 6.0, 4.0, 2.0, 1.0, 0.5 and 0.25 ng/ml were used, and measurements were made with a Titertek Multiskan Plus MKII spectrophotometer at 450 nm following the procedure described in Obrist et al. (2006a).

4.3.3 Insect rearing

Larvae of *O. nubilalis, S. nonagrioides, M. unipuncta,* and *H. armigera* were from the culture maintained in the entomology laboratory at the UdL, renewed every three or four generations with insects collected in the field in the Lleida area. The larvae were reared on a semi-artificial diet (see **4.3.5** for composition). Larvae were maintained at 25°C with high humidity (>60%) under a 16:8h light:dark photoperiod.

4.3.4 Bioassays

4.3.4.1 Study 1

In study 1, four different corn pest species (lepidoptera family) were used: the stem borers *O. nubilalis* and *S. nonagrioides,* the leaf feeder *M. unipuncta* and the earworm *H. armigera.* Four independent plants were used as replicates for each of the three corn varieties: 4Bt plants, expressing *cry1Ac*; M37W (near-isogenic line, as a negative control); MON810 (expresses the Cry1Ab toxin, positive control). Leaves from 40-day-old plants were washed in water, dried, and 4-5cm sections were placed in a container lined with moist filter paper. Each replicate consisted of ten containers; five second-instar (L2) larvae were released into each container. The containers were maintained at 25°C and high humidity (>60%) under a 16:8h light: dark photoperiod. Every 48/72h, insect mortality was recorded for one week. In the case of *M. unipuncta* a second assay was performed using L5 larvae, recording the mortality rate until larvae died or pupated. Pupal weight was also recorded. Experiments performed in Study 1 are summarized in **Table 1**.

Plant Variety	Diet	Species	Instar stage	Variables measured
M37W (- control) 4Bt MON810 (+ control)	Leaves	*O. nubilalis* *S. nonagrioides* *H. armigera*	L2	Mortality 48/72 h Pupal weight
		M. unipuncta	L2 L5	

Table 1: Table summarizing the experiments performed to evaluate the efficacy of *cry1Ac* in 4Bt corn against four lepidopteran species (Study 1).

4.3.4.2 Study 2

In study 2 the stem borer *O. nubilalis* and the leaf feeder *M. unipuncta* were chosen as a *Bt* target and non-target species, respectively, to test 4 plant lines: the wild-type white endosperm variety M37W, the high-carotenoid transgenic line Carolight^{R} (from now on named HC), expressing corn *psy1* and *Pantoea annatis crtI* in a M37W genetic background (Zhu et al., 2008), 4Bt, and 4BtxHC. L2 larvae were fed with two different diets based on leaves or grains. For leaf feed, four independent plants were used as replicates for each of the four varieties. In grain diet experiment, replicates were four different insect populations. Each replicate consisted of 50 five second-instar (L2) larvae, divided in 10 containers for *O. nubilalis* species and in individual containers for *M. unipuncta* species, to avoid episodes of cannibalism. For the leaf diet, leaves from 40-day-old plants were washed in water, dried and

4-5cm sections were placed in a container lined with moist filter paper. In the case of the grain diet, a cube of diet was placed in each container (see **4.3.5** for diet composition).

The containers were maintained at 25°C and high humidity (>60%) under a 16:8h light: dark photoperiod. Every 48/72h, insect mortality was recorded. Pupal weight and duration of period from L2 to pupation were determined. Furthermore, in the leaf diet experiment, degree of feeding was determined by measuring the consumed area of each leaf with ImageJ software (http://imagej.net). Experiments performed in Study 2 are summarized in **Table 2**.

Plant Variety	Diet	Species	Instar stage	Variables measured
M37W (- control) HC 4Bt 4BtxHC	Leaves Grains	*O. nubilalis* *M. unipuncta*	L2	Mortality Pupal weight Consumed leaf area Duration of L2 to pupation development

Table 2: Summary of experiments to test the efficacy of *cry1Ac* in the high carotenoid maize line 4BtxHC (Study 2).

4.3.5 Grain diet

Grain diet components are listed in **Table 3.** To prepare the diet, water and agar were mixed, taken to boiling point, poured into a 1 liter plastic beaker and allowed to cool down to 65^0C.

Milled dry corn seeds, nutritional yeast and wheat germ were then added and the mixture was homogenized with a blender. When temperature reached 55^0C, acids were added (see Table 3). The homogenized mixture was then poured into an appropriate container, allowed to solidify and stored at 4^0C.

	Weight (in grams)
Milled dry corn seeds	27.5
Nutritional yeast	7.5
Wheat germ	7.5
Ascorbic acid	1.25
Sorbic acid	0.5
Agar agar	4.0
Water	175.0

Table 3: Grain diet composition

4.3.6 Statistical analysis

The influence of each variety on the variables we measured was analyzed by means of one way (variety) ANOVA and means compared by LSD. All statistical analyses were done using JMP software (http://www.jmp.com/en_us/software.html). For all comparisons $P \leq 0.05$ was considered significant. Percentages were transformed by ASIN(SQRT(%/100) to normalize data.

4.4 Results

4.4.1 Protein accumulation in transgenic corn plants

We analyzed Bt toxin concentration in insect diet using an ELISA kit. Cry1Ab/Cry1Ac toxin concentration in the leaves of MON810, 4Bt and 4BtxHC varieties was not significantly different, with a mean value of 47±6 µg/g FW. Cry1Ac concentration in an average grain (35±3 µg/g FW) was lower compared to average concentration in leaves and it was similar between 4Bt and 4BtxHC plants. In general, we detected a great variability in Cry1Ab/c toxin concentration between different plants of the same variety, as well as between grains of the same plant. Grain diets were designed and prepared in order to have the same amount of Bt Cry1Ac concentration. The diet prepared with grains from 4Bt plants contained same amount of Cry1Ac toxin than the one prepared with 4BtxHC grains (2.8±0.1 µg/g FW), and was significantly lower compared to toxin concentration in leaves or grains, since plant material is diluted in the other diet components.

4.4.2 Study 1- Toxicity of 4Bt plants

4.4.2.1 Efficacy of 4Bt leaves against L2 larvae of four lepidopteran species

O. nubilalis was highly susceptible to 4Bt, with a rate of mortality after 5 days (second evaluation) of 100%, same as with MON810 (**Fig. 1a)**. *S. nonagrioides* displayed a significantly higher (44% *vs* 0%) mortality when fed on MON810 compared to M37W leaves after 3 days; no statistically significant differences were measured between 4Bt and MON810 groups or between 4Bt and M37W **(Fig. 1b)**. At five days from the start of the experiment 100% of mortality was recorded for larvae fed on both 4Bt and MON810 leaves, while larvae

fed on M37W leaves had 0% of mortality. We measured a significantly higher mortality for *H. armigera* larvae fed on 4Bt and MON810 leaves compared to those fed on M37W **(Fig. 1c)**, both at 3 and 5 days from the start of the experiment. No statistically significant differences were found between mortality caused by MON810 and 4Bt leaves. For *M. unipuncta,* which is less susceptible than the other species to Cry1A toxins, similar levels of mortality to those recorded for the previous species were measured after two additional days of feeding (7 days). In this case, a significantly lower mortality was recorded for larvae fed on 4Bt compared to MON810 leaves (15% lower) **(Fig. 1d)**. In general, all four species fed in a significantly higher degree on non-Bt compared to Bt leaves (**Fig.2**).

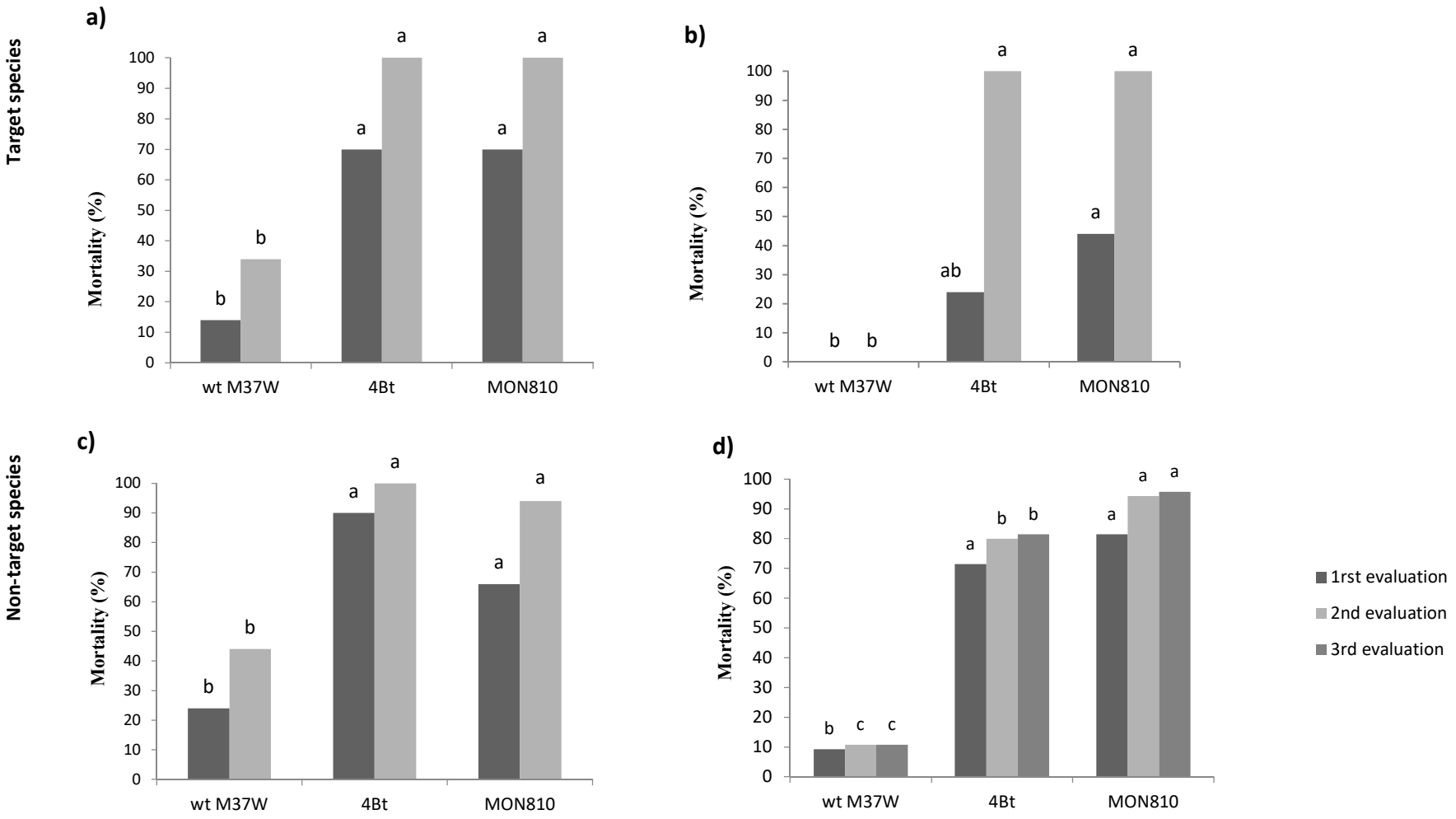

Target species
Non-target species
a)
b)
c)
d)
Mortality (%)
wt M37W
4Bt
MON810
1rst evaluation
2nd evaluation
3rd evaluation

Figure 1: Efficacy of 4Bt in controlling L2 larvae of the corn borers a) *O. nubilalis*, b) *S. nonagrioides*, c) the earworm *H. armigera*, d) the leaf feeder *M. unipuncta* at 3 (first evaluation), 5 (second evaluation) and 7 days (third evaluation only for *M. unipuncta*) from the beginning of the experiment. Wild-type (M37W) was used as a negative control and MON810 was used as a positive control. Different letters indicate statistically significant differences in mortality between the varieties ($P \leq 0.05$).

M37W **4Bt** **MON810**

Figure 2: Feeding behavior of L2 larvae of the corn borer *S. nonagrioides* at 3 days after the beginning of the experiment with transgenic corn plants 4Bt. M37W was used as a negative and MON810 as a positive control.

4.4.2.2 4Bt corn is as effective as MON810 in controlling L5 *M. unipuncta* larvae

At two weeks after the beginning of the experiment, the same degree of mortality was measured for L5 *M. unipuncta* larvae fed on 4Bt and MON810 leaves. Larvae fed on M37W leaves had a significantly lower mortality rate (~20% *vs* ~90%) than *Bt*-fed groups **(Fig. 3a)**. Weight of the surviving pupae was recorded. Pupae from larvae which had survived after ingestion of the *Bt* toxin (both from 4Bt and MON810 leaves) weighted significantly less (~40% less) compared to the ones fed on M37W leaves. There were no statistically significant differences on the weight between the pupae from larvae fed on 4Bt or MON810 (**Fig. 3b**).

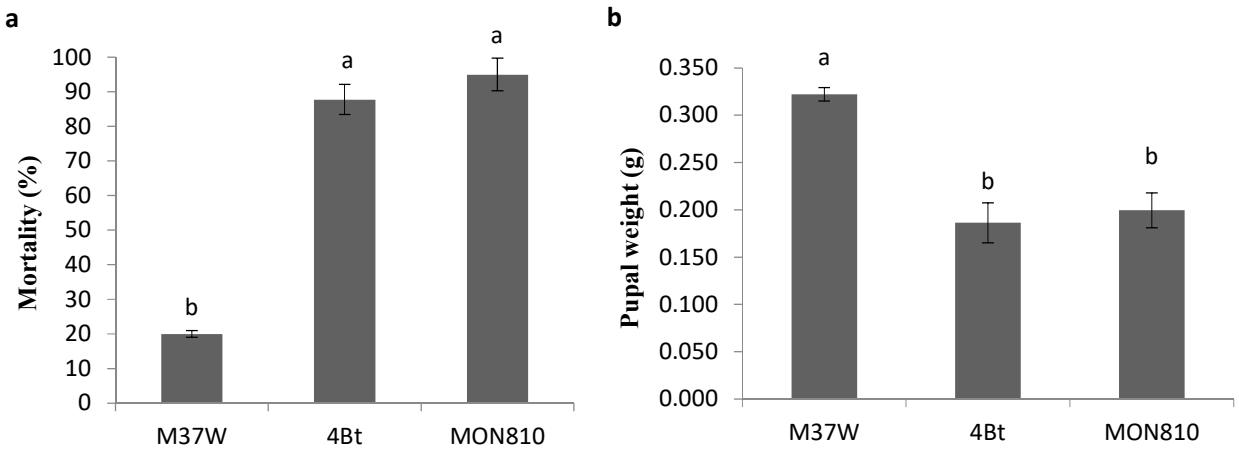


Figure 3: a) Efficacy of 4Bt in controlling L5 larvae of the leaf feeder *M. unipuncta*. Wild-type (M37W) was used as a negative control and MON810 was used as a positive control. b) Weight of *M. unipuncta* pupae fed on 4Bt plants. Wild-type (M37W) was used as a negative and MON810 as a positive control. Different letters indicate statistically significant differences between the varieties ($P \leq 0.05$). Bars represent Standard Error of the Mean (SEM).

4.4.3. Study 2- Efficacy of *cry1ac* in leaves of a high carotenoid maize line against L2 larvae of *O. nubilalis* and *M. unipuncta*

4.4.3.1 4Bt and 4BtxHC leaves are equally effective against L2 larvae of *O. nubilalis*

4Bt and 4BtxHC caused similar mortality after 3 and 5 days of feeding, with a rate of mortality at 5 days of 100% for 4Bt and 95% for 4BtxHC **(Fig.4a)**. After 7 days, all larvae from both groups were dead. This confirms the efficacy of the *Bt*-containing plants in controlling *O. nubilalis*. No differences in mortality were measured at three days after the beginning of the experiment between M37W- and HC-fed larvae, while at five days larvae fed on M37W leaves showed a significantly higher mortality compared to those fed on HC leaves (~60% higher) **(Fig.4a)**. Larvae consumed significantly lower quantities of *Bt* than non-*Bt* leaves at 3 and 5 days. While there were no statistically significant differences between the degree of feeding on 4Bt and 4BtxHC leaves, there was a tendency for larvae to consume higher quantity of M37W than HC leaf material, with a statistically significant difference at three days after the beginning of the experiment (~55% increase) **(Fig.4b** and **5)**. This difference in feeding behaviour on M37W and HC plants, however, was not reflected on weight or survival of two week-old larvae. In fact, two weeks after the beginning of the experiment, there were no significant differences in mortality **(Fig.6a)** or in the weight **(Fig.6b)** between larvae fed on M37W and HC leaves.

