Understanding and modulating vitamin C biosíntesis in corn and generating insect resistant corn plants expressing simultaneously multiple insecticidal genes

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Doctoral dissertation

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Paul Christou i Ludovic Bassié, professors del Departament de Producció Vegetal i Ciència Forestal de la Universitat de Lleida, i directors de la tesi realitzada per la senyora Georgina Sanahuja Solsona, "Understanding and modulating vitamin C biosynthesis in corn and generating insect resistant corn plants expressing simultaneously multiple insecticidal genes",

INFORMEM:

Que el treball d'investigació es considera ja finalitzat i que compleix les condicions exigibles a la legislació vigent per optar al grau de Doctor. Per tant autoritzem la seva presentació perquè pugui ser jutjada pel Tribunal corresponent a la Universitat de Lleida.

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Abstract

Malnutrition is a significant global challenge, particularly in the developing world which lacks the compensatory measures often found in industrialized countries (e.g. a varied diet, fortification schemes and dietary supplements). However, fundamental advances in molecular biology and plant transformation now allow us to produce new traits in agriculturally-important crops, helping to improve human food and animal feed quality as well as offering protection against pests and pathogens.

Vitamin deficiencies affect impoverished people in developing countries in a disproportionate manner because poor people cannot afford a diversified diet. Plants provide nearly all the vitamins and minerals required to maintain health and well-being in humans, but staple crops such as cereal grains tend to be poor sources of essential nutrients, including vitamin C (ascorbic acid). Biochemical analysis and metabolic engineering in maize show us not only how to improve the nutrient content of seeds but also how the metabolic pathways work. I therefore analyzed ascorbic acid metabolism and the genetic basis of L-galactose biosynthesis in maize to determine how ascorbic acid accumulation is regulated. I also investigated the developmental profile of ascorbic acid accumulation in the endosperm of three diverse genotypes and a transgenic line expressing rice dehydroascorbate reductase, which enhances ascorbic acid recycling. Furthermore, I initiate a program to establish a combinatorial transgenic maize population comprising different combinations of genes related to ascorbic acid metabolism, aiming to provide insight into the interactions among alternative ascorbic acid biosynthesis pathways and help in the development of strategies to boost the accumulation of ascorbic acid in the endosperm. I discuss the vitamin content of nutritionally-enhanced crops developed by conventional breeding and genetic engineering, focusing on dosage and bioavailability, the latter often being overlooked.

Many of our crop plants are attacked by insect pests, and devastating losses occur throughout the world due to pest infestations either in the field or during storage. Therefore, I generated transgenic maize plants engineered with multiple insecticidal genes derived from the bacterium *Bacillus thuringiensis* as a first step in developing plants with durable resistance to insect pests. Such plants will be crossed with multivitamin maize and other nutritionally enhanced lines prior to their deployment in the field to enhance pest resistance.

Resum

La malnutrició és un problema molt important al qual la societat actual té que trobar solucions, sobretot per als països en vies de desenvolupament on no es poden aplicar les mateixes mesures preses per afrontar-la que als països desenvolupats (dieta diversificada, sistemes de fortificació i supplements alimentaris). Els avanços en biologia molecular i l'aplicació de programes de millora genètica amb l'objectiu d'obtenir plantes que presenten noves característiques nutricionals, poden ajudar a la millora de la qualitat dels productes obtinguts, ja siguin per consum humà o per a la producció de pinso per consum animal; fins i tot aquestes tècniques poden ajudar en la protecció dels cultius contra plagues i patògens.

Les deficiències en vitamines afecten majoritàriament a la població pobra dels països en vies de desenvolupament degut a la manca d'una dieta variada. Les plantes proporcionen la majoria de les vitamines essencials per mantenir una bona salut i aconseguir el benestar en les persones, però la majoria dels cultius alimentaris, i en particular els cereals, en són deficients, com per exemple en vitamina C (àcid ascòrbic). La modificació de les rutes metabòliques mitjançant enginyeria genètica aplicada al panís, i el posterior anàlisi bioquímic dels teixits enginyats ens pot ajudar tant a l'hora de millorar el contigut nutricional en la llavor com a l'hora d'entendre el funcionament d'aquestes rutes metabòliques. Per aquesta raó, en la meva tesis, he investigat el contingut en vitamina C en el panís i el mecanisme genètic de regulació d'una de les rutes de biosíntesis de la vitamina C, la ruta de la L-galactosa. També he investigat en detall l'acumulació de vitamina C durant el desenvolupament del endosperma de la llavor en quatre genotips diferents de panís, un d`ells, un panís transgènic que conté el gen dehydroascòrbic àcid reductasa, el qual fa augmentar el contingut en vitamina C. A més a més, amb l'objectiu d'entendre les interconnexions de les diferents rutes de síntesis de la vitamina C i així, ajudar a trobar una estratègia per augmentar el contingut d`aquesta vitamina en el endosperma de la llavor, he començat un programa de generació de plantes de panís transgènic. Aquestes plantes contenen diferents combinacions de gens de la ruta metabòlica de la vitamina C. Un altre element que he tractat en la meva tesis doctoral, ha estat la biodisponibilitat de les vitamines en els cultius millorats nutricionalment ja sigui per millora convencional o per enginyeria genètica.

Finalment, donat el fet de que la majoria dels nostres cultius són atacats per plagues que causen danys severs tant en el camp com en l'emmagatzemament del fruit, resultant en perdues econòmiques molt importats, i que les plantes regenerades fins ara al nostre laboratori careixen de protecció contra l'atac del cuc barrinador, he generat plantes de panís transgèniques que contenen múltiples gens derivats de la bactèria *Bacillus thuringiensis* amb activitat insecticida. Aquestes plantes seran creuades amb el blat de moro multivitamínic genèticament modificat i amb altres línies de blat de moro amb altres trets nutricionals. Així obtindrem un panís millorat nutricionalment i resistent a insectes abans de que sigui cultivat al camp.

Resumen

La malnutrición es uno de los problemas más importantes que debe solucionar la sociedad actual, sobre todo en los países en vías de desarrollo, donde no se pueden aplicar las mismas medidas paliativas que las tomadas para solucionarla en los países desarrollados (dieta diversificada, sistemas de fortificación y suplementos alimenticios). Sin embargo, los avances en biología molecular y la mejora de plantas mediante ingeniería genética, nos permite obtener cultivos con nuevas características, facilitando la mejora de la calidad de los productos que se obtienen de ellas, ya sean para consumo humano o para la fabricación de pienso para consumo animal. La aplicación de estas tecnologías puede extenderse a la obtención de cultivos que estarán protegidos contra plagas y patógenos.