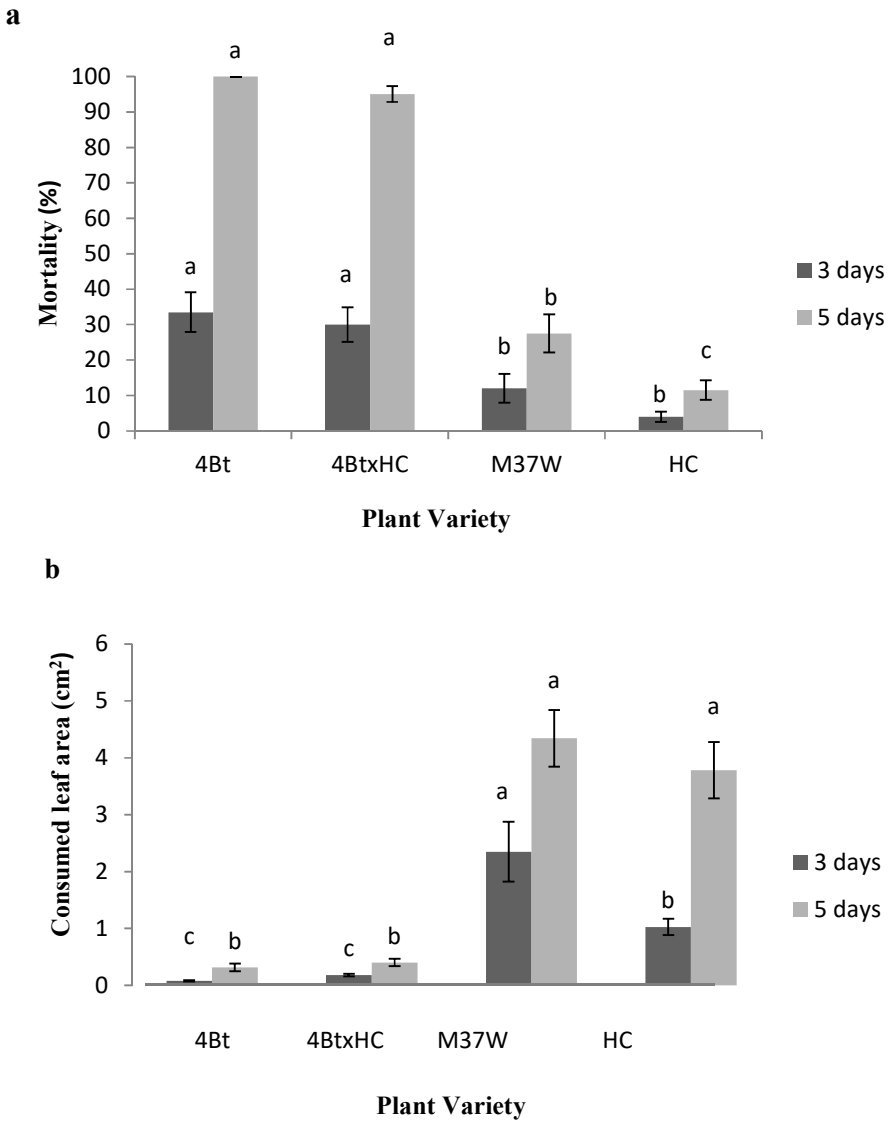


Figure 4: a) Efficacy of 4Bt and 4BtxHC in controlling L2 larvae of the corn borer *O. nubilalis*, at 3 and 5 days after the beginning of the experiment. Wild-type (M37W) and HC plants were used as controls. **b)** Leaf consumption of *O.nubilalis* larvae three and five days after the beginning of the experiment. On the y axes, cm^2 of consumed leaf area is shown. Bars represent Standard Error of the Mean (SEM). Different letters indicate statistically significant differences between the varieties ($P \leq 0.05$).

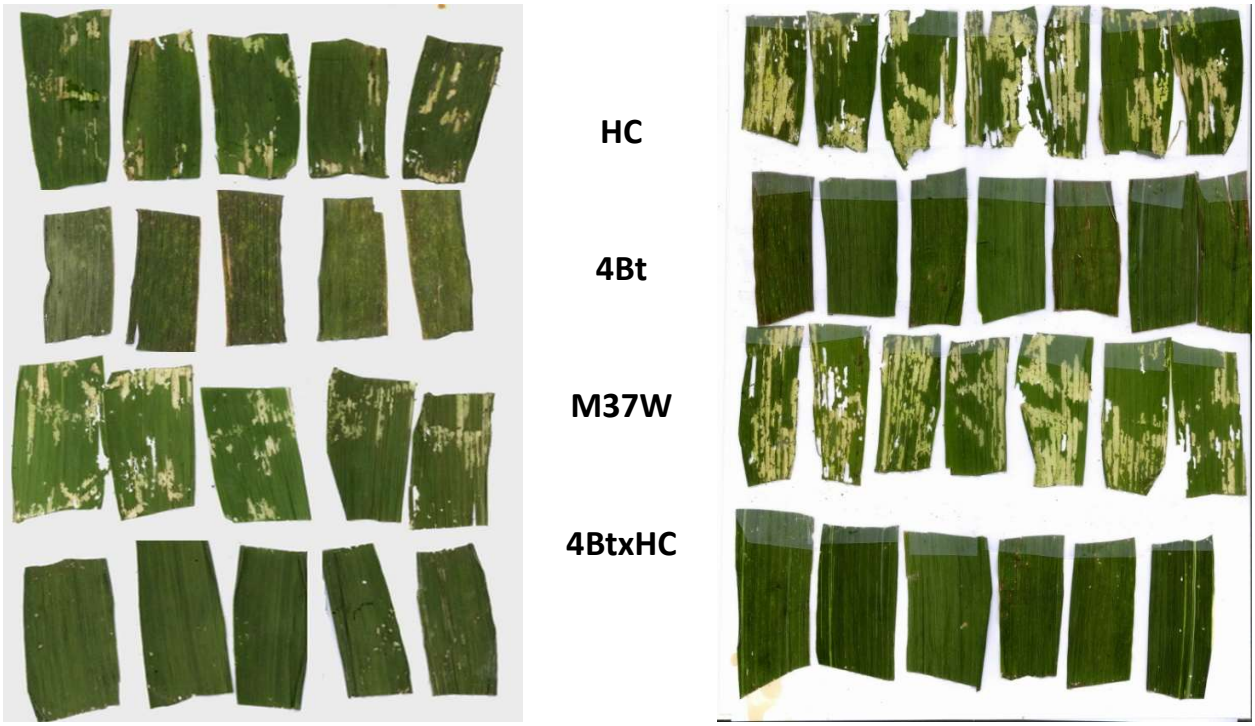


Figure 5: Feeding behaviour of *O.nubilalis* larvae on the four plant varieties HC, 4Bt, M37W and 4BtxHC three and five days after the beginning of the experiment.

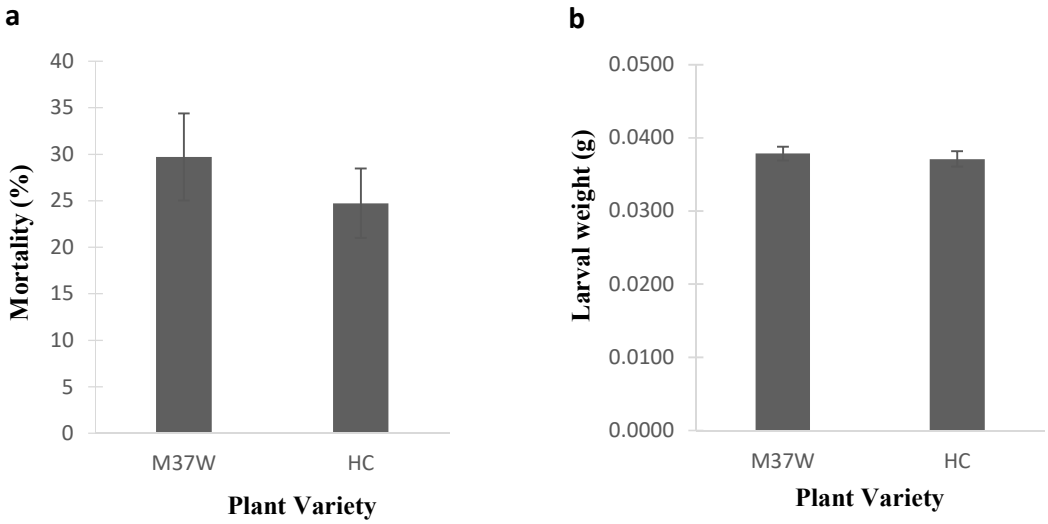


Figure 6: Effect of M37W and HC plants on the development of *O. nubilalis* larvae two weeks after the beginning of the experiment **a)** Mortality rate and **b)** Weight (in grams). Bars represent Standard Error of the Mean (SEM).

4.4.3.2 Mortality of *M. unipuncta* on a leaf diet is similar for all 4 plant lines

We measured no statistically significant differences in the mortality rate of L2 larvae of *M. unipuncta* fed on M37W, HC, 4Bt and 4BtxHC **(Fig.7).**

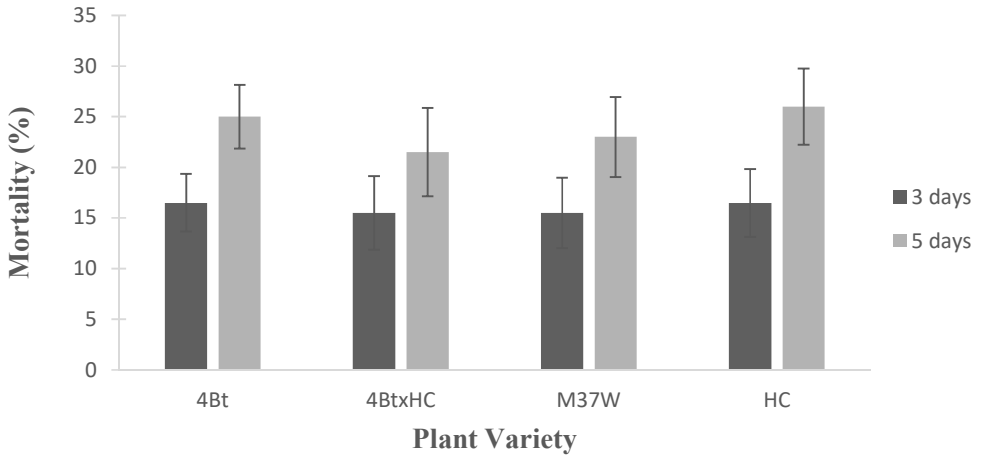


Figure 7: Mortality of *M. unipuncta* larvae three and five days after the beginning of the experiment. Bars represent Standard Error of the Mean (SEM).

However, 4Bt and 4BtxHC plants had a sub-lethal effect on the larvae which weighed significantly less than those fed on non-*Bt* leaves (ca: 75 % less) **(Fig. 8)**.

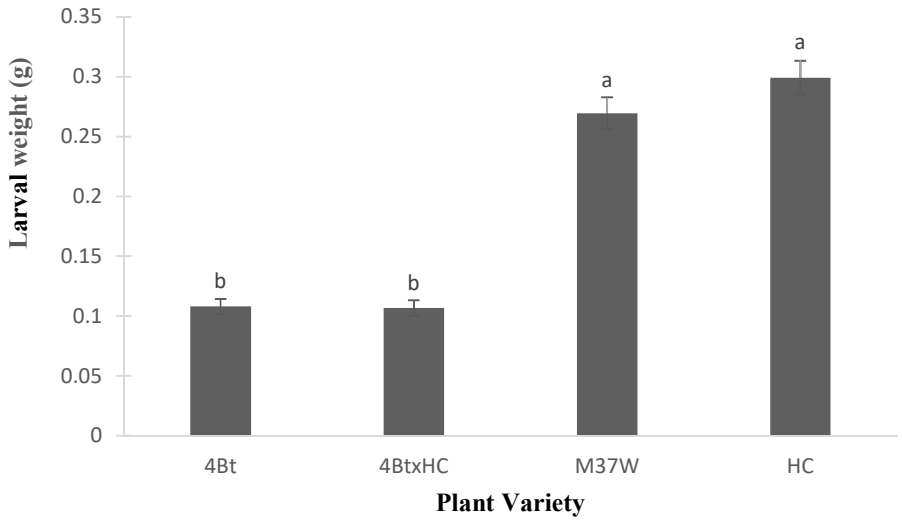


Figure 8: Weight (in grams) of *M. unipuncta* larvae fed for 2-weeks on M37W, HC, 4Bt and 4BtxHC plant leaves. Different letters indicate statistically significant differences ($P \leq 0.05$). Bars represent Standard Error of the Mean (SEM).

We measured the degree of feeding 3 days after the beginning of the experiment and found that larvae consumed a statistically significant higher amount of M37W leaves compared to the other varieties. They consumed a statistically significant higher amount of M37W than 4Bt (~40% increase), 4BtxHC (~55% increase) or HC (~30% increase) leaf area. Larvae ate a statistically significant higher amount of HC than 4BtxHC leaves (~40% increase) **(Figs.9** and **10)**. At 5 days, a statistically significant higher degree of feeding was measured for M37W-fed larvae compared to HC- (~30% higher) or 4BtxHC- (~50% higher) fed larvae. No statistically significant differences were found between feeding on 4Bt and 4BtxHC **(Fig. 9).**

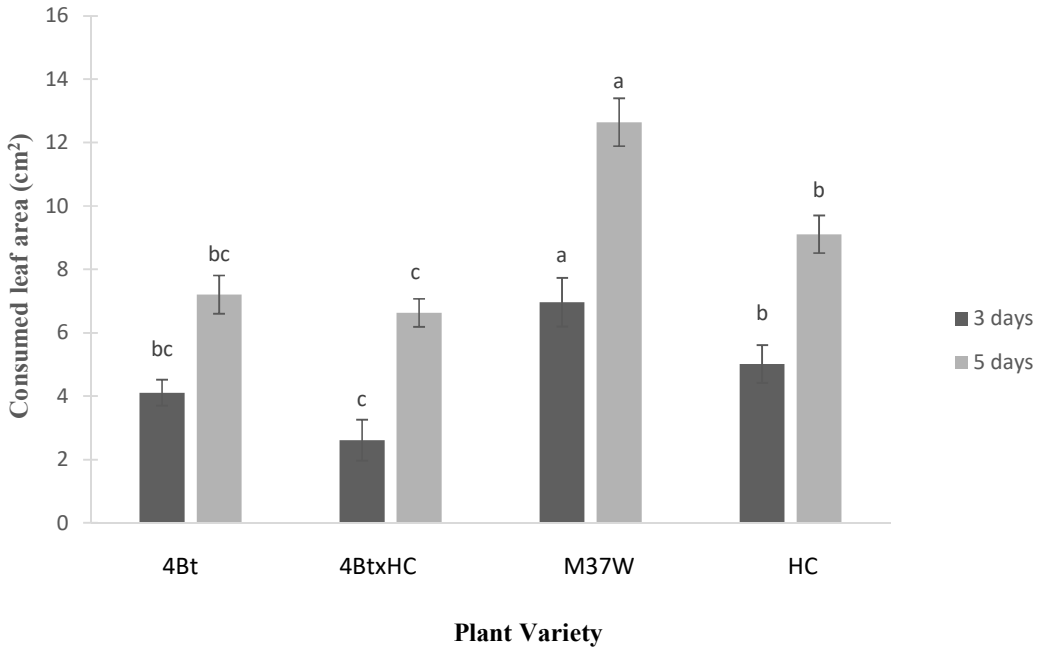


Figure 9: Consumed leaf area (in cm^2) by *M. unipuncta* larvae fed on 4Bt, 4BtxHC, M37W and HC plants, three and five days after the beginning of the experiment. Different letters indicate statistically significant differences between the varieties ($P \leq 0.05$). Bars represent Standard Error of the Mean (SEM).

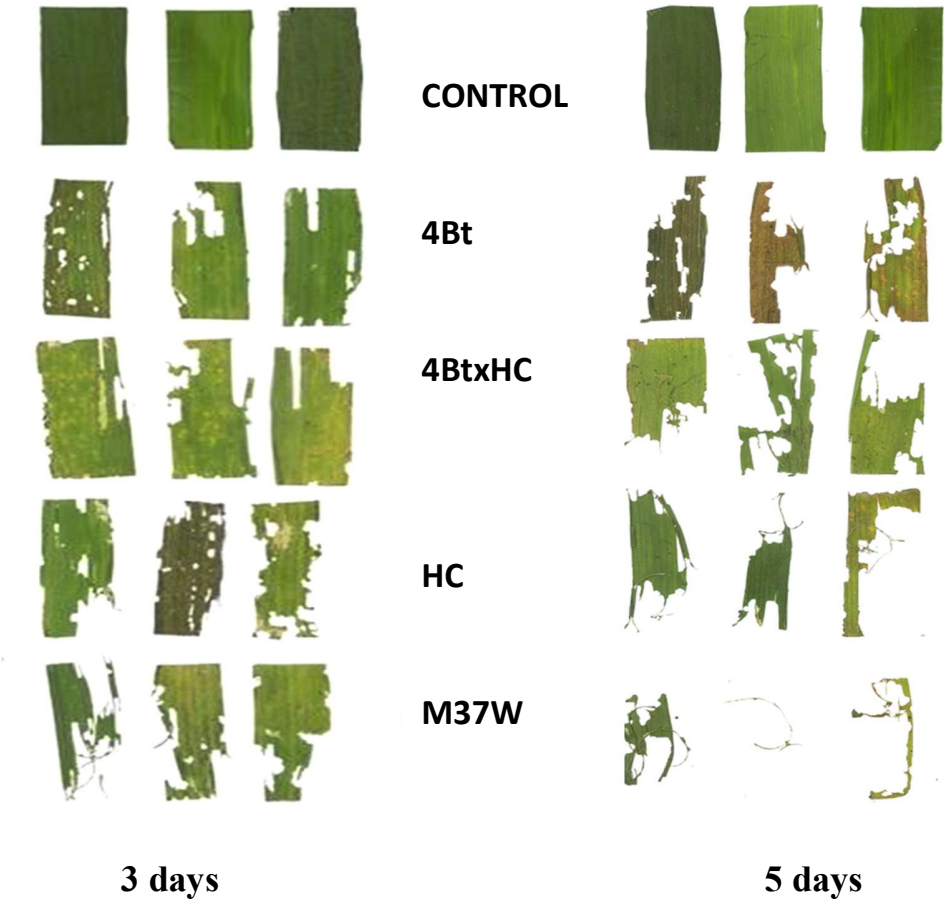


Figure 10: Feeding behavior of *M. unipuncta* larvae on leaves of two *Bt* (4Bt and 4BtxHC) and two non-*Bt* (M37W, HC) maize lines three and five days after the beginning of the experiment. In the upper row three leaves before the beginning of the feeding experiment are shown as leaf area control (CONTROL).

4.4.4 Study 2- Efficacy of *Bt cry1ac* in grains of a high carotenoid maize line against L2 larvae of *O. nubilalis* and *M. unipuncta*

4.4.4.1 4Bt grain diet is more toxic than 4BtxHC against L2 larvae of *O. nubilalis*

Mortality of *O. nubilalis* larvae fed on diets supplemented with grains of the 4 plant lines is shown separately for the non-*Bt*- and the *Bt*-containing diets to emphasize important differences between the mortality caused by diets supplemented with 4Bt and 4BtxHC grains. L2 larvae had a mortality rate (8 weeks after the beginning of the experiment) of ~8% for M37W and HC fed larvae **(Fig. 11)** probably due to manipulation or other factors.

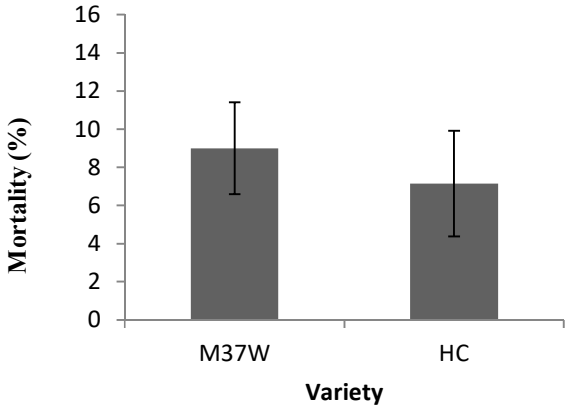


Figure 11: Mortality (in %) of *O. nubilalis* larvae fed on diet prepared with grains from M37W and HC plants, 8 weeks after the beginning of the experiment. Bars represent Standard Error of the Mean (SEM).

A significantly higher mortality rate was measured for larvae fed on *Bt*-containing diets, as expected. At two weeks after the beginning of the experiment, ~60% mortality was recorded

for 4Bt and 4BtxHC fed larvae, with no statistically significant differences between the two groups **(Fig. 12a)**. However, from 3 weeks until the end of the experiment we measured a significantly higher mortality rate (~17%) for larvae fed on 4Bt compared to larvae fed on 4BtxHC diet **(Fig. 12 b-d)**.

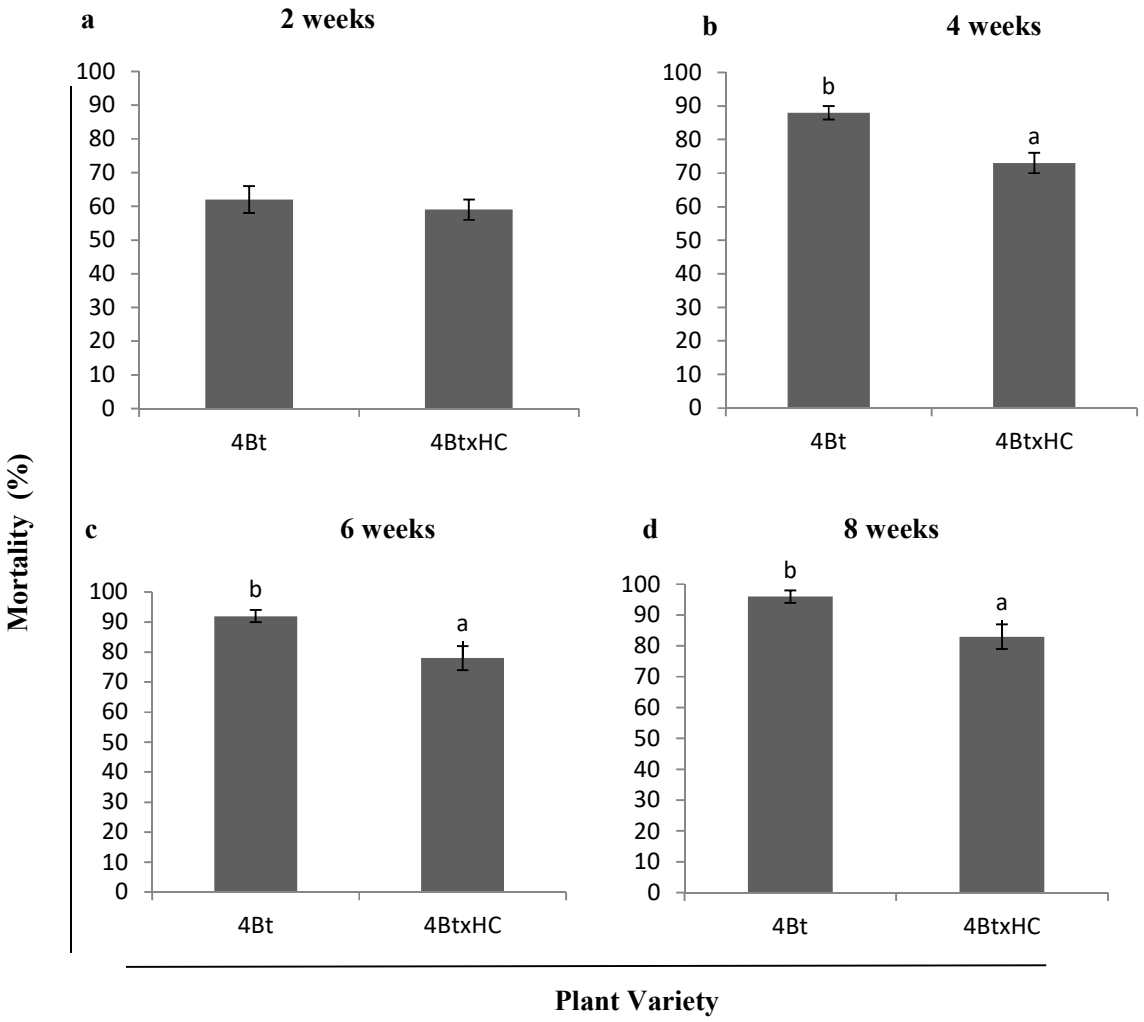


Figure 12: Mortality rate for *O. nubilalis* larvae fed on diets prepared with grains from 4Bt or 4Bt x HC plants at **a)** 2 weeks **b)** 4 weeks **c)** 6 weeks **d)** 8 weeks after the beginning of the experiment. Different letters indicate statistically significant differences between the varieties ($P \leq 0.05$). Bars represent Standard Error of the Mean (SEM).

Within addition to increased survival, 5-week-old larvae fed on the 4BtxHC diet weighted ~60% more compared with those fed on the 4Bt diet **(Fig. 13).**

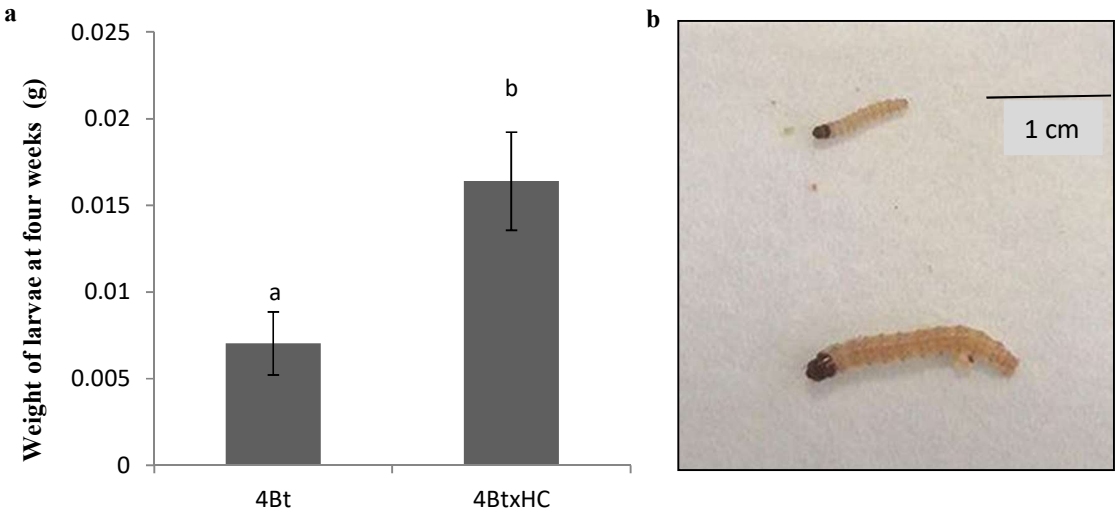


Figure 13: Development of *O. nubilalis* larvae fed for 5 weeks on diets prepared with 4Bt or 4BtxHC grains. **a)** Weight of larvae; **b)** larvae fed on 4Bt (on top) and on 4BtxHC grain diet (on the bottom). Different letters indicate statistically significant differences ($P \leq 0.05$). Bars represent Standard Error of the Mean (SEM).

All surviving larvae from the M37W and HC groups reached pupation, with no statistically significant differences in the pupation rate (~92±1%), pupal weight (855±9 mg) or L2 to-pupation period (18.9±0.07 days) **(Fig. 14).** Larvae fed on 4Bt supplemented diets failed to pupate, while 2% of 4BtxHC fed larvae were able to pupate. Duration of L2 to pupation was significantly extended (more than double) and weight was significantly decreased (~ 60% less) for larvae fed on 4BtxHC compared to non-*Bt* fed larvae **(Fig. 14).**

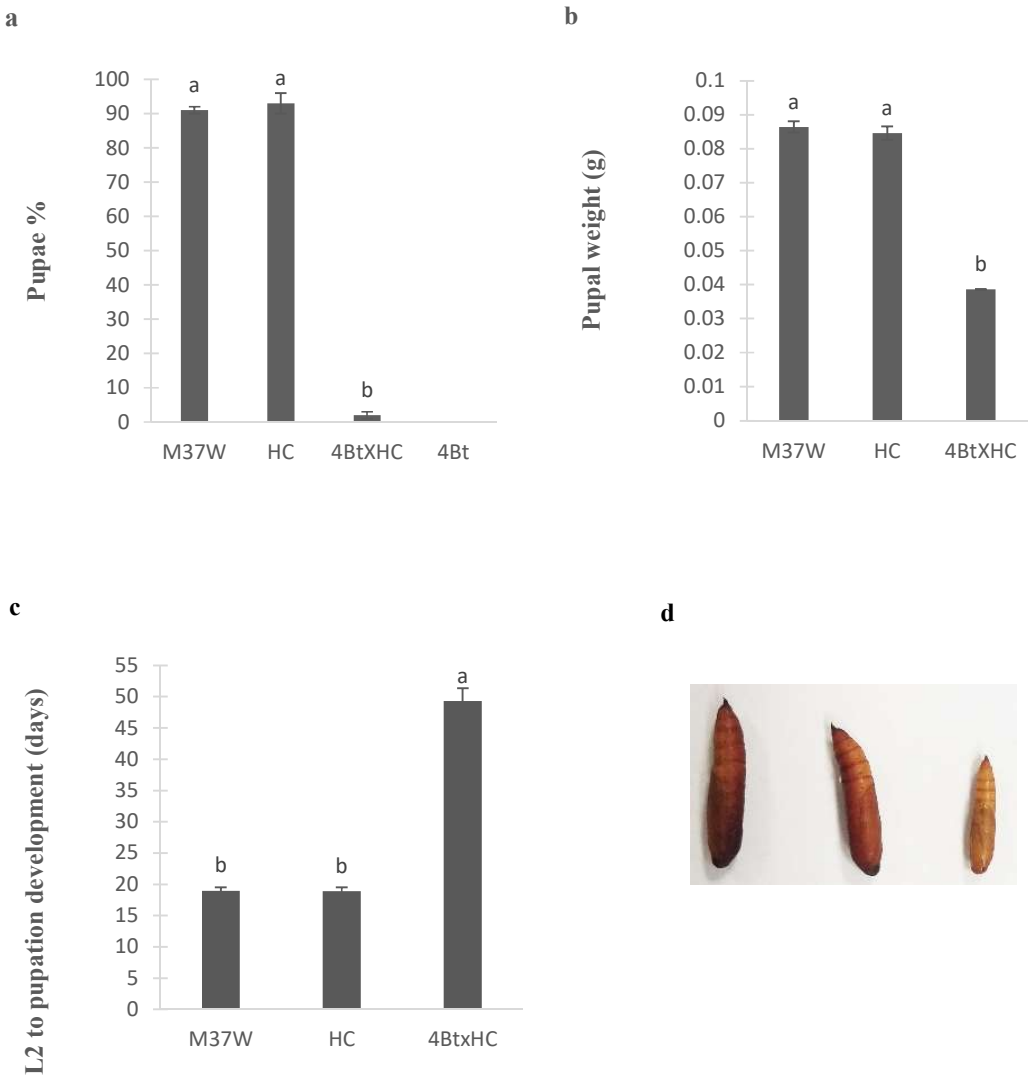


Figure 14: Development of *O.nubilalis* larvae fed on diets supplemented with grains from M37W, HC, 4BtxHC and 4Bt plants. **a)** Percentage of pupation; **b)** pupal weight; **c)** L2 to pupation development (days); d) pupae of larvae fed on M37W, HC and 4BtxHC supplemented diets (from left to right). Different letters indicate statistically significant differences between the varieties ($P \leq 0.05$). Bars represent Standard Error of the Mean (SEM).