Las deficiencias vitamínicas se detectan mayoritariamente en la población pobre de los países en vías de desarrollo al no poder permitirse una dieta variada. Las plantas proporcionan la mayoría de las vitaminas esenciales para tener buena salud y lograr el bienestar de las personas. Sin embargo, la mayoría de los cultivos alimenticios, y en particular los principales cereales son deficientes en su contenido, como por ejemplo la vitamina C (ácido ascórbico). Los análisis bioquímicos y las modificaciones de las rutas metabólicas mediante ingeniería genética en las plantas de maíz nos pueden ayudar a mejorar el contenido nutricional de la semilla y/o nos ayudaran a entender el funcionamiento de estas rutas metabólicas. Con el objetivo de aumentar los conocimientos en este campo, he analizado en maíz el contenido en vitamina C y el mecanismo que regula genéticamente una de las rutas de biosíntesis de la vitamina C, la ruta de la L-galactosa. También he investigado en profundidad la acumulación de esta vitamina durante el desarrollo del endospermo en la semilla de maíz de cuatro genotipos diferentes, uno de ellos de un maíz transgénico que contiene un gen, el dehydroascórbico ácido reductasa, que hace aumentar el contenido de vitamina C. Además, con el objetivo de facilitar la comprensión de las interconexiones de las diferentes rutas de síntesis de la vitamina C, y así poder encontrar una estrategia para aumentar el contenido de esta vitamina en el endospermo de maíz, he iniciado la generación de maíz transgénico con diferentes combinaciones de genes de la ruta metabólica de la vitamina C. Otro factor importante que he analizado durante mi tesis doctoral, ha sido la biodisponibilidad de las vitaminas en los cultivos mejorados nutricionalmente ya sea por mejora convencional o per ingeniería genética.

La mayoría de los cultivos son atacados por plagas que causan daños tanto en el campo como en el almacenaje de los frutos, por lo que en esta parte del trabajo he generado plantas transgénicas que contienen múltiples genes derivados de la bacteria *Bacillus thuringiensis* con actividad insecticida. Estas plantas serán cruzadas con el maíz multivitamínico genéticamente modificado y también serán cruzadas con otras líneas de maíz con otros rasgos nutricionales. Así obtendremos maíz mejorado nutricionalmente y resistente a insectos antes de su previo cultivo en el campo.

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List of Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
µg	Micrograms
µl	Microliters
µm	Micrometres
Anti-DIG-AP	Anti-Digoxigenin-AP Fab fragments
BAP	6-Benzylaminopurine, synthetic cytokinin
Bt	*Bacillus thuringiensis*
CaMV35S	Cauliflower Mosaic Virus 35S promoter
cDNA	Complementary DNA
CSPD	Disodium3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'- chloro)tricycle [3.3.1.13,7]decan}-4-yl)phenyl phosphate
cv.	Cultivar
DNA	Deoxyribonucleic acid
Dap	Days after pollination
DRI	Dietary Reference Intake
DW	Dry weight
EDTA	Ethylene Diamino Tetra Acetic Acid
EFSA	European Food Safety Authority
E.coli	Escherichia coli
FAO	Food and Agriculture Organization
g	Grams
GE	Genetic engineering
IOM	US Institute of Medicine
ISAAA	International Service for the Acquisition of Agri-biotechnological Applications
KDa	KiloDalton
mg	Milligrams
ml	Mililiter
mM	MiliMolar
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
MS	Murashige and Skoog media
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADP	Phosphate nicotinamide adenine dinucleo
ng	Nanograms
PCR	Polymerase chain reaction
QTLs	Quantitative trait loci
RAE	Retinol Activity Equivalent
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
ssp.	Multiple species
SSC	Saline Sodium Citrate
T-DNA	Transferred DNA
Ubi-1	Maize ubiquitin 1
UNICEF	The United Nations Children's Fund
UL	Tolerable Upper intake Level
US	United States

USDA	United States Department of Agriculture
v/v	Volume to volume ratio
w/v	Weight to volume ratio
WHO	The World Health Organization
Zm	*Zea mays*

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1. General introduction

In the developing world, almost one billion people are chronically undernourished, regularly consuming less than 2000 calories per day (FAO, 2006). A further two billion people consume enough calories but nevertheless lack essential nutrients. This means that up to half the world's population at any one time may suffer from malnutrition (Graham et al., 2001).

Food insecurity is defined as the lack of access to sufficient amounts of safe and nutritious food to maintain an active and healthy life. One of the persistent myths concerning food insecurity is that a shortfall in food production is to blame. There is actually plenty of food to go around, at least at present. The reasons for food insecurity are complex, but one of the main factors is poverty (Barrett, 2010). More than one billion people live on less than $US 1 per day with another two billion only marginally better off (World Bank, 2000; 2001) and most of these people are rural subsistence farmers in developing countries, where they can account for 70% or more of the population. In contrast, farmers account for only 1% of the US population (World Bank, 2000; 2001).

Subsistence farmers have limited purchasing power, and generally cannot irrigate their crops or buy fertilizers, herbicides and pesticides. This leads to soil exhaustion and falling yields, and the crops become susceptible to pests, diseases and natural disasters such as drought (Ramessar et al., 2009a). Many poor farmers are eventually forced to abandon their land and move to cities, adding to the growing problem of urban poverty and hunger (DFID, 2002). It is now thought that half the world's population lives in cities, so any disruption to agriculture could precipitate an urban food crisis in a matter of days. The markets can also increase the prevalence of food insecurity, i.e. when food prices increase but incomes are low or unemployment increases (FAO/WFP, 2009). Any long-term strategy to address food insecurity in the developing world must therefore tackle the underlying problem of poverty by increasing the level of rural employment-based income through increased agricultural productivity (Christou and Twyman, 2004).

Given projected population increases, fuel price hikes, falling reserves of fresh water and increased urbanization, the only sustainable solution to address food insecurity is to increase the yields of major food crops (particularly cereal grains) using the land that is currently available using less water, and to diversify the uses of crops to facilitate the creation of wealth. A variety of approaches can be envisaged, including the efficient use

of organic and inorganic fertilizers, irrigation strategies, soil and water conservation, pest and disease management and the production of improved plant varieties with higher yields or novel products (Ramessar et al., 2009a).