4.4.4.2 Mortality rate of *M. unipuncta* L2 larvae caused by grain diets is similar for all 4 lines

We measured a similar mortality rate for *M. unipuncta* larvae reared on the four diets. L2 larvae are very sensitive to manipulation consequently an average mortality of 25±4% was recorded which we attributed to handling manipulations **(Fig.15)**.

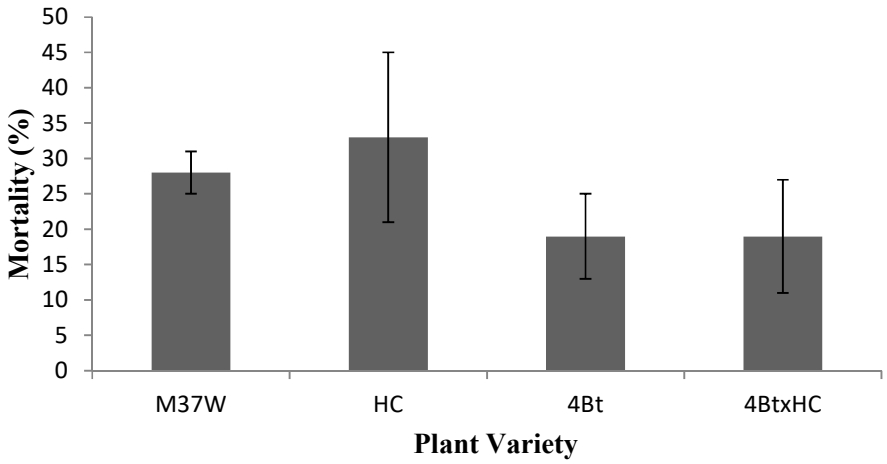


Figure 15: Mortality of *M. unipuncta* L2 larvae fed on M37W, HC, 4Bt and 4BtxHC diets. Different letters indicate statistically significant differences ($P \leq 0.05$). Bars represent Standard Error of the Mean (SEM).

No statistically significant differences were measured in the duration of L2 to pupation **(Fig 16a)** or in pupal weight for larvae fed on the four diets **(Fig 16b)**.

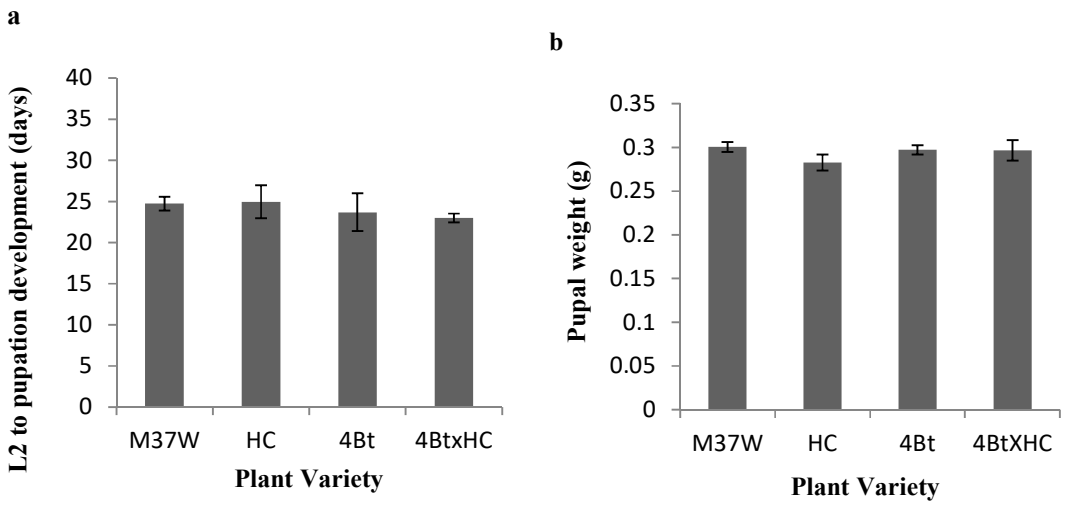


Figure 16: a) L2 to pupation development (days) and **b)** pupal weight of *M. unipuncta*'s larvae fed on M37W, HC, 4Bt and 4BtxHC diets.

4.5 Discussion

4.5.1 Toxicity of Cry1Ac (in 4Bt plants) to four lepidopteran species

Cry1Ac toxin is structurally similar to Cry1Ab, which is efficient in controlling different lepidopteran maize pests, such as the European corn borer, *O. nubilalis* (Comas et al., 2013) and *S. nonagrioides* (González-Núñez et al., 2000). Cry1Ac expressing cotton is effective towards the control of *H. armigera* (Wu et al. 2008), and a recent study reported that a maize line expressing Cry1Ac showed significant efficacy not only against larvae of *H. armigera* but also against *O. furnacalis* (Chen et al., 2016). Wang et al. (2014) reported that a transgenic maize line expressing *cry1Ac* provides good resistance to *O. furnacalis* while the first commercial Bt soybean (MON 87701 × MON 89788) expressing Cry1Ac (recently approved for cultivation in Brazil) is effective against the soybean looper *Chrysodeixis includes* (Yano et al., 2015). In general little is known regarding the efficacy of *cry1Ac* to control insect pests in corn, in particular *O. nubilalis* and *S. nonagrioides*, which are more common in our region of study (Catalonia, Spain). We provide evidence that corn plants expressing *cry1Ac* (4Bt), with a toxin concentration of 50 µg/g FW (fresh weight) are as effective as corn plants that express *cry1Ab* (MON810) in controlling L2 larvae of the target species *O. nubilalis* and *S. nonagrioides*. This is consistent with previous studies reporting that Cry1Ab and Cry1Ac compete with high affinity for the same binding sites in *O. nubilalis* midgut (Li et al., 2004; Hernandez-Rodriguez et al., 2013) and for one binding site in *S. nonagrioides* (González-Cabrera et al., 2006). Furthermore, we measured susceptibility to both Cry1Ac and Cry1Ab toxins for the non-target species *H. armigera*, similarly to what has been recently reported (Chen et al., 2016). *M. unipuncta* was found to be susceptible to Bt toxins, in particular Cry1Ab, to a lower extent compared to the other three species. *Cry1Ab*

containing Bt maize plants have a much lower efficacy in controlling *M. unipuncta* than *O. nubilalis* or *S. nonagrioides* (González-Cabrera et al., 2013; Eizaguirre et al., 2010). L5 larvae of *M. unipuncta* were susceptible to the Bt plants (both 4Bt and MON810) with a mortality of ca: 90% and a reduced weight of surviving pupae compared to those fed on non-Bt leaves (40% less weight). It is extremely important to study the effect of Bt maize on secondary pests, in particular *M. unipuncta*, since L5 larvae can migrate from other crops and grassy areas to corn fields during the growing season (Pilcher et al., 1997) and this behaviour might cause substantial damage if plants are not protected against L5 *M. unipuncta* larvae.

4.5.2 Toxicity of a transgenic *Bt* plant enriched with carotenoids (4BtxHC): leaf feed

The carotenoid pathway introgressed into 4BtxHC did not influence leaf toxicity against *O. nubilalis* which displayed a similar mortality rate when fed on 4Bt or 4BtxHC plants. However, we measured a significantly different mortality between M37W and HC groups at 5 days after the beginning of the experiment. After 3 days of feeding, larvae consumed a significantly higher amount of M37W leaves compared to HC. Nevertheless, this did not result in a higher larval weight (measured 2 weeks after the beginning of the experiment), which was similar between larvae fed on the two varieties. *M. unipuncta* L2 larvae used in this study (performed almost two years later than Study 1) were a different population from the one used in Study 1) since populations are periodically renewed by collecting new individuals from crop fields. 4Bt or 4BtxHC leaves were not effective in killing L2 larvae of *M. unipuncta*, and caused a mortality similar to HC and M37W plants, even though a sub-lethal effect was noted: the weight of 2-week old larval was significantly reduced for larvae fed on Bt corn, with a weight decrease of ~40% compared to non-Bt (both HC and M37W).

Lower weight of larvae influences the weight of the corresponding pupae, which can in turn affect adult fecundity (hypothesis to be tested). It would not be surprising that this more recent population could have acquired some degree of resistance to *Bt* toxins. Pérez-Hedo et al. (2012) reported that Cry1Ab concentration in field plants could represent a "low toxin dose" for *M. unipuncta*, which is able to survive and complete its development on Bt corn thus favouring the onset of resistance through two main types of mechanisms: proteolytic activation of protoxin and binding of the active toxin to receptors in larval gut (Ferré and Van Rie, 2002). It is possible that the same mechanisms could have decreased the susceptibility of *M. unipuncta* to Cry1Ac as well. It is also possible that the higher mortality observed for *M. unipuncta* in Study 1) compared to Study 2) is due to episodes of cannibalism. During Study 1) and other experiments in our laboratory, we observed that *M. unipuncta* can exhibit a cannibalistic behavior when two or more larvae are put together in the same container, especially if the provided diet is not ideal for the insect (such as Bt-containing leaves). In Study 2) we therefore individualized larvae, to avoid mortality due to factors other than plant toxicity.

Similarly to what we observed with *Ostrinia*, we measured a statistically significant lower degree of feeding on HC leaves compared to M37W. It has previously been reported that *S. nonagrioides* female adults prefer to lay eggs on M37W plants rather on Carolight^{R} (HC) (Cruz and Eizaguirre, 2015). This was attributed to the fact that insects preferred the odours emitted by M37W compared to HC host plants, the volatile composition of which may change due to the production of carotenoids. Even if carotenoid content in M37W and Carolight^{R} leaves is the same (Ferrio et al., personal communication) Carolight^{R} leaves were less pigmented than M37W ones, with chlorophyll-B concentration being significantly lower

(Ferrio et al., personal communication), suggesting a knock-on effect of carotenoid metabolism in the seeds which affects the leaves and, more specifically, how attractive they are to insect pests. This could explain the lesser degree of feeding for *O. nubilalis* and *M. unipuncta* larvae on Carolight^{R} leaves compared to M37W. It is not to exclude that the difference in feeding behaviour are due to unintended effects of the genetic transformation itself, similar to the pleiotropic effects in transgenic plants found by Saxena and Stotzy (2001). In their study, they reported 33-97% higher lignin content in the stems of various transformed Bt maize varieties compared to their near isogenic lines. This could explain the lesser degree of feeding for *O. nubilalis* larvae on Carolight^{R} leaves compared to M37W ones. Lignin is indeed relatively indigestible and reduces the ability of herbivours to digest plant materials, thus affecting their rates of feeding (Gardner et al., 1999). Further studies are needed to evaluate the actual causes (or combination of) behind the differential feeding behaviour on wild type and HC plants.

4.5.3 Toxicity of a transgenic *Bt* plant enriched with carotenoids (4BtxHC): grain diet

Insect diets prepared with grains from Bt varieties (4Bt and 4BtxHC) had a substantially lower toxin content compared to the corresponding leaf diet. L2 *O. nubilalis* larvae fed on M37W and HC grain diets showed minimal mortality (~8%) which was probably due to handling, young larval age (L2) and/or other causes. A significantly higher mortality was measured for larvae fed on Bt grain diet (both 4Bt and 4BtxHC) compared to non-Bt, with 60% of dead larvae two weeks after the beginning of the experiment, with no statistically significant difference between the two Bt groups. In the case of leaf diet, mortality had already reached more than 60% after just 4 days of feeding on 4Bt or 4BtxHC plants, due to

the higher toxin concentrations in these plants. From 4 weeks of feeding until the end of the experiment, *O. nubilalis* larvae fed on 4BtxHC grain diet showed a higher degree of survival (~17%) compared to the ones fed on the 4Bt grain diet. An important impact of nutrition in insect susceptibility to Bt toxins has been reported (Deans et al., 2016). In particular, an antagonistic effect between antioxidants and Bt toxins has been observed in more than on study (Broderick et al., 2010; Li et al., 2015). Broderick et al. suggested that Bt toxins cause larval mortality by three different mechanisms: direct toxaemia, developmental arrest and starvation, and incitation of an overblown innate immune response in the larval gut (Broderick et al., 2010). The permeabilization of the larval gut epithelium after Bt ingestion allows the enteric microbial community to reach the hemocoel, where enteric bacteria incite the onset of innate immune response which will finally result in sepsis and larval death (Broderick et al., 2006). A dramatic decrease in susceptibility to Bt toxin was measured when larvae of different lepidopteran species were treated with antibiotics before feeding on Bt toxins. Susceptibility was restored by subsequent feeding of larvae with *Enterobacter* sp., a Gram negative bacterium normally found in the gut of many lepidopteran species (Broderick et al., 2009). Antioxidants are thought to suppress immune response by interacting with reactive nitrogen and oxygen species, which play a role in the incitation of the immune response. This might explain the dose-dependent extended larval survival that Broderick et al. reported when they exposed larvae of gypsy moth (*Lymantria dispar dispar*) to Bt toxin together with different antioxidant compounds versus the Bt toxin alone (Broderick et al., 2010). A similar antagonism between a flavonoid (quercetin) and Cry1Ac toxin was reported in neonate and third instar larvae of *H. armigera* (Li et al., 2015). A decrease in the severity of Cry1Ac effect on larval weight, larval development time, pupation success and pupal weight was reported when quercetin was introduced at various concentrations into the diet

together with the Cry1Ac toxin (Li et al., 2015). Similarly, together with increased survival, we also measured a higher larval weight (more than double) and pupation success (2% for 4BtxHC *vs* 0% for 4Bt) for *O. nubilalis* larvae fed on 4BtxHC grain diet compared to the 4Bt alone. Larvae fed on HC or M37W grain diet had a similar percentage of pupation, pupal weight and duration of the development from L2 to pupation. All parameters differed significantly for larvae fed on the 4BtxHC and the 4Bt diets. Cry1Ac delayed pupation of L2 larvae, caused a weight pupal loss and decreased pupation success in larvae fed on 4BtxHC compared to the non-Bt grain diet.

Toxin concentration in the grain diets was probably too low to exert any effect on *M. unipuncta* L2 larvae fed on the 4Bt or the 4BxHC diets, which had very similar survival and development as larvae fed on non-Bt diets. Thus the four plant varieties resulted in similar mortality, pupal weight and duration of the development from L2 to pupae.

In conclusion, I demonstrated that Cry1Ac toxin at a concentration of 50 µg/g FW is useful in the control of the Bt-target species *O. nubilalis* and *S. nonagrioides*. A certain degree of susceptibility to both Cry1Ab and Cry1Ac toxins has also been shown for the secondary pests *H. armigera* and *M. unipuncta*. I also bring evidences that the high carotenoid content in Carolight^{R} can influence insect susceptibility to Bt toxins, both by making plant leaves less attractive to feeding and by increasing larval survival if the Bt toxin is present at a low concentration (3 µg/g FW) in the kernels. Further studies are needed to confirm these results, especially since very little is known about the interaction between Bt toxins and carotenoids in transgenic Bt plants.

4.6 References

- Angus, T. A. (1956). Association of toxicity with protein crystalline inclusions of *Bacillus* sotto *Ishiwata*. *Can. J. Microbiol*. **2**: 122-131.
- Berliner, E. (1911). Uber de schlaffsucht der Mehlmottenraupe. *Zeitschrift fur das Gesamstadt*. **252**:3160-3162.
- Broderick, N.A., Raffa, K.F., Handelsman, J. (2006). Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *Proc Natl Acad Sci USA*. **103**:15196-15199.
- Broderick, N.A., Robinson, C.J., McMahon, M.D., Holt, J., Handelsman, J., Raffa, K.F. (2009). Contributions of gut bacteria to Bacillus thuringiensis-induced mortality vary across a range of Lepidoptera. *BMC Biology*. **9**:7-11.
- Broderick, N.A., Raffa, K.F., Handelsman, J. (2010). Chemical modulators of the innate immune response alter gypsy moth larval susceptibility to *Bacillus thuringiensis*. *BMC Microbiol*. **10**:129.
- Caceres, L. A. Lakshminarayan, S., Yeung, K.K., McGarvey, B.D., Hannoufa, A, Sumarah, M.W., Benitez, X., Scott, I.M. (2016). Repellent and Attractive Effects of alpha-, beta-, and Dihydro-beta- Ionone to Generalist and Specialist Herbivores. *J Chem Ecol.* **42:**107-117.
- Chen, H.X., Yang, R., Yang, W., Zhang, L., Camara, I., Dong, X.H., Liu, Y., Shi, W.P. (2016). Efficacy of Bt maize producing the Cry1Ac protein against two important pests of corn in China. *Environ Sci Pollut Res Int*. doi: 10.1007/s11356-016-7340-7.

- Christensen, A.H. & Quail, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res*. **5**:213–8.
- Christou, P., Ford, T. & Kofron, M. (1991). Production of transgenic rice *(Oryza sativa*L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Nat Biotechnol.***9:**957-962.
- Comas, J., Lumbierres, B., Pons, X., Albajes, R. (2013). Ex-ante determination of the capacity of field tests to detect effects of genetically modified corn on non-target arthropods. *J Econ Entomol*. **106**:1659-1668.
- Cruz, D. & Eizaguirre, M. (2015). Do *S. nonagrioides* (Lepidoptera; Noctuidae) Gravid Females Discriminate between *Bt* or Multivitamin Corn Varieties? Role of Olfactory and Visual Cues. *J Insect Sci.* doi: 10.1093/jisesa/iev018.
- Deans, C.A., Sword, G.A. & Behmer, S.T. (2016). Nutrition as a neglected factor in insect herbivore susceptibility to *Bt* toxins. *Current Opinion in Insect Science*. **15**:97-103.
- Edwards, K., Johnstone, C. & Thompson, C. A (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res*. **19**:1349.
- Eizaguirre, M., Madeira, F. & Lopez, C. (2010). Effects of *Bt* maize on non-target lepidopteran pests. *IOBC/WPRS Bull*. **52**:49–55.
- Ferré, J. & Van Rie, J. (2002). Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annu. Rev. Entomol*. **47**:501-533.

- Gardner, P.T., Wood, T.J., Chesson, A., Stuchbury, T. (1999). Effect of degradation on the porosity and surface area of forage cell walls of differing lignin content. *J Sci Food Agr*. **79**:11-18.
- Gonzalez, J. M., Brown, B. J. & Carlton, B. C. (1982). Transfer of *Bacillus thuringiensis* plasmids coding for δ- endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proc. Natl. Acad. Sci. USA* **79**:6951-6955.
- González-Cabrera, J., Farinós, G.P., Caccia, S., Díaz-Mendoza, M., Castañera, P., Leonardi, M.G. (2006). Toxicity and mode of action of *Bacillus thuringiensis* Cry proteins in the Mediterranean corn borer, *Sesamia nonagrioides* (Lefebvre). *Appl Environ Microbiol.* **72**:2594-2600.
- González-Cabrera, J., García, M., Hernández-Crespo, P., Farinós, G.P., Ortego, F., Castañera, P. (2013). Resistance to *Bt* maize in *M. unipuncta* (Lepidoptera: Noctuidae) is mediated by alteration in Cry1Ab protein activation. *Insect Biochem Molec*. **43**:635-643.
- González-Núñez, M., Ortego, F., Castañera, P. (2000). Susceptibility of Spanish populations of the corn borers *Sesamia nonagrioides* (Lepidoptera: Noctuidae) and *Ostrinia nubilalis* (Lepidoptera: Crambidae) to a *Bacillus thuringiensis* endotoxin. *J Econ Entomol*. **93**:459-463.
- Hernandez-Rodriguez, C., Hernandez-Martinez, P., Van Rie, J., Escriche, B. & Ferre, J. (2013). Shared midgut binding sites for Cry1A. 105, Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa proteins from *Bacillus thuringiensis* in two important corn pests, *Ostrinia nubilalis* and *Spodoptera frugiperda*. PLoS One 8, e0068164.

- Husz B. (1930). Field experiments on the application of *Bacillus thuringiensis* against the corn borer. Int. *Corn Borer Invest*. *Sci. Rep*. **3**:91-98.
- Lambert, B. & Peferoen, M. (1992). Insecticidal promise of *Bacillus thuringiensis*. Facts and mysteries about a successful biopesticide. *BioScience.* **42**:112-122.
- Li, H., Gonzalez-Cabrera, J., Oppert, B., Ferre, J., Higgins, R.A., Buschman, L.L., Radke, G.A., Zhu, K.Y., Huang, F. (2004). Binding analyses of Cry1Ab and Cry1Ac with membrane vesicles from *Bacillus thuringiensis*-resistant and -susceptible *Ostrinia nubilalis*. *Biochem. Biophys. Res. Commun*. **323**:52–57.
- Li, Z., Guan, X., Michaud, J. P., Zhanga, Q., Liua, X. (2016). Quercetin interacts with Cry1Ac protein to affect larval growth and survival of *H. armigera. Pest Manag Sci.* **72**:1359-1365.
- Naqvi, S., Zhu, C., Farre, G., Ramessar, K., Bassie, L., Breitenbach, J. (2009). Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proc Natl Acad Sci U S A*. **106**:7762-7767.
- Obrist, L.B., Dutton, A., Albajes, R., Bigler, F. (2006). Exposure of arthropod predators to Cry1Ab toxin in *Bt* corn fields. *Ecol Entomol*. **31**:143-154.
- Peter, W. & Russell, R. (2001). Carotenoid-derived aroma compounds: an introduction. *American Chemical Society*. **1**:1-17.
- Pilcher, C.D., Rice, M.E., Obrycki, J.J., Lewis, L.C. (1997) Field and laboratory evalua-tions of transgenic Bacillus thuringiensis corn on secondary lepidopteran pests (Lepidoptera: Noctuidae). *J. Econ. Entomol*. **90**:669-678.

- Sambrook, J., Fritsch & E.F., Maniatis, T. (1989). Molecular cloning: a laboratory manual (Second ed). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, US.
- Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., Christou, P. (2011). *Bacillus thuringensis*: a century of research, development and commercial applications. *Plant Biotechnol. J.* **9**:283-300.
- Saxena, D., & Stotzky, G. (2001). *Bt* corn has a higher lignin content than non-*Bt* corn. *Am. J. Bot.* **88**:1704-1706.
- Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev.* **62**:775-806.
- Vogel, J.T., Tan, B., Mccarty, D.R. & Klee, H.J. (2008). The carotenoid cleavage dioxygenase 1 enzyme has broad substrate specificity, cleaving multiple carotenoids at two different bond positions. *J Biol Chem*. **283**:11364-11373.
- Wu, K. Lu, Y.H., Feng, H.Q., Jiang, Y.Y., Zhao, J.Z. (2008). Suppression of cotton bollworm in multiple crops in China in areas with *Bt* toxin-containing cotton. *Science*. **321**:1676-1678.
- Yano, S.A.C., Specht, A., Moscardi, F., Carvalho, R.A., Dourado, P.M., Martinelli, S., Head, G.P., Sosa-Gómez, D.R. (2016). High susceptibility and low resistance allele frequency of *Chrysodeixis includens* (Lepidoptera: Noctuidae) field populations to Cry1Ac in Brazil. *Pest Manag Sci*. **72**:1578-84.

- Wang, Y.Q., He, K.L.; Fan. J, Wang, Y.D., Zhang, T.T., Wang, Z.Y., Bai, S.X. (2014). Resistance of transgenic *Bt* corn variety BT799 to the Asian corn borer. *Ying Yong Kun Chong Xue Bao*. **51**:636-642.
- Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P., Capell, T. (2008). Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in corn. *Proc Natl Acad Sci U S A*. **105**:18232-18237.

Chapter 5

FREEDOM-TO-OPERATE ANALYSIS OF A TRANSGENIC MULTIVITAMIN CORN VARIETY

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Chapter 5:

Freedom-to-operate analysis of a transgenic multivitamin corn variety

5.1 Abstract

In this chapter, we explore the intellectual property (IP) landscape relevant to the production and commercialization of our most complete version of Carolight^{R}, a transgenic multivitamin corn variety created on humanitarian grounds to address micronutrient deficiencies in low-and-middle-income countries. The successful production of this variety requires IP rights risk management because there is a strong protection on inventions and processes via patent portfolios in both developing and industrialized countries. The IP framework is complex, and specialist patent lawyers are usually employed to perform such analysis, but the costs cannot always be met by small, publicly funded projects. We report an alternative strategy, a do-it-yourself patent analysis, to produce a review with limited legal value that can nevertheless lay the foundations for a subsequent more in-depth professional freedom-to-operate opinion.

5.2 Introduction

An improved version of the high carotenoid corn Carolight^{R} was created, having three different vitamin metabolic pathways modified simultaneously, which was achieved by transforming a white kernel corn variety with four genes: *Zmpsy1* (corn phytoene synthase 1) and *PacrtI* (bacterial phytoene desaturase) representing the carotenoid pathway, *Osdhar* (rice dehydroascorbate reductase) representing the ascorbate pathway and *EcfolE* (bacterial GTP cyclohydrolase 1) representing the folate pathway. The endosperm thus accumulates 169-fold more β-carotene (pro-vitamin A) as well as other nutritionally relevant carotenoids such as lycopene, lutein and zeaxanthin, six-fold more ascorbate (vitamin C) and double the normal amount of folate, in each case compared with the equivalent non-transgenic variety, M37W (Naqvi et al., 2009; Zhu et al., 2008). Carolight™ is also resistant to lepidopteran insect pests due to the presence of three *Bacillus thuringiensis* (Bt) genes (*cry1Ac*, *cry1Ca* and *vip3Aa*), which makes it both nutritionally and economically beneficial in LMICs. The additional health benefits of multivitamin Carolight™ reflect the accumulation of the two additional vitamins together with the high carotenoid content. Ascorbate (vitamin C) is an enzyme cofactor required for the synthesis of collagen, carnitine, cholesterol and some hormones and is normally acquired by the consumption of fresh fruit and vegetables (Pérez-Massot et al., 2013). This may not be possible in LMICs resulting in higher levels of ulceration diseases such as scurvy. Similarly, folate (vitamin B9) is the source of tetrahydrofolate, an essential molecule for DNA synthesis and many other core metabolic reactions. Folate deficiency causes macrocytic anaemia in adults but is particularly dangerous during pregnancy, where a low intake is associated with neural tube defects such as spina bifida (Houghton et al., 2011). The deployment of Carolight™ in LMICs could therefore

address the high level of preventable diseases, particularly those affecting children and pregnant women, even if it is impossible for the population to access safe and nutritious fruit and vegetables. Carolight™ was developed at the University of Lleida, Spain, on humanitarian grounds, as a vehicle to alleviate micronutrient deficiencies in developing countries. The intention was to transfer a technology package to the Council for Scientific and Industrial Research, Pretoria South Africa (CSIR), and Tamil Nadu Agricultural University Coimbatore, India (TNAU). This technology package would consist of the Carolight™ seed, together with a dossier that included relevant technical know-how and a freedom-to-operate (FTO) analysis covering the intellectual property (IP) landscape surrounding the technology so that the CSIR and TNAU could take the subsequent steps towards addressing any IP management issues that may be relevant should they undertake commercialization and distribution of the technology. The FTO analysis presented here is based on the screening of publically available patent databases and provides a strong foundation from which additional legal expertise can be used to address any IP barriers that may be encountered. Before Carolight™ seed can be introduced into local agricultural systems, any IP issues relevant to the deployment of the seed must be addressed. These IP issues are not confined to developed countries because the Agreement on Trade-Related Aspects of Intellectual Property Rights administered by the World Trade Organisation (WTO) requires all WTO members including LMICs to conform to certain agreed norms of IP protection. This has led to higher protection thresholds for all technologies, including agricultural biotechnology in countries such as South Africa and India. It is therefore necessary to address the challenges of technology transfer from the developed world to LMICs, and part of the challenge involves the analysis of patents and other IP aspects. At a later stage, it may be necessary to obtain negotiated licences so that technology can be

incorporated smoothly into local agricultural practices. Our FTO analysis aimed to identify patents (both granted patents and patent applications) that could affect Carolight™ research, development and commercialization in target countries in order to avoid, to the best of our ability, any infringement of existing IP rights. Mapping the IP landscape relevant to Carolight™ also helps partners involved in the growth and distribution of this variety, as well as other varieties of biofortified maize. The FTO review can also form a sound basis for a forward-looking IP strategy, especially if carried out at the early stages of development. The FTO analysis should ideally be updated at different stages of product development to accommodate both internal and external developments. An FTO review in itself has limited legal value, but serves as an important preliminary basis for a more formal FTO Opinion, the latter usually provided by legally trained professionals. The pro-active FTO review/analysis described herein was conducted by scientists and one paralegal IP expert, providing a cost-effective foundation for a subsequent professional FTO Opinion. Intellectual property landscapes are evolving continuously. According to the World Intellectual Property Organization (WIPO), nearly 100,000 biotechnology and pharmaceutical patent applications are filed worldwide every year, and the trend appears to be increasing. Furthermore, patents expire, lapse or are withdrawn, sold or licensed, or their validity and scope can be altered by legal challenges. Patent applications are typically published 18 months after filing, so many new applications may enter the system during the initial FTO analysis and regular updating is necessary. Unlike an FTO Opinion, this FTO review takes into account only the literal interpretation of the specification and claims. It does not attempt to consider legal interpretations, for example the validity of claims, or the potential strength or weakness of a patent. Even granted patents can be invalidated if challenged, and as part of the FTO analysis, we documented events such as actual or calculated expirations, lapses, invalidations,

revocations and other legal events where known. However, unlike an FTO Opinion, our analysis reports the data as presented and does not offer any analysis of the strength or weakness of patents and their claims. Even so, such an FTO review provides a comprehensive basis to guide commercial decisions. Another consideration is that the patent landscape pertaining to a particular technology can vary among countries due to the territorial nature of IP. A technology patented in one country may not be protected in another. In LMICs, it is often difficult to find comprehensive patent information even in commercial databases, and the information accessible to the public can therefore be severely limited.

5.3 Methods

The Carolight™ product had to be deconstructed into individual components, including materials such as genetic elements, and processes such as genetic transformation (Kowalski and Kryder, 2002; Kryder et al., 2000). Searches were carried out to reveal patents with claims relevant to each of the components or combinations of components. We documented all the components by analysing publications, nucleotide sequences and laboratory notebooks, and by conducting interviews with the scientists involved in the invention process. Patent searches were carried out using online databases such as WIPO's Patentscope (http://patentscope.WIPO.int/search/en/search.jsf), EPO's Espacenet (http://worldwide.espacenet.com/?locale=en_EP) and Cambia's The Lens (http://www.lens.org/lens/) as well as PatSeq Finder (Cambia, https://www.lens.org/lens/bio/sequence), which covers claimed nucleotide and protein sequences.

Combinations of International Patent Codes, search words, Boolean operators and wild cards were used to find relevant applications. Different strategies were tested to ensure maximum coverage, such as searching not only in the title and/or the abstract but also widening the search to the claims and the description (Miralpeix et al., 2014). A strictly literal interpretation of the claims was applied to select potentially relevant patents that may constrain the commercial deployment of Carolight™. We also documented the legal status or grant status of patents, and geographical coverage by patent families where such data were available.