Food insecurity also increases malnutrition, which reflects the combined impact of poverty, poor access to food, inefficient food distribution infrastructure and an overreliance on subsistence agriculture based on individual cereal crops that lack essential nutrients. Malnutrition is therefore endemic in developing countries where the lack of a diverse diet means that many individuals are exposed to the risk of deficiency diseases (Pérez-Massot et al., 2013). Plant biotechnology provides a range of tools that can contribute significantly and sustainably to humanitarian efforts in developing countries where much of the economy is based on subsistence agriculture.

1.1 Crop improvement through biotechnology

Fundamental advances in molecular biology have increased our understanding of biochemical processes and metabolic pathways in plants. Therefore, the modulation of metabolic pathways, which a few years ago could be achieved only in microbes, is rapidly becoming applicable to plants. The advent of genome sequencing and functional genomics has led to the discovery of many new plant genes related to primary and secondary metabolism. Coupled with improvements in plant transformation, this technology can now be used to produce new traits in agriculturally important crops (Yuan et al., 2011, **fig. 1**). Such traits include the improvement of human food and animal feed quality (Zhu et al., 2008; Naqvi et al., 2009), the protection against pests and pathogens (Christou and Twyman, 2004), the enhancement of abiotic stress tolerance (Peremarti et al., 2009) and the production of pharmaceuticals and other value-added substances (Ramessar et al., 2008).

The application of metabolic engineering in plants will teach us not only how to engineer biochemical changes but also about how the metabolic pathways work. This approach will allow us to identify limitations and rate-determining steps by providing the means to experimentally define the control points. Transgenic approaches will also allow us to test directly whether a particular gene product is of interest or, more appropriately, important to the plant.

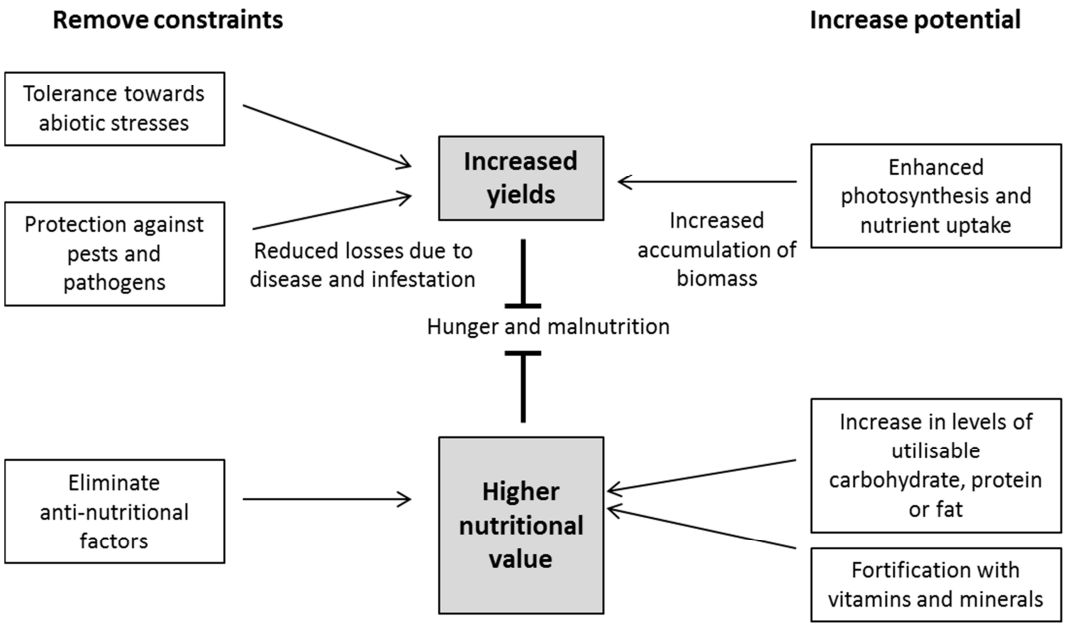

Figure 1. Transgenic strategies to tackle hunger and malnutrition (Christou and Twyman, 2004). Strategies on the left help to eliminate constraints and thus realise the yield or nutritional potential of the crop. Strategies on the right help to raise the yield ceiling by releasing unlocked potential.

1.1.1 Genetic engineering targets: increasing the nutritional value of food

Access to sufficient, safe and nutritious food depends not only on the quantity of food available but also on its nutritional quality. Unfortunately, the poorest individuals in the world are faced with a limited choice, and generally rely on a single staple food crop for their energy intake. Most plants are deficient in some essential amino acids, vitamins and minerals but a balanced diet provides adequate quantities of all. Problems arise when the diet is restricted to a single protein source, which is often the case for both human populations and domestic animals in developing countries (Graham et al., 2001). For example, cereal storage proteins are deficient in lysine and threonine while legumes tend to lack the S-containing amino acids methionine and cysteine. A diet solely comprising one of these protein sources will therefore be deficient for one or more essential amino acids. Milled cereal grains are also deficient in several vitamins and minerals, the most important of which are vitamin A, E, C, folic acid, Fe and Zn. Genetic engineering strategies to provide nutrient fortification represent one of the most straightforward approaches to alleviate malnutrition.

Biofortification means that nutrients and other health-promoting compounds are incorporated while the plant is still growing, and are therefore present in the harvested material and at all subsequent stages en route to the consumer. Biofortification programmes generally focus on essential micronutrients, which are either organic compounds (vitamins) or minerals required in amounts <1 mg/day. These compounds act as cofactors or metabolic precursors and are required for specific biological processes, such that insufficient intake results in characteristic deficiency diseases such as blindness for vitamin A deficiency or scurvy for vitamin C deficiency (Zhu et al., 2007; Gomez-Galera et al., 2010). Some essential nutrients also act as antioxidants or promote the activity or availability of antioxidants, which help to prevent diseases that result from or that are exacerbated by the accumulation of oxidative damage to cells, including cancer, cardiovascular disease and neurodegenerative disorders (**Table 1**). A key example of such a 'dual-purpose nutrient' is vitamin A, which is obtained in the diet either as esters of retinol from meat and dairy products or as pro-vitamin A such as β-carotene from plants. Vitamin A is converted into the visual pigment rhodopsin (retinal), in the retina of the eye, and acts as co-regulator of gene expression (retinoic acid); β-carotene is also an antioxidant, as are many other (non-essential) carotenoids. Similarly vitamin C (ascorbic acid) is an essential cofactor for several enzymes and vitamin E (tocochromanol family) is a regulator of protein kinase activity and gene expression, but their potent antioxidant activities are arguably just as important as their essential and non-replaceable functions (Zhu et al., 2013) (**Table 1**). Even metal ions, which are usually regarded as pro-oxidants, can be important to maintain antioxidant activity in humans, because they act as cofactors for certain antioxidant enzymes, for example iron as a cofactor for catalase (Zhu et al., 2013).