Many of the materials used in the development of Carolight™ are also likely to be classed as technical property, the use of which may be restricted by contracts such as material transfer agreements (MTAs). Such materials may include, for example, vectors or their genetic elements. Materials are often exchanged informally between researchers despite the existence of MTAs that may restrict such downstream transfers. To the best of our ability, we sought to audit such transfers where possible **(Figure 1).**

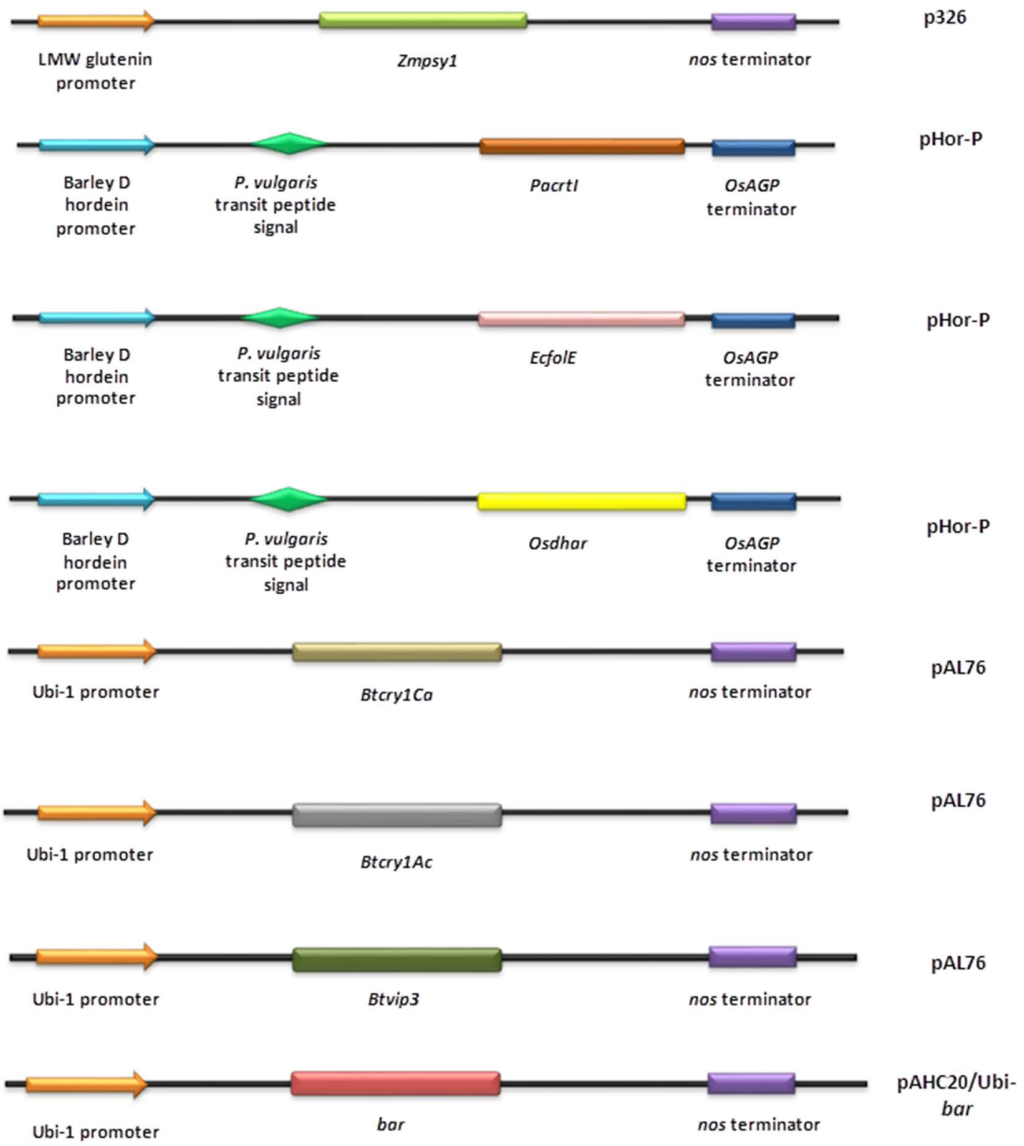

Figure 1: Composite DNA constructs (plasmids) and their essential elements used to generate the Carolight™ seed product. *Promoters/terminators*: wheat LMW glutenin promoter; barley D-hordein promoter; *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase; corn Ubi-1 promoter; nopaline synthase terminator; rice ADP-glucose pyrophosphorylase terminator. *Genes*: *Zmpsy1*, *Zea mays* phytoene synthase 1; *PacrtI*, *Pantoea ananatis* phytoene desaturase; *Osdhar*, *Oryza sativa* dehydroascorbate reductase; *EcfolE*, *Escherichia coli GTP cyclohydrolase I*; *Btcry1Ca*, *Bacillus thuringiensis*

gene codingCry1Ca crystal endotoxin; *Btcry1Ac*, *B. thuringiensis* gene coding Cry1Ac crystal endotoxin; *Btvip3*, *B. thuringiensis* gene coding Vip3 vegetative insecticidal protein; *bar,* gene from *Streptomyces hygroscopicus* confering resistance to the herbicide bialaphos. Ubi-1 promoter is followed by intron 1 of maize Ubi-1 gene in all constructs.

The elements identified through the deconstruction process were subdivided into four main categories: (i) plants and seeds; (ii) cloning vectors; (iii) genetic elements including genes, promoters and terminators; and (iv) transformation, plant regeneration and other processes **(Table 1)**.

Elements	Components	Sub-components
Plant/seed source	South African elite inbred variety M37W seeds were directly obtained from South Africa (CSIR, Pretoria, S Africa)	
Gene constructs and their individual elements	pGEM®-T easy (Promega) used for subcloning	
	plasmid p326 (for *Zmpsy1* gene)	- LMW glutenin promoter
		- Nopaline synthase terminator region (nos) terminator
	plasmid pHor-P (for *crtI*, *folE* and *dhar* genes)	- Transit peptide signal from *Phaseolus vulgaris*
		- *Oryza sativa* ADP glucose phosphorylase terminator
	plasmid pAL76 (for *cry1C*, *vip3* and *cry1Ac* genes)	- Ubiquitin fusion protein-1 promoter
		- Nopaline synthase terminator region (nos) terminator
	pIA2	- Ubiquitin fusion protein-1 promoter
		- Nopaline synthase terminator region (nos) terminator
	pAHC20/Ubi-bar	- Ubiquitin fusion protein-1 promoter
		- *bar* gene
		- Nopaline synthase terminator region (nos) terminator
Processes	Vector cloning	
	Combinatorial genetic transformation	
	Particle bombardment	
	Plant regeneration and selection	

Table 1: Elements identified through the product deconstruction process

Although DNA amplification steps were used during construct assembly, it is clear that the manufacturers of DNA polymerases do not claim 'reach through' licences that impede downstream work; thus, patents for polymerases and associated amplification processes were excluded from the FTO review. A schematic diagram of the methodology used in the FTO analysis of Carolight™ is shown in **Figure 2**.

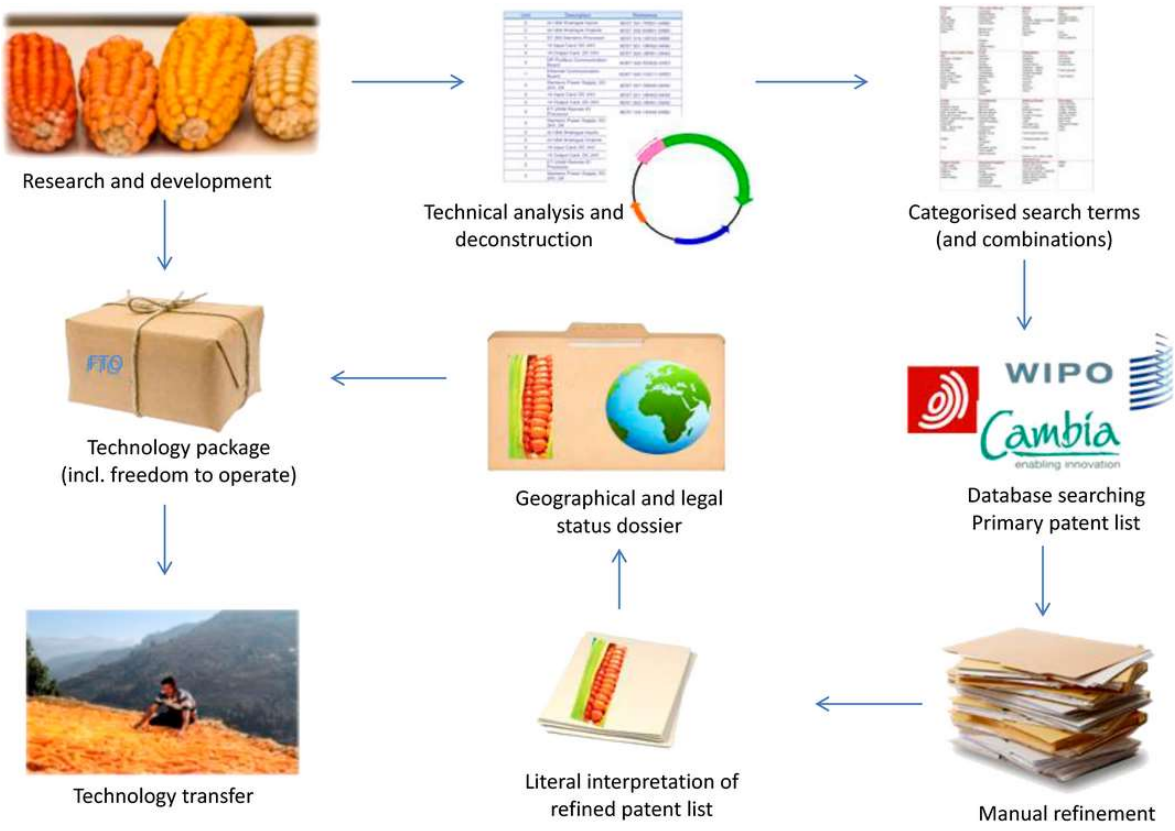

Figure 2: Freedom-to-operate (FTO) review strategy for Carolight™.

5.4 Results

5.4.1 Overview

Due to the complex nature of the invention and the large number of genes introduced, we initially identified 36 patents or patent families that potentially affect our FTO (**Tables 2** and **3**). This initial group was identified based on content and therefore was not screened to determine whether the patents had expired or whether the protection had been sought or granted in Europe/Spain (place of development) or South Africa/India (principal places of intended deployment).

Component	Patent document number (one of a patent family where applicable)	Patent family members in other countries	Legal status (where data is available) (Updated on 17.03.15)	Applicants	Priority date	Calculated expiry date
1. Plant material M37W seeds Seed were obtained from Southafrica (CSIR, Pretoria, S Africa)						
2. Gene construct ○ pGEM®-T easy(Promega)	US4766072 Vectors for in vitro production of RNA copies of either strand of a cloned DNA sequence	US		Promega Corp. US	17.07.1985	17.07.2005
○ Promoters for seed specific expression	AU612326 Seed Specific Transcriptional Regulation	AU, CN, DE, EP, ES, IN, NZ, US ES2054676 IN170125	Lapse because of expiration of protection	Calgene Inc. US	31.07.1986	31.07.2006
Promoters for seed specific expression	EP0781849 Method employing tissue specific promoter	AT, AU, CA, DE, DK, EP, ES, JP, US, WO ES2293649	Lapse because of non-payment of due fees	Sapporo Breweries JP	05.07.1995	05.07.2015
Promoters for seed specific expression	WO1999014314 Regulation of gene expression in plants	AT, AU , CA, ES, EP, JP, NZ, US, WO ES2321248	Lapsed in Spain on 08/10/2014	Commonwealth Scientific and Industrial Research Organisation AU	12.09.1997	12.09.2017

				The Australian National University AU Goodman Fielder Ltd AU Groupe Limagrain Pacific Pty Ltd. AU		
	EP1019517 Production of proteins in plant seeds	AT, AU, CA, DE, EP, ES, JP, US, WO ES2276475	Granted in ES	University of California US	30.09.1997	30.09.2017
○ Ubiquitin fusion protein-1 promoter	EP1210446 Modified Ubiquitin Regulatory System	AT, AU, CA, CZ, DE, DK, EP, ES, HU, PL, PT, US, WO, ZA ES2265978 ZA200201758	Granted in ES and ZA (Year of Fee Payment: 15)	Monsanto Ltd UK	09.09.1999	09.09.2019
Ubiquitin fusion protein-1 promoter	**US5510474** Plant ubiquitin promoter system	AT, CA, DE, EP, ES, JP, US ES2060765	Granted in Spain 01.12.1994	Mycogen Plant Science Inc. US	17.05.1988	17.05.2008
Ubiquitin fusion protein-1 promoter	WO0194394 Novel Plant Promoter Sequences And Methods Of Use For Same	AU, US, WO	EP: PCT application did not enter european phase	Prodigene Inc. Us	09.06.2000	09.06.2020
○ Nopaline synthase terminator region (*nos* terminator)	None found					
○ Transit peptide signal from	None found					

Phaseolus vulgaris						
○ Oryza sativa ADP glucose phosphorylase terminator	None found					
○ *Zmpsy1* gene	EP1002117 Methods for producing carotenoid compounds and specialty oils in plant seeds	AR, AU, CA, CN, EP, IN, JP, US, WO IN189320	EP application deemed to be withdrawn in 12.08.2003	Calgene Inc. US	08.08.1997	08.08.2017
Zmpsy1 gene	WO2001088169 Method for producing carotenoid compounds in plants	AR, AU, US, WO	EP: PCT application did not enter european phase	Monsanto Technology LLC US	12.05.2000	12.05.2020
Zmpsy1 gene	EP1608759 Enhanced accumulation of carotenoids in plants	AT, AU, CA, DE, EP, ES, JP, WO ES2298733	Granted in ES	Syngenta Ltd GB	24.03.2003	24.03.2023
Zmpsy1 gene	WO2014070646 Transformed plants having increased beta-carotene levels, increased half-life and bioavailability and methods of	US, WO	EP: the EPO has been informed by wipo that ep was designated in this application Recent patent	Pioneer Hi-Bred International Inc. US	31.10.2012	31.10.2032
Zmpsy1 gene	WO9506128 Fertile, transgenic maize plants and	AR, AU, BR, CA, EP, HU, IL, JP, US, ZA ZA9700399	EP application withdrawn in 11/05/2011 Granted in ZA	DeKalb Genetics Corp. US	25.08.1993	25.08.2013

	methods for their production					
Zmpsy1 gene	EP1159428 Method for improving the agronomic and nutritional value of plants	AR, AT, AU, BR, CA, CN, DE, EA, ES, EP, HU, ID, JP, MX, PL, US, WO, ZA ES2269110 ZA200106948	Granted in ES and ZA	Greenovation Pflanzenbiotechnologie GmbH DE Syngenta Participations AG.	05.03.1999	05.03.2019
Zmpsy1 gene	EP0471056 Biosynthesis Of Carotenoids In Genetically Engineered Hosts	CA, DE, DK, EP, JP, US, WO	Patent expired after termination of 20 years	Amoco Corp. US	19.02.1992	19.02.2012
Zmpsy1 gene	WO1999055888 Carotenoid biosynthesis enzymes	AR, AU, BR, EP, US, WO	EP application withdrawn in 16/03/2005	E. I. du Pont de Nemours and Company US	04.11.1999	04.11.2019
○ *PacrtI* gene	EP1608759 Enhanced accumulation of carotenoids in plants	AT, AU, CA, DE, EP, ES, JP, US, WO ES2298733	Granted in ES	Syngenta Ltd GB	24.03.2003	24.03.2023
PacrtI gene	WO2014070646 Transformed plants having increased beta-carotene levels, increased half-life and bioavailability and methods of	US, WO	EP: the EPO has been informed by wipo that ep was designated in this application Recent patent	Pioneer Hi-Bred International Inc. US	31.10.2012	31.10.2032
PacrtI gene	WO9506128 Fertile, transgenic maize plants and	AR, AU, BR, CA, EP, HU, IL, JP, US, ZA	EP application withdrawn in 20/07/2011	DeKalb Genetics Corp. US	25.08.1993	25.08.2013

	methods for their production	ZA9700399	Granted in ZA			
PacrtI gene	EP1159428 Method for improving the agronomic and nutritional value of plants	AR, AT, AU, BR, CA, CN, DE, EA, ES, EP, HU, ID, JP, MX, PL, US, WO, ZA ES2269110 ZA200106948	Granted in ES and ZA	Greenovation Pflanzenbiotechnologie GmbH DE Syngenta Partecipations AG	05.03.1999	05.03.2019
PacrtI gene	WO1991013078 Biosynthesis Of Carotenoids In Genetically Engineered Hosts	CA, DE, DK, EP, JP, US, WO	EP patent granted	Amoco Corp. US	02.03.1990	02.03.2010
○ *EcfolE* gene	WO2006034501 Materials and methods for folate biofortification in plants	US, WO	PCT application did not enter european phase	University of Florida US	23.09.2004	23.09.2024
EcfolE gene	WO2009000733 Fortification of plants with folates by metabolic engineering	CN, EP, US, WO	Entry into national phase in US in 16/12/2009	Universiteit Gent BE	22.06.2007	22.06.2027
EcfolE gene	US20100251416 Plants with increased tolerance and/or resistance to environmental stress and increased biomass production	AR, AU, BR, CA, CN, DE, EP, MX, US, WO	EP application deemed to be withdrawn in 25/02/2015	BASF Plant Science GmbH DE	22.05.2007	22.05.2027
○ *Osdhar* gene	WO2011074959	US, WO		Holman Edwin NL	15.12.2009	15.12.2029

	Transgenic ozone-resistant plants			Henricus Antonius NL		
Osdhar gene	US20030215949 Dehydroascorbate Reductase ("*dhar*") Genes And Their Uses	US	Expired due to failure to pay maintenance fee	The Regents of the University of California US	28.05.2002	28.05.2022
Osdhar gene	WO9964612 Enhanced storage organ production in plants	AU, CA, IL, US, WO	EP: PCT application did not enter european phase in 07/11/2001	University of Guelph CA	11.06.1998	11.06.2018
○ Bt genes (*cry1Ac*, *cry1Ca* and *vip3Aa*) Plasmids containing *cry1Ca* and *vip3* were obtained from Dr. Raj Bhatnagar, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India *cry1Ac* gene was made available from the Rockefeller Foundation's program on rice biotechnology, gratis for humanitarian applications	WO9506128 Fertile, transgenic maize plants and methods for their production	AR, AU, BR, CA, EP, HU, IL, JP, US, ZA ZA9700399	EP application withdrawn in 20/07/2011 Granted in ZA	DeKalb Genetics Corp. US	25.08.1993	25.08.2013
Bt genes	EP2513314 Combined use of *cry1Ca* and *cry1Ab* proteins for insect resistance management	AR, AU, CA, CN, CO, EP, JP, KR, MX, RU, US, WO	-	Dow Agrosciences LLC US	16.12.2009	16.12.2029

Bt genes	EP2288708 Armyworm insect resistance management in transgenic plants	AR, CA, EP, MX, US, WO	EP application deemed to be withdrawn in 02/10/2013	Bayer Bioscience NV BE	01.05.2008	01.05.2028
Bt genes	EP2298067 Novel pesticidal toxins	AS, AU, BR, CN, EP, HU, US, WO	EP application deemed to be withdrawn in 17/09/2014	Syngenta Partecipations AG CH	01.04.2002	01.04.2022
Bt genes	EP2297189 Toxin genes and methods for their use	AR, AU, CN, CO, EA, EP, IN, JP, MX, NZ, PE, US, WO	EP application deemed to be withdrawn in 16/07/2014	Athenix Corp. US	25.06.2009	25.06.2029
Bt genes	EP2087120 Novel *Bacillus thuringiensis* crystal polypeptides, polynucleotides, and compositions thereof	AR, BR, CA, CL, CN, EA, IN, MX, US, WO, ZA ZA200903784	EP application deemed to be withdrawn in 23/05/2012 Granted in ZA	Pioneer Hi Bred International US	19.06.2008	19.06.2028
Bt genes	EP0408403 Prevention Of Resistance Development Against *Bacillus thuringiensis* Insecticidal Crystal Protein.	AT, AU, CA, CL, DE, DK, EP, ES, HK, JP, US, WO ES2060975	Patent expired after termination of 20 years	Plant Genetic Systems Nv BE	31.05.1989	31.05.2009
3. Processes ○ Vector Cloning	EP0286200	AT, DE, ES, EP, US, WO ES2065333	Granted in ES	Stratagene Inc. US	12.01.1987	12.01.2007

	DNA cloning vector with in vivo excisable plasmids					
○ Maize transformation with biolistics	EP0772687 Transgenic cereal plants	AU, BR, CA, EP, HU, JP, MX, NZ, US, WO	EP application deemed to be withdrawn in 04/09/2002	Pioneer Hi-Bred International Inc. US	28.07.1995	28.07.2015
Maize transformation with biolistics	WO2009090284 Method for creating a combinatorial population of transgenic plants which express and gather together diverse valuable metabolites	AR, ES, WO ES2340119	Granted in ES but withdrawn in 18/10/2011	Universitat de Lleida ES ICREA ES	23.07.2009	23.07.2029
Maize transformation with biolistics	WO2009093200 Production of progenitor cereal cells	AP, AU, US, WO, IN	EP: PCT application did not enter european phase	Pioneer Hi-Bred International Inc. US CSIR ZA	22.01.2009	22.01.2029
Maize transformation with biolistics	US5489520 Process of producing fertile transgenic zea mays plants and progeny comprising a gene encoding phosphinothricin acetyl transferase	US	Granted in US	DeKalb Genetics Corp. US	17.04.1990	17.04.2010
Maize transformation with biolistics	US4945050 Method For Transporting	US	Granted in US	Cornell Research Foundation Inc US	13.11.1984	13.11.2004

	Substances Into Living Cells And Tissues And Apparatus Therefor					
○ Maize regeneration	EP0256165 Process for regenerating corn	AU, CA, EP, JP, US	EP application withdrawn in 03/10/1990	Sungene Technologies Corp. US	18.08.1986	18.08.2006
Maize regeneration	US6946587 Method for preparing fertile transgenic corn plants	US	Expired due to failure to pay maintenance fee	DeKalb Genetics Corporation US	22.01.1990	22.01.2010
○ Selection with *bar* gene	WO9506128 Fertile, transgenic maize plants and methods for their production	AR, AU, BR, CA, EP, HU, IL, JP, US, ZA ZA9700399	EP application withdrawn in 20/07/2011 Granted in ZA	DeKalb Genetics Corp. US	25.08.1993	25.08.2013
Selection with *bar* gene	EP0814166 Methods and compositions for the production of stably transformed fertile monocot plants and cells thereof	AP, AT, AU, BR, DE, EP, ES, HU, JP, WO ES2110417	EP application deemed to be withdrawn in 15/09/2004	DeKalb genetics Corporation US	09.08.1989	09.08.2009
Selection with *bar* gene	US5489520 Process of producing fertile transgenic *Zea mays* plants and progeny comprising a gene encoding phosphinothricin acetyl transferase	US ZA9406488 ZA9604217	Granted in US	DeKalb genetics Corporation US	17.04.1990	17.04.2010

Table 2: Possibly relevant patent applications. Calculated expiry date is 20 years from the priority date (this method of calculation is not necessarily correct for US patents)

CODE	COUNTRY	CODE	COUNTRY
AP	African Regional Industrial Property Organization	**GT**	Guatemala
AR	Argentina	**HK**	Hong Kong (S.A.R.)
AT	Austria	**HU**	Hungary
AU	Australia	**ID**	Indonesia
BE	Belgium	**IL**	Israel
BR	Brazil	**IN**	India
CA	Canada	**JP**	Japan
CH	Switzerland	**KR**	Korea (South)
CL	Chile	**MX**	Mexico
CN	China	**NL**	Netherlands
CO	Colombia	**NZ**	New Zealand
DE	Germany	**PE**	Peru
CZ	Czech Republic	**PL**	Poland
DK	Denmark	**RU**	Russian Federation
EA	Eurasian Patent Organization	**US**	United States of America
EP	European Patent Office	**WO**	World Intellectual Property Organization (WIPO)
ES	Spain	**ZA**	South Africa
GB	United Kingdom		

Table 3: World Intellectual Property Organization Country Codes. Country codes consist of two letters (e.g. GB) indicating the country or organization where the patent application was filed or granted.

These 36 patents were analysed in detail by considering the descriptions, claims and also where appropriate using BLAST searches to compare our sequences with sequences listed under the patent claims. As a measure of caution, we compiled a full list of those patents (which include both patent applications and granted patents irrespective of legal status) where the claims appeared to pertain to the elements or processes used in the construction of Carolight™. Although we excluded patents whose claims obviously were not relevant or infringed, we retained all patents with ambiguous claims that may be considered relevant. These distinctions are expanded in the following sections.

5.4.2 Carotenogenic genes

Patent application WO2001088169 ('Method for producing carotenoid compounds in plants'), assigned initially to Calgene Inc. and from 2000 to Monsanto Technology LLC, claims

"A method for altering the carotenoid content in seed of a host corn plant comprising: transforming cells of a host corn plant with a construct comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA sequence derived from a carotenoid biosynthesis gene coding region, and a transcriptional termination region, producing a transformed host corn plant from said transformed cells, and growing said transformed host corn plant or progeny thereof containing said construct under conditions whereby seed is produced having an altered carotenoid content." (claim 1)

All the elements described in this claim are included in vector p326, which was used to introduce the *Pacrt*I gene into corn plants during the development of Carolight™, making this claim and therefore the patent relevant. However, a claim is only infringed if

all elements are included in an alleged infringing process or product (Durham, 2013). Therefore, the *Zmpsy1* is not within the scope of this patent claim because the pHor-P plasmid contains all the claimed elements *except* a plastid transit peptide. This PCT application has not entered the European phase, and corresponding applications were only found in AR, AU and USA. These patents, if granted, will expire in May 2020.

Another potentially relevant patent is EP1159428 ('Method for improving the agronomic and nutritional value of plants'), assigned to Greenovation Pflanzenbiotechnologie GmbH, with Syngenta Participations AG, which claims

"An isolated DNA molecule comprising a nucleotide sequence providing one or more expression cassettes capable of directing production of one or more enzymes specific for the carotenoid biosynthesis pathway selected from the group consisting of: (i) phytoene synthase derived from plants, fungi or bacteria, (ii) phytoene desaturase derived from plants, fungi or bacteria, (iii) ζ-carotene desaturase derived from plants, and (iv) lycopene cyclase derived from plants, fungi or bacteria, under the proviso that an expression cassette capable of directing production of phytoene synthase alone is excluded." (claim 1)

The *Pacrt*I gene included in Carolight™ acts as a phytoene desaturase and is derived from a bacterium (*Pantoea ananatis*), whereas the *Zmpsy1* gene encodes a phytoene synthase and is derived from corn plants, so we conclude that the claim is relevant to Carolight™. This patent needs legal scrutiny particularly because this is a granted EP patent, and protection is extended through family members in South Africa and India. The expiry date for this patent is March 2019.

5.4.3 Folate biosynthesis genes

PCT application WO2006034501 ('Materials and methods for folate biofortification in plants'), assigned to University of Florida, claims

"A method for increasing levels of a folate in a plant, said method comprising incorporating in a plant a polynucleotide that encodes an enzyme that catalyzes the synthesis of dihydroneopterin-PPP from GTP, wherein said enzyme is not under feedback control of the plant." (claim 1)

This potentially affects our product because the *EcfolE* gene encodes the enzyme GTP cyclohydrolase I. However, this application has been filed in the USA only and has not entered the national phase in Europe (i.e. protection is not extended through a corresponding European patent). Although patents may be filed directly in individual European countries, our searches did not reveal any such counterpart filings.

5.4.4 Ascorbic acid biosynthesis genes

Patent application WO9964612 ('Enhanced storage organ production in plants'), assigned to the University of Guelph, claims

"A method for increasing the mass of a storage organ of a plant comprising transforming the plant with at least one heterologous gene that encodes an enzyme that results in NAD(P)H consumption." (claim 1)

Although the *dhar* gene in Carolight™ encodes an enzyme that increases the consumption of NAD(P)H, the transgenic plants do not have a higher seed biomass, making this patent irrelevant. Furthermore, the application has not entered the European

phase, and considering its priority date, the patent family members will expire in June 2018.

5.4.5 *Bt* genes

We identified several patents covering the use of *Bt* genes, mainly assigned to Monsanto Technology LLC, Dow Agrosciences LLC, Bayer Bioscience N.V. or Syngenta AG. However, only a few appeared to be relevant because the majority claim synthetic genes, modified or hybrid proteins, polynucleotides or combinations of different *Bt* genes that are not present in our transgenic line.

An example of a relevant patent application is EP2288708 ('Armyworm insect resistance management in transgenic plants'), assigned to Bayer Bioscience N.V., which claims

"A method of controlling *Spodoptera frugipera* infestation in transgenic plants while securing a slower buildup of *S. frugiperda* insect resistance development to said plants, comprising expressing a combination of (i) a VIP3 protein insecticidal to said insect species and (ii) a Cry1A or Cry1 F protein insecticidal to said insect species, in said plants." (claim 1)

Carolight™ expresses both Cry1Ac and Vip3Aa; thus, this patent is relevant. The EPO application was deemed to be withdrawn in 2013. The corresponding family members will expire in May 2028.

Another relevant patent is EP2297189 ('Toxin genes and methods for their use'), assigned to Athenix Corp., which claims

"An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of: (i) the nucleotide sequence of any of SEQ ID NO: 1–60 and 124–

132; (ii) a nucleotide sequence having at least 90% sequence identity to the nucleotide sequence of any of SEQ ID NO: 1–60 and 124–132, wherein said nucleotide sequence encodes a polypeptide having pesticidal activity; (iii) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence of any of SEQ ID NO: 61–121 and 133–141; and, (iv) a nucleotide sequence encoding a polypeptide having at least 90% amino acid sequence identity to the amino acid sequence of any of SEQ ID NO: 61–121 and 133–141, wherein said polypeptide has pesticidal activity." (claim 1)

We used BLAST to compare the protected sequences with those used in Carolight™ and discovered that sequence 46 (axmi112) encodes a Cry1Ab protein with 91% identity to the Cry1Ac protein expressed in our plants, bringing it within the scope of the claim. The latest update in 2015 shows that the EP application is deemed to be withdrawn, but there is also an Indian patent in this family with a calculated expiry date of June 2029.

A similar analysis was carried out to determine the relevance of patent EP2087120 ('Novel *B. thuringiensis* crystal polypeptides, polynucleotides and compositions thereof'), assigned to Pioneer Hi-Bred, which claimed

"An isolated nucleic acid molecule comprising a nucleotide sequence that is at least 99% homologous to the nucleotide sequence of any of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or a complement thereof." (claim 1)

We found that the *cry1Ca* gene in Carolight™ was 90% identical to sequence 7 covered by the patent (GM662610.1), not enough to infringe claim 1. The European application was withdrawn in 2011 but as above there is a corresponding application in India, which will expire in June 2028.

Some patents covering *Bt* genes, especially the older ones, have wide claims. One example is patent EP0408403 ('Prevention of resistance development against

B. thuringiensis insecticidal crystal protein'), assigned to Plant Genetic Systems N.V., which claims a method to prevent resistance against *Bt* proteins by simultaneously expressing two *Bt* genes in the same plant cell, which have two different modes of action and target sites in the mid-gut of the larvae. This patent is relevant to Carolight™ because three different *Bt* genes are expressed. This patent was granted in Spain, but protection expired after 20 years in June 2010.

5.4.6 Maize transformation and regeneration under selection using the *bar* gene

Several patents cover technical processes relevant to the development of Carolight™ although most are only in force in the USA. In contrast, European Patent EP0772687 ('Transgenic cereal plants'), assigned to Pioneer Hi-Bred, specifies and claims the production of a transgenic crop plants (including maize) by introgressing foreign DNA into an embryo through biolistic bombardment. The embryo (early proembryo, mid-proembryo, late proembryo, transitional or early coleoptilar stage) must be grown in the presence of hormones to induce the formation of a plantlet which can be grown into a transformed plant that will transmit said foreign DNA to progeny. The patent specifies a shoot multiplication medium containing a cytokinin and a low level of auxin, which is the same combination of hormones used to regenerate Carolight™ plants, but the precise combination of hormones and tissues involved in the transformation process is unique so this claim is unlikely to be relevant. The patent also specifies an osmoticum medium containing high levels of cytokinins, whereas in the Carolight™ development process, the osmoticum medium contained a small amount of auxin. The European application was withdrawn in 2002 and the patent expired in July 2015.

Another relevant patent is US4945050 ('Method for transporting substances into living cells and tissues and apparatus therefor'), assigned to Cornell Research Foundation Inc., because it covers the transformation of monocotyledonous plant species by particle bombardment. This patent claims

"A method for introducing particles into cells comprising accelerating particles having a diameter sufficiently small to penetrate and be retained in a preselected cell without killing the cell, and propelling said particles at said cells whereby said particles penetrate the surface of said cells and become incorporated into the interior of said cells." (Claim 1)

The subsequent claims specify more detailed characteristics of such particles, including gold spheres 0.1–4 μm in diameter, comprising biological particles (such as nucleic acids), which have biological activity within the cells after penetrating them. Although particles covered under this claim were used in our transformation process, this patent was only filed and granted in the USA and there were no corresponding applications in Europe, India or South Africa.