Biological antioxidant	**Solubility**	**Molecular structure and molecular forms**	**Source**	**Regeneration properties**	**Health benefits as antioxidant**	**References**
Ascorbic acid (Vitamin C)	Water soluble	Ascorbic acid/MHA /DHA	Plants (fruits, vegetables, leafy greens)	Glutathione recycles ascorbic acid by the dehydroascorbate reductase	Immune enhancing, Potential protection against oxidative diseases	Gülçin, 2012; Loewus, 1988; Maggini et al., 2010; Levine et al., 2011
Carotenoids (Vitamin A)	Fat soluble	Tetraterpenoid organic pigments, such as β-carotene	Plants, algae, many bacteria, and fungus		Prevention of cancer, Prevention of blindness, Maintenance of the immune system	Tanaka et al., 2008; Nancy et al., 2010; Dutta et al., 2005
Vitamin E	Fat soluble	A set of eight related tocopherols and tocotrienols	Plants (fruits, vegetable, nuts, cereal), milk	Cell signaling, Effect on gene expression	Decrease age related macular Degeneration, decrease osteoporosis	Reboul et al., 2006; Herrera et al., 2001; Traber et al., 2007

Table 1. Essential nutrients which also act as antioxidants or promote the activity or availability of antioxidants, and help to prevent diseases (Zhu et al., 2013).

1.1.1.1 Biofortification and bioavailability of vitamins

Vitamins, such as carotenoids (vitamin A), tocochromanols (vitamin E), ascorbic acid (vitamin C) and folic acid (vitamin B9), are synthesized de novo by plants, so engineering strategies aiming to enhance the availability of such compounds involve the modulation of endogenous plant metabolism (Capell and Christou, 2004). This can be achieved using a variety of strategies as outlined in **figure 2**. The most common strategy has been to overexpress a known rate-limiting enzyme thus alleviating a metabolic bottleneck, preferably using an enzyme devoid of feedback inhibition (Shewmaker et al., 1999; Ye et al., 2000; Zhu et al., 2008). The first committed step in a metabolic pathway is often a suitable intervention point because this ensures flux is delivered to all downstream steps (Enfissi et al., 2005; Morris et al., 2006). However, it is also possible to overexpress multiple enzymes to ensure there is adequate flux throughout the entire pathway (Ravanello et al., 2003; Zhu et al., 2008), or express regulatory proteins to coordinately induce an entire endogenous pathway without the introduction of heterologous enzymes (Butelli et al., 2008; de Vos et al., 2000). Alternative strategies to achieve the accumulation of a specific desired metabolite include the suppression of a competitive pathway or branch point to ensure flux is directed in the appropriate direction (Diretto et al., 2006; Yu et al., 2008) or the creation/enlargement of a metabolic sink, which reduces feedback inhibition and allows the desired product to accumulate in a stable manner (Lopez et al., 2008; Lu et al., 2006).

Although much progress has been made towards vitamin enhancement in staple crops, it is important to establish if the total vitamin content in food can be absorbed and used by the body (bioavailability). Several *in vitro* and *in vivo* methods can be used to assess bioavailability (Lemmens et al., 2011; Granado et al., 2006). Typically, nutrient bioaccessibility (defined as the fraction of the ingested nutrients that is released from the food matrix and is available for intestinal absorption) is determined by *in vitro* methods, whereas nutrient bioavailability (which includes nutrient absorption, tissue distribution and metabolism) can be assessed by *in vitro* and *in vivo*. The bioavailability of nutrients in engineered crops is a more important indicator of its nutritional quality (Hirschi, 2008). However, the factors affecting bioavailability must also be taken into account for each particular vitamin, e.g. food processing techniques, meal components and preparation techniques can modify plant foods in ways that either promote or reduce the amount of bioavailable nutrients (Michaelsen and Friis, 1998).

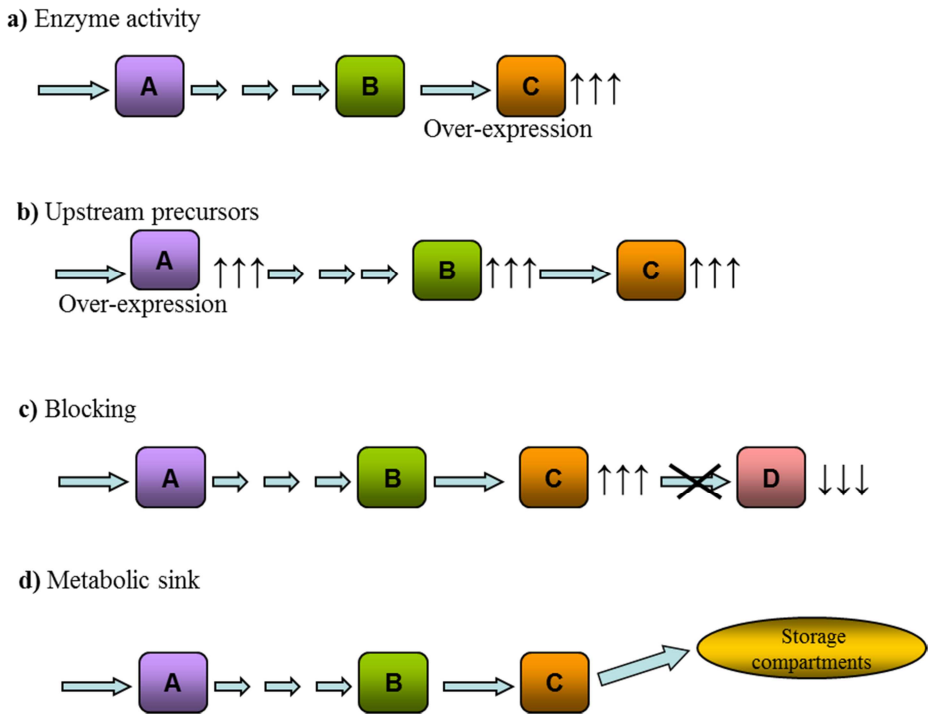

Figure. 2 Strategies to modulate organic compound levels in plants (Zhu et al., 2013). These strategies comprise the modification of: (a) activity of enzymes implicated in rate limiting steps in target pathways by modulation of one or two key enzymes, or multiple enzymes; (b) upstream precursors to increase flux through the pathway by overexpressing the enzyme that catalyzes the first committed step of the target pathway; (c) pathway branch points by blocking and relieving feedback inhibition by RNA interference or antisense; (d) enhancement of accumulation of target metabolite(s) by increasing sink compartments to store target compounds.

The food matrix plays an important role in the bioavailability of organic and inorganic compounds. For example, 12 mg of β–carotene in a food matrix must be ingested to gain the same benefit as 1 mg of pure β-carotene dissolved in oil. Similarly, vitamin E absorption requires the presence of bile salts, pancreatic enzymes and oils or fats to promote solubility (Jeanes et al., 2004), and the bioavailability of ascorbic acid is enhanced by copresentation with proteins in the food matrix (Vinson and Bose, 1988). Recently, it has been demonstrated that β-carotene in biofortified rice (Golden Rice) and maize has good bioavailability as a plant source of vitamin A in humans (Li et al., 2010; Muzhingi et al., 2011; Tang et al., 2009) and toxicity assessment in mice fed with

multivitamin corn (Naqvi et al., 2009) showed no adverse health effects and did not induce any clinical sign of toxicity (Arjo et al., 2012).

1.1.2 Genetic engineering targets: Insect pest, insect-borne diseases and the consequences of pest infestations

Many of our crop plants are attacked by insect pests, and devastating losses occur throughout the world due to pest infestations either in the field or during storage. In the developing world, about half of all crop production is thought to be lost to insects, 15% of these losses occurring due to post-harvest consumption and spoilage (Christou et al., 2006). Insects not only cause direct yield losses by damaging and consuming plants but also act as vectors for many viral diseases, and the damage they inflict encourages bacterial and fungal infections, the latter resulting in contamination with mycotoxins (Yuan et al., 2011). A good example of the positive impact of plant biotechnology is the development of pest-resistant crops expressing insecticidal toxin genes from the soil bacterium *Bacillus thuringiensis* (Bt). Different strains of Bt produce different toxins which are both potent and highly specific against narrow taxonomic groups of insects, making them inocuous to mammals and to non-target beneficial insects (Sanahuja et al., 2011). In developing countries, Bt crops have been extraordinarily successful and beneficial, increasing yields, reducing the use of pesticides and the fuel needed for spraying, and improving the economic status of farmers while at the same time preserving biodiversity (James, 2010; Brookes and Barfoot, 2010). The adoption of Bt crops in India provides strong support for the role of plant biotechnology in progress towards eradicating extreme poverty and hunger (Yuan et al., 2011). In 2009, more than 5.5 million small-scale farmers planted a total of 8.4 million hectares of Bt cotton, representing nearly 90% of the national total (James 2010). More than half of these crops contained multiple Bt genes providing resistance against different pests, and for the first time locally developed varieties were planted instead of varieties developed in the US, therefore keeping all the agricultural profits within India's economy rather than servicing foreign royalty payments. India is now the world's largest cotton exporter (having been a net importer at the beginning of the decade), and it is estimated that rural farmers have benefitted from the technology through yield improvements to a total amount exceeding US $5 billion. Net yields per hectare have doubled in 10 years while agrochemical inputs have halved (APCoAB, 2006; Manjunath, 2008). The widespread adoption of Bt cotton in India has also helped to address the concerns of critics, who highlight the potential for resistant pests to evolve under intense selection pressure.

Against these expectations, the first generation of Bt crops has maintained efficacy against nearly all targeted pest populations for more than a decade (Bourguet, 2004). The scarcity of resistant populations despite the lack of integrated pest management suggests that resistance may attract a fitness penalty in the absence of the Bt toxins (Sanchis and Bourguet, 2008).

Resistant populations have appeared for a small number of pests, such as pink bollworm (*Pectinophora gossypiella*) which has evolved resistance to Bollgard I cotton (expressing the Cry1Ac toxin) in the Amreli, Bhavnagar, Junagarh and Rajkot areas of Gujarat (Yuan et al., 2011). Resistance is anticipated because each toxin binds to a specific receptor in the brush border of midgut epithelial cells, and point mutations affecting toxin/receptor interactions would be strongly favored under selection. However, no resistance has been observed in fields growing the Bollgard II variety, which expresses the Cry1Ac and Cry2Ab toxins simultaneously (Monsanto, 2010). These toxins bind different receptors, and the likelihood of mutations occurring in genes for both receptors is much lower than the likelihood of a single mutation, so this strategy of 'pyramiding' resistance genes (i.e. expressing multiple toxins with different targets in the pest) is a very powerful approach to prevent the evolution of resistant pest populations.

In the last year, Indian regulatory authorities also approved Bt brinjal (eggplant), India's first biotechnology derived major food crop. Eggplant is a profitable crop but is extremely susceptible to pests, which cause up to 70% yield losses. Pest control normally requires repeated generous pesticide applications, up to 40 applications in 120 days, which many farmers cannot afford resulting in less intense treatments that are ineffective (Jayaraman, 2010). The Bt variety has the potential to increase net yields by 33% while reducing pesticide use by up to 80%, thus lifting another 1.4 million farmers out of poverty (James, 2010), but the regulatory approval was overruled by the government after lobbying by activists, and Bt brinjal is now subject to an indefinite moratorium pending additional safety data (Balga, 2010). The technology behind Bt eggplant was freely donated by Maharashtra Hybrid Seeds Company Ltd. (MAHYCO), who co-developed the product with Monsanto, to public sector institutions in India, Bangladesh and the Philippines for use by small resource poor farmers, with 18 varieties awaiting final approval. These farmers will now be deprived of an opportunity to increase their economic prosperity for the foreseeable future (Jayaraman 2010).

As well as the direct impact of insect pests on crop yields, insects also act as vectors for viruses and fungal spores, encouraging crop diseases and fungal colonization of stored grains. One of the indirect benefits of Bt technology has been to reduce the level of mycotoxin contamination in grains such as maize by reducing damage and spore transmission (Brookes, 2008; Wu, 2007). Mycotoxins such as aflatoxin, deoxynivalenol, fumonisin and zearalenone are secondary metabolites produced by fungi that act as antinutritional factors when present at low doses in food, therefore preventing humans gaining the full benefit of the calories they consume (Wu, 2007). Mycotoxins also affect domestic animals (Miller and Marasas, 2002), so they have a compound impact on food security by limiting weight gain in farm animals as well as directly affecting humans. The consumption of mycotoxins also carries a disease burden because they are carcinogenic and can also suppress the immune system, e.g. fumonisin has been revealed as an exacerbating factor in susceptibility to HIV (Williams et al., 2010). It is therefore important to realize that poor nutrition and disease can have a synergic effect on the welfare of the world's poorest people, particularly the combination of limited calories, mycotoxin-contaminated grain, HIV and other diseases in sub-Saharan Africa, where maize is a staple crop. Bt maize shows a consistently lower level of mycotoxin contamination and can therefore help to address this compound effect. There is also evidence that the lower levels of mycotoxin contamination specifically attract a price premium in some developing countries, providing another impetus to lift farmers out of poverty (Yorobe, 2004).

1.2 Conclusions

Advances in plant transformation and gene expression technology allow the introduction of novel traits into crop plants. Genetically engineered crops have been used commercially for more than 16 years, although all varieties to date provide either herbicide tolerance, pest resistance or both. Impact studies show that these crops are beneficial to farmers and consumers and produce significant aggregate welfare gains (Qaim, 2009). Biotechnology has the potential to develop crops with additional valuable traits, such as better nutritional properties (second generation of g*enetically engineered crops*), or the production of added-value molecules such as vaccines, fuels and industrial enzymes (third generation of *genetically engineered crops*; Zhu et al. 2007; Ramessar et al. 2009b; Godwin et al. 2009; Farre et al. 2010). These new traits can help to address malnutrition and to fight food insecurity worldwide. However, no biofortified crops have yet been approved for cultivation even though Golden Rice was developed in 1999 (Potrykus 2010). This reflects that it is time to implement sensible and rational policies that do not introduce unnecessary delays in the approval process. The implementation of such policies would allow those most in need to benefit from biofortified genetically engineered crops.

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AIMS AND OBJECTIVES

Aims

The overall aim of my research program was to develop an in-depth understanding of ascorbic acid metabolism and its regulation in maize. I also explored the creation of plants with multiple insecticidal genes as a first step in developing maize plants already engineered to accumulate vitamins protected against several pests.

Objectives

1. To investigate the maize L-galactose pathway and to evaluate the relationship between ascorbic acid accumulation and the L-galactose pathway.

2. To investigate the L-galactose and ascorbic acid recycling pathways in the endosperm of wild-type maize (three different genetic backgrounds) and a transgenic line with enhanced ascorbic acid recycling in order to gain insight into the key components of the ascorbic acid regulatory network.

3. To generate maize plants expressing specific transgenes involved in ascorbic acid metabolism for further analysis.

4. To engineer maize plants containing and expressing multiple insecticidal genes for further analysis.

CHAPTER 2

THE MAIZE L-GALACTOSE PATHWAY

Chapter 2: The maize L-galactose pathway

2.1 Abstract

Ascorbic acid (vitamin C) is synthesized from D-glucose in plants and functions as an antioxidant and enzyme cofactor in metabolic pathways and in response to abiotic stress and pathogens. Plants also provide the major source of vitamin C in the human diet. Maize (*Zea mays* L.) is a model for the analysis of crop genetics, evolution and domestication. Ascorbic acid metabolism and the genetic mechanisms controlling ascorbic acid accumulation in maize are poorly understood. Biochemical analysis indicates that ascorbic acid is synthesized mainly from L-galactose in *Arabidopsis thaliana*, so understanding how maize ascorbic acid metabolism is controlled should provide a basis for engineering or otherwise enhancing its accumulation. In this chapter, I describe the analysis of gene copy number and expression profiles in the maize L-galactose biosynthesis pathway, in comparison to ascorbic acid levels in the same tissues in order to gain insight into the regulation of ascorbic acid metabolism in maize.

2.2 Introduction

Ascorbic acid (AsA) is the most abundant water-soluble antioxidant in higher plants and has many different functions in diverse physiological processes. It is involved in the regulation of development, cell division and cell expansion (Davey et al., 2000; Smirnoff, 2000a), thus *Arabidopsis thaliana* (Arabidopsis) *vtc* mutants (AsA-deficient) and transgenic plants with disrupted AsA metabolism show abnormal growth and development (Veljovic-Jovanovic et al., 2001; Alhagdow et al., 2007). AsA also protects plant tissues against damage caused by reactive oxygen species (ROS) reflecting its potent antioxidant activity (Noctor and Foyer, 1998). ROS are toxic because of their ability to damage proteins, lipids and DNA, and are produced by environmental stresses such as drought, extreme temperatures, intense light or nutrient deprivation (Foyer et al., 1994). AsA therefore promotes resistance to numerous environmental stresses including ozone (Sanmartin et al., 2003), water stress (Wang et al., 2010), excess light (Yabuta et al., 2007; Li et al., 2009) and pathogens (Pavet et al., 2005).

2.2.1 Ascorbic acid biosynthesis in plants

There are several AsA biosynthesis pathways in plants (**Fig. 1**) but the prevalence of these pathways in different tissues and/or developmental stages is largely unknown (Davey et al., 2000; Jain and Nessler, 2000; Valpuesta and Botella, 2004). The first AsA biosynthesis pathway to be described was the L-galactose pathway (Wheeler et al., 1998). The phenotypic analysis of Arabidopsis *vtc* mutants with low AsA levels and transgenic plants expressing L-galactose pathway genes provides strong evidence that this is the main AsA biosynthesis pathway in this species (Conklin et al., 1999; Dowdle et al., 2007; Gatzek et al., 2002; Tabata et al., 2002). D-glucose is converted into AsA via the following intermediate compounds: GDP-D-mannose, GDP-L-galactose, L-galactose-1-phosphate, L-galactose and L-galactono-1,4-lactone (**Fig. 1**). All the corresponding genes have been characterized in plants, including those encoding GDP-D-mannose pyrophosphorylase (GMP) (Conklin et al., 1999), GDP-D-mannose-3',5'-epimerase (GME) (Gibson et al., 2008; Wolucka and Van Montagu, 2003), GDP-L-galactose phosphorylase (GGP) (Dowdle et al., 2007; Laing et al., 2007), L-galactose-1-phosphate phosphatase (GPP) (Laing et al., 2004), L-galactose dehydrogenase (GDH)

(Gatzek et al., 2002) and L-galactono-1,4-lactone dehydrogenase (GalLDH) (Imai et al., 1998; do Nascimento et al., 2005). Biochemical analysis has shown that GDP-L-gulose is an alternative product of GME (Wolucka and Van Montagu, 2003), which establishes the L-gulose branch of the pathway (**Fig. 1**). The subsequent steps in the branch have not yet been described in plants. A further pathway starting with D-galacturonate was proposed following the identification of a D-galacturonate reductase (*GalUR*) gene in strawberry (Agius et al., 2003) (**Fig. 1**). Finally, a pathway starting with *myo*-inositol was proposed when the Arabidopsis *myo*-inositol oxygenase gene was cloned (Lorence et al., 2004) (**Fig. 1**).

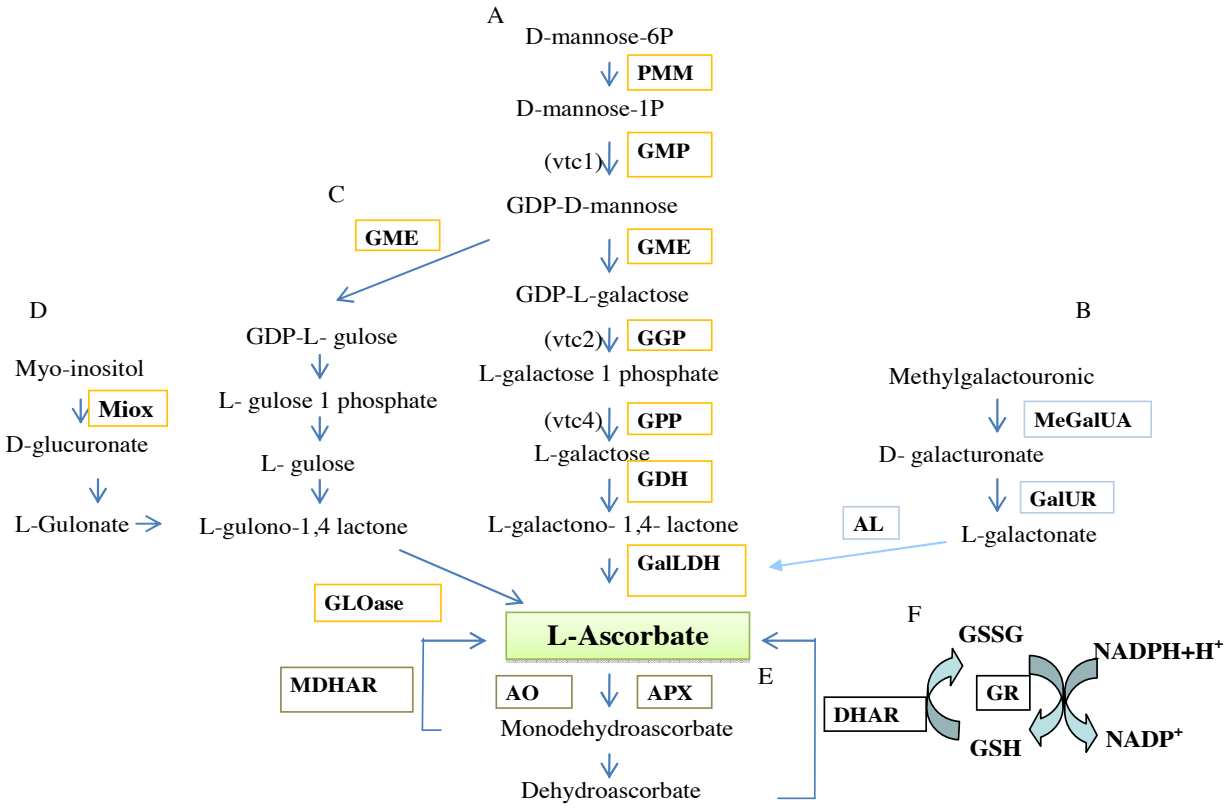


Figure 1 Postulated ascorbic acid biosynthesis and recycling pathways in plants (Ishikawa et al., 2006; Ishikawa and Shigeoka, 2008). Entries in parentheses indicate the names of Arabidopsis *vtc* (ascorbic acid-deficient) mutants. Enzymes: (A) The L-galactose pathway: phosphomannomutase (PMM), GDP-mannose pyrophosphorylase (GMP), GDP-mannose-3',5'-epimerase (GME), GDP-L-galactose phosphorylase (GGP), L-galactose 1-phosphate phosphatase (GPP), L-galactose dehydrogenase (GDH), L-galactono-1,4-lactone dehydrogenase (GalLDH). (B) The D-galacturonic pathway: methylesterase (MeGalUA), D-galacturonate reductase (GalUR), aldonolactonase (AL). (C) The L-gulose pathway. (D) The *myo*-inositol pathway: *myo*-inositol oxygenase (MIOX), L-gulonolactone oxidase (GLOase). (E) The ascorbic acid recycling pathway: ascorbate oxidase (AO), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR).

2.2.2 Ascorbic acid recycling in plants

The AsA pool is generated not only by de novo synthesis, but also by recycling through the ascorbate-glutathione cycle, which involves the enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Halliwell and Gutteridge, 1989; Noctor and Foyer, 1998). APX planes a key role in ROS scavenging in higher plants (Shigeoka et al., 2002). It converts hydrogen peroxide (H_2O_2) to H_2O and O_2. The detoxification of H_2O_2 by APX is followed by a set of reactions catalyzed by MDHAR, DHAR and GR. AsA is oxidized to monodehydroascorbate (MDHA) by APX or ascorbate oxidase (AO) (**Fig. 1**). MDHA is an unstable radical that can spontaneously undergo disproportionation to AsA and DHA, or it can be reduced back to AsA by MDHAR, which requires NAD(P)H as a reductant. Dehydroascorbate (DHA) can be reduced back to AsA by DHAR, using reduced glutathione (GSH) as the reductant (**Fig. 1**). The oxidized glutathione (GSSG) is then reduced by GR (Loewus, 1988; Smirnoff, 1995) (**Fig. 1**). The ascorbate-glutathione cycle is found in different cellular compartments including chloroplasts, peroxisomes and the cytosol, protecting them against oxidative damage. This role is critical in chloroplasts because it removes the large amounts of H_2O_2 generated by photosynthesis (Loewus and Loewus, 1987; Smirnoff et al., 2001). Phylogenetic and molecular analysis of APX genes indicates that the different APX isoforms arose through a complex evolutionary process involving several gene duplications, suggesting a close relationship among proteins located in the same subcellular compartment (Teixeira et al., 2004; 2006). The final subcellular localization of the APX isoenzyme is determined by the presence of organelle-specific targeting peptides and transmembrane domains that are found at the N-terminal and C-terminal regions of the protein (Shigeoka et al., 2002; Teixeira et al., 2004).

2.2.3 Ascorbic acid content of plant tissues

AsA accumulation in plants is influenced by many factors, including light (Tabata et al., 2002; Badejo et al., 2009; Li et al., 2009), age (Bartoli et al., 2000), tissue (Lorence et al., 2004) and cell compartment (Davey et al., 2000; Zechmann et al., 2011). Light affects the AsA content of leaves more than fruits (Massot et al., 2012) and AsA tends to concentrate in photosynthetic tissues, fruits and meristems rather than non-

photosynthetic tissues such as roots (Davey et al., 2000; Alhagdow et al., 2007). However, sink tissues such as fruits and tubers require AsA for the regulation of cell cycle transitions, signaling, cell division and cell elongation (Smirnoff, 2000b). Substantial changes in the AsA content of plant tissues and organs are also observed in relation to developmental processes such as seed germination (Pallanca and Smirnoff, 1999; Tommasi et al., 2001), fruit development (Davey et al., 2004; Agius et al., 2003; Li et al., 2010a) and post-harvest storage (Lee and Kader, 2000; Davey and Keulemans, 2004). In addition, it has been shown that AsA is actively transported between the leaves and seeds as shown in Arabidopsis, *Medicago truncatula* and potato (Franceschi and Tarlyn, 2002; Tedone et al., 2004).

2.3 Aim

The aim of the work described in this chapter was to develop an in-depth understanding of the regulation of ascorbic acid metabolism in different maize tissues. We investigated the maize L-galactose pathway at the mRNA and ascorbic acid/dehydroascorbate levels in order to evaluate whether ascorbic acid accumulation is related to L-galactose biosynthesis.

2.4 Materials and methods

2.4.1 *In silico* analysis of maize genes in the L-galactose biosynthesis pathway

Maize cDNA sequences representing genes in the L-galactose pathway (*gme*, *ggp, gpp, gdh* and *galldh*) were sought in GenBank (www.ncbi.nlm.nih.gov/Entrez/) and the maize genomic database MaizeGDB (www.maizegdb.org) using known Arabidopsis, rice and tobacco sequences as queries. The maize genomic database was also used to find the chromosomal loci of the genes. Intron/exon boundaries were determined by aligning the maize cDNA and genomic sequences using Vector NTI Suite 8.0 (Invitrogen). ESTs and conceptual translation were used to distinguish gene paralogs.

2.4.2 Plant material

Maize plants (*Zea mays* L. cv. B73) were grown in a growth chamber and in the greenhouse 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. Roots at 4 and 10 days after germination, young leaves (1 month old), mature leaves (4 months old) and endosperm at 20, 25 and 30 days after pollination (dap) were frozen in liquid nitrogen and stored at –80ºC.

2.4.3 DNA analysis of endogenous maize genes

Genomic DNA was isolated from maize leaves by phenol extraction (Edwards et al., 1991) and 20-μg aliquots were digested overnight with different restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII and/or *Xba*I). The DNA was fractionated by 0.8% agarose gel electrophoresis (Sambrook et al., 1989), transferred to a positively-charged nylon membrane (Roche, UK) and fixed by UV cross-linking. DIG-labeled probes generated by PCR using the exon-specific primers listed in **Table 1** were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) and denatured at 95ºC for 10 min prior to hybridization overnight at 42ºC. Membranes were washed at high stringency (twice for 5 min in 2x SSC, 0.1% SDS at room temperature, twice for 25 min in 0.5x SSC, 0.1% SDS at 68ºC, once for 15 min in 0.2x SSC, 0.1% SDS at 68ºC, and once for 10 min in 0.1x SSC, 0.1% SDS at 68ºC) prior to chemiluminescent detection using the DIG

Luminescent Detection Kit (Roche, UK) according to the manufacturer's instructions. After washing, the membranes were incubated with CSPD chemiluminescent substrate (Roche, UK) and exposed on BioMax light film (Kodak, Rochester, NY, USA) at 37ºC for 2 h at room temperature (or overnight if the signal was weak).

Gene	Forward primer sequence	Reverse primer sequence
gme	5'-CGAGAAGCTGCGGGTCTCCATCACC-3'	5'-ACCTCTTGACGCCGTTAATTC-3'
ggp	5'-CAGGCGTACTACTTGTCAGTGC-3'	5'-GTTGTCTTGCAGCCAAATGC-3'
gpp	5'-CAGGTGGATTTGGTGACGGAG-3'	5'-GTGGTCCCGTCGAGGGGATCG-3'
gdh	5'-GGTGGAGGAGAATGTGGCGGC-3'	5'-GTCCCCTTCCATCAATGACCA-3'
galldh	5'-GGACGAACTTGCCGAACTCC-3'	5'-GACAGCCAGGGTAGGGTCCC-3'

Table 1. Primer sequences used to amplify the exons of endogenous maize genes in the L-galactose biosynthesis pathway.

2.4.4 Ascorbic acid measurement

Total AsA and reduced AsA levels were measured in aliquots of the same extracts using the method described by Gillespie and Ainsworth (2007), allowing the DHA content to be deduced by subtraction. Five independent biological replicates of each sample were used for AsA measurements. Approximately 0.2 g of tissue was homogenized under liquid nitrogen, extracted in 6% trichloroacetic acid (TCA) and centrifuged for 5 min at 13,000 x g and 4ºC. The supernatant was mixed with 75 mM phosphate buffer (pH 7.0) and aliquots were removed for testing. To determine the total AsA content, the aliquot was supplemented with 50 mM dithiothreitol (DTT) to reduce DHA to AsA, and excess DTT was removed by adding 0.5% N-ethylmaleimide (NEM). To determine the specific AsA content, the aliquot was left untreated. Both aliquots were mixed with 10% TCA, 43% H_3PO_4, 4% 2,2'-bipyridine and 3% $FeCl_3$, and were incubated at 37°C for 1 h. The absorbance at 525 nm was then determined using a Unicam UV2 spectrophotometer (Speck & Burke Analytical, Clackmannanshire, Scotland). An AsA standard curve was used for calibration between 2.5 and 40 μM.

2.4.5 Isolation of total mRNA

Total RNA was extracted from roots at 10 days after germination, young and mature leaves and endosperm at 20, 25 and 30 dap. Approximately 1.2 g of tissue was ground to a powder under liquid nitrogen