Monsanto Technology LLC was granted US patent US5489520 ('Process of producing fertile transgenic *Zea mays* plants and progeny comprising a gene encoding phosphinothricin acetyl transferase') claiming

"A process for producing a fertile transgenic *Z. mays* plant comprising the steps of (i) establishing a regenerable culture from a *Z. mays* plant to be transformed, (ii) transforming said culture by bombarding it with DNA-coated microprojectiles, wherein said DNA comprises a selectable marker gene encoding for phosphinothricin acetyl transferase, (iii) identifying or selecting a transformed cell line and (iv) regenerating a fertile transgenic *Z. mays* plant it is therefrom, wherein said DNA is transmitted through

a complete sexual cycle of said transgenic plant to its progeny, wherein said progeny comprises said selectable marker gene encoding phosphinothricin acetyl transferase, and wherein said gene is chromosomally integrated."

Although this patent is relevant because we used the same selection marker to produce Carolight™, it is another example of a patent that was only filed and granted in the USA with no corresponding applications in Europe, India or South Africa.

PCT application WO2009093200 ('Production of progenitor cereal cells'), assigned to Pioneer Hi-Bred and CSIR South Africa, claims

"A process for the production of pluripotent and/or totipotent progenitor cereal cells, the process comprising the steps of: selecting a population of cells including undifferentiated cereal callus cells; and culturing the undifferentiated cereal callus cells in a primary plant tissue culture medium containing at least one auxin and at least one cytokinin to produce pluripotent and/or totipotent progenitor cereal cells." (claim 1)

The transformation of the undifferentiated callus cells and/or the progenitor cereal cells is also described. Although this patent is relevant to Carolight™, our primary tissue culture medium contains auxin but no cytokinin so our product does not fall within the scope of the claim. This application has not entered the European phase, but it has entered the national phase in India.

5.4.7 Promoters and plant varieties

Several different promoters were used to generate Carolight™, including endosperm-specific promoters for the metabolic genes and constitutive promoters for the selectable marker genes and *Bt* genes, all of which were used without modifications. One example

is the corn ubiquitin-1 promoter, which is the subject matter in a patent assigned to Dow Agro Sciences (with granted patent family members in Europe, the USA, Japan and Canada), a patent assigned to Monsanto (granted in the USA, South Africa, Australia, Europe and Canada) and one to Prodigene (one member granted in the US and one filed in Australia). However, none of these patents refer to the unmodified promoter, but instead claim components of the ubiquitin regulatory system (such as the heat shock elements) which have been modified by genetic engineering. For example, the Monsanto patent claims excised heat-shock elements, whereas the Prodigene patent claims a modified ubiquitin regulatory system in which the two overlapping heat-shock elements are adjacent to each other. The maize variety used for transformation, that is the South African inbred M37W, no longer enjoys plant variety protection under plant breeder's rights, and this variety has been available for general use (including research) for more than 20 years.

5.5 Discussion

We have identified 36 patents, some of which represent a family of related patents with geographical coverage in multiple territories, whose claims may potentially constrain the commercialization of Carolight™. Careful analysis suggests that many of these claims are not relevant and that there is little likelihood of infringement. Nevertheless, as a measure of caution, this list of 36 patents allows third parties to use the information for more detailed analysis by patent experts and provides a database that can be updated as the patent landscape evolves.

Freedom-to-operate clearance may be required to commercialize Carolight™ in South Africa and India, where preliminary FTO analysis is underway. The most straightforward approach would be to negotiate appropriate licences from the owners of protected materials and processes, if geographic protection extends to these countries. This project has a humanitarian purpose: to address nutritional imbalances in poor populations. Therefore, it is not a commercial project in the normal sense of the word, and it is hoped that royalty-free licences could be negotiated for humanitarian use, as demonstrated in the case of GoldenRice™ where many companies publicly declared their willingness to make their technologies available for free (Kowalski and Kryder, 2002; Kryder *et al*., 2000). It is unclear from the previous FTO analysis of GoldenRice™ whether the geographical coverage of many of the relevant patents extended to the target LMICs (and consequently whether any permissions were actually necessary) because patent data in many LMIC jurisdictions are difficult to acquire. We encountered the same issues and could only make definitive statements about the extent of protection in South Africa, India and other LMICs in a few cases.

It is also possible that some agricultural biotechnology patents may not be granted or may be revoked if granted in some LMICs even if applications are filed, due to the pressure of public opposition.

A list of tangible property (TP) used is provided in **Table 1**. The table lists both genetic elements and plasmid constructs and their sources. Where known, the primary and secondary sources are listed. The primary source typically is the person or entity where the components (elements/constructs) were isolated or constructed. A secondary source is typically where the component was obtained from. TP may sometimes be just as important as patents because protection is often through MTAs that impose restrictions on the scope of use of the TP. Very often there is no restriction on 'research use', but limitations are imposed on further distribution, commercial use and sometimes modification. MTAs and their terms are usually negotiated in advance, and thus define the rights of both the provider and the recipient of the materials. Such rights may relate to not only the original materials but derivatives of the materials as well. In academic institutions, materials are often passed on through collegial courtesy from one person/entity to another without regard to any rights that may be defined by MTAs or even patents. Paper trails over time are hard to follow retrospectively. Where possible, we have tried to document any MTAs and the conditions attached. It is likely that if any material has been used in a manner that is not authorized by the corresponding MTA, then the terms of the MTA may have to be renegotiated. It is worth noting that while patents have a limited period of exclusivity, TP and their corresponding MTAs may not be time restricted. In the case of constructs, one way of getting around ownership issues is to make similar (but not identical) constructs from scratch. However, this is time-consuming and utility needs to be compared to the original construct. There is little use if utility is limited, or construction is time-consuming or difficult. Also it should be borne in mind

that some constructs, and/or their elements may be patent protected, and patent claims can be wide and ambiguous or open to interpretation. In the case of TP that has passed through several hands and the paper trail is lost or no MTAs exist at the end of the chain, liability for any infringing activity (inadvertent infringement) is unclear, and to the best of our knowledge, no significant case law exists. However, if protected by patents, then one may be infringing. At this stage, we have decided to defer legal matters relating to use of materials incorporated in the seed to the recipient organizations, although to the best of our ability, we will provide all information relating to TP that can be identified and documented.

We have described the relevant patents in detail and offered an opinion as to whether the commercial development of Carolight™ may infringe the pertinent claims. Although scrutiny by legal experts is necessary for a definitive FTO Opinion, the most important aspects of the analysis comprise the following: (i) a literal interpretation of the claims (without a legal interpretation or opinion on the validity of the claims); (ii) relevant geographical coverage (Europe, South Africa and India); and (iii) the calculated expiry date of 20 years from the priority date for all patent documents except US patents. Therefore, the following patent documents and relevant family members warrant special attention: PCT application WO2001088169 (carotenogenic genes); EP2297189 and EP2087120 (*Bt* genes, specifically the corresponding Indian filing). Consequently, the following assignees may be most important in case appropriate clearances need to be negotiated: Monsanto Technology LLC (carotenogenic genes), Athenix Corp. and Pioneer Hi-Bred (*Bt* genes).

We have chosen not to patent any of the technologies involved in the production of Carolight™ so there will be no upstream barriers to research and development if others choose to pursue related scientific paths. However, the line has been registered as a plant

variety and a trademark has been sought. This ensures that the product quality can be guaranteed and trusted by governments during their implementation of programmes that use Carolight™ and it reduces the potential for counterfeit or substandard substitutes. It is pertinent to note that although the scope of our analysis is restricted to IP issues, several other issues need to be addressed before successful commercialization, including ethical concerns, support from the public and farming community, government engagement and regulatory issues. Only a holistic approach will make commercialization possible. We hope that the product will be successfully deployed to address the serious and sometimes fatal nutritional imbalances that are so prevalent in many LMICs.

5.6 Lessons learned and recommendations

At the start of this project, we did not have the resources to conduct an FTO in advance. Our recommendation to funders of projects that have humanitarian impact is that they allocate some funding at least for basic patent searches and documentation of TP and MTAs. It might appear that this is a function of an institution's technology transfer office. However, in reality they are overstretched, and many institutions do not have a well-resourced technology transfer office. Performing an FTO in advance will help the institution and researchers foresee at least some IP barriers that may arise subsequently. Often during research that has a commercial endpoint, less stringent licensing terms, preferably royalty free, can be negotiated during the early stages of research compared with the end stages when a product is nearly market-ready. If done in advance or at the early stages of a project, some issues that a researcher may have to consider include: Do existing MTAs have any restrictions such as ‘research only use’? Are there any ‘reach through’ restrictions (meaning the external institution will claim downstream IP generated by the researcher)? Renegotiating MTAs will resolve many potential

difficulties. There may be 'workaround solutions' if negotiations prove to be difficult, thus circumventing unreasonably restrictive IP. In our case, the humanitarian nature of Carolight™ and the limited territories in which it is deployed is likely to convince the patent holder that their legitimate commercial interests are not threatened. It is unlikely that they would be successful in extracting royalties from relatively poor countries and technology that is not likely to be lucrative.

For patents, it is useful to initiate a preliminary FTO analysis to identify patents and potential licensors at the early stages of a research programme. As the project results crystallize (perhaps by mid-project), it may be useful to reach out to holders of IP, and inform them of commercial intentions so they have plenty of time to evaluate whether there is any potentially adverse effect on the licensor's own interests. Generally most licensors may not apply a 'squeeze' on a project that is humanitarian in nature by being unreasonable (Kryder *et al*., 2000). For technologies that are relatively lucrative or likely to generate significant income, or are deployed in OECD countries, licensing terms may not be very generous, as one of the uses of IP is to provide a return on investment, typically through patent protection. There may be instances where the licensor may not agree to license the technology for use, or may impose extortionate terms. As a last resort, a potential user of a technology who has had unsuccessful negotiations may apply for a compulsory licence. A compulsory licence, once granted by a national government, allows the potential user to proceed to use the technology without the authorization of the patent holder. Compulsory licences have been used for essential patented pharmaceuticals by India, Brazil and other LMICs (Stirner and Thangaraj, 2012). Therefore, commercially oriented research does not necessarily have to halt during the early research stage of a project if it is not possible to obtain permissions on reasonable terms. Compulsory licences are a legitimate (albeit onerous) way of rectifying such situations.

While any negotiated agreement is usually done before a product is commercialized or transferred to another entity for deployment or commercialization, this usually happens 'after the event'. Perhaps the most straightforward way is to negotiate a non-assert agreement as this is usually simple in its construction. A typical non-assert agreement that would be relevant to Carolight™ is for the patent holder to not assert patent rights in the recipient countries (India, South Africa) if the relevant inventions are patented in those countries, further stipulating that the non-assert would be applied to Carolight™ for humanitarian purposes. There are multiple ways of agreeing negotiated terms for a licence/agreement and non-asserts are only one of them. Another typical arrangement would be for the technology to be made royalty free or for a reasonable royalty, combined with other terms that relate to field of use or territorial restrictions.

In conclusion, it would have been useful to perform an FTO analysis and negotiate the IP landscape early on during the project. However, as discussed earlier, this was not practical in the case of Carolight™. Furthermore, it is advisable to document thoroughly the MTA paper trail and renegotiate terms where necessary, or circumvent IP barriers by working around patent claims and MTAs provided that this is not unduly inefficient, time-consuming or resource intensive to do so.

4.7 References

- Bai, C., Twyman, R.M., Farré, G., Sanahuja, G., Christou, P., Capell, T. and Zhu, C. (2011). A golden era-pro-vitamin A enhancement in diverse crops. *In Vitro Cell. Dev. Biol. Plant*. **47**: 205–221.

- Durham, A.L. (2013). Patent Law Essentials: A Concise Guide. Section 10.4 (Direct and Indirect Infringement), pp. 180. Praeger: Praeger Publishers Inc, ISBN-10: 1440828784. ISBN-13: 978-1440828782.

- Harrison, E.A. (2005). Mechanisms of digestion and absorption of dietary vitamin A. *Annu. Rev. Nutr.* **25**: 87–103.

- Houghton, L.A., Gray, A.R., Rose, M.C., Miller, J.C., Hurthouse, N.A. and Gregory, J.F.I.I.I. (2011). Long-term effect of low-dose folic acid intake: potential effect of mandatory fortification on the prevention of neural tube defects. *Am. J. Clin. Nutr*. 94: 136–141.

- Kowalski, S.P. & Kryder, R.D. (2002). Golden rice: a case study in intellectual property management and international capacity building. *Risk*. **13**: 47–67.

- Kryder, R.D., Kowalski, S.P. & Krattiger, A.F. (2000). The Intellectual and Technical Property Components of pro-Vitamin A Rice (GoldenRice^{TM}): A Preliminary Freedom-To-Operate Review, pp. 56. *ISAAA Briefs* No. 20. Ithaca, NY: ISAAA. ISBN: 1-892456-24-9.

- Miralpeix, B., Sabalza, M., Twyman, R.M., Capell, T., Christou, P. (2014). Strategic patent analysis in plant biotechnology: terpenoid indole alkaloid metabolic engineering as a case study. *Plant Biotechnol. J.* **12**:117–134.

- Naqvi, S., Zhu, C., Farre, G., Ramessar, K., Bassie, L., Breitenbach, J., Perez Conesa, D. (2009). Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proc. Natl. Acad. Sci. USA*. **106**:7762–7767.

- Pérez-Massot, E., Banakar, R., Gómez-Galera, S., Zorrilla-López, U., Sanahuja, G., Arjó, G., Miralpeix, B. (2013). The contribution of transgenic plants to better health through improved nutrition: opportunities and constraints. *Genes Nutr*. **8**: 29–41.

- Stirner, B. & Thangaraj, H. (2012). Learning from practice: compulsory licensing cases and access to medicines. *Pharm. Pat. Anal.* **1**: 555–575.

- Zhu, C., Sanahuja, G., Yuan, D., Farré, G., Arjó, G., Berman, J., Zorrilla-López, U. (2013). Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies. *Plant Biotechnol. J*. **11**:129–141.

General Conclusions

GENERAL CONCLUSIONS

- The agronomic performance of Carolight^{R} in the field was indistinguishable from that of its near isogenic line M37W. The insertion of a transgenic cassette for high carotenoid accumulation did not affect general crop performance, including grain yield and its main components.
- Endogenous carotenogenic gene and transgene expression in Carolight^{R} endosperm was similar in greenhouse- and field-grown plants.
- The proportions of the major endosperm carotenoids varied during development, although differences disappeared by maturity and the carotenoid composition in mature kernels in greenhouse- and field-grown plants was similar. This demonstrates that the transgenic trait in Carolight^{R} is stable under different growing conditions.
- The Cry1Ac Bt toxin, at a concentration of 50 µg/g of fresh weight in 4Bt leaves, has been shown to be as effective as Cry1Ab in MON810 in controlling L2 larvae of the Bt-target species *O. nubilalis* and *S. nonagrioides.*
- Susceptibility to both Cry1Ab and Cry1Ac toxins has been observed in L2 larvae of Bt non-target species *M. unipuncta* and *H. armígera*. *M. unipuncta* was the least susceptible to Bt toxins between the four studied lepidopteran species, and was more subsceptible to Cry1Ab in MON810 than to Cry1Ac in 4Bt leaves.
- The high carotenoid content in the endosperm of 4Bt influences the response of insects to the Bt toxin at a concentration of 3 µg/g of fresh weight. A 17% decrease in mortality was measured for L2 *O. nubilalis* larvae when fed on a diet

supplemented with grains from 4BtxHC plants, compared to larvae fed on a 4Bt supplemented diet.

- Carotenoids may suppress the immune response elicited by Bt toxins in the insect gut. This has been demonstrated to be one of the mechanisms responsible for larval death after toxin ingestion.
- *M. unipuncta* and *O. nubilalis* consumed less Carolight^{R} leaves compared to M37W. I attributed this behaviour to a knock-on effect of carotenoid metabolism in the seeds which affects the leaves and, more specifically, how attractive they are to insect pests. Further studies need to be performed to validate this hypothesis.
- Through a detailed analysis of the intellectual property landscape relative to production and commercialization of Carolight^{R}, I identified three relevant patents which would require particular attention in future commercialization activities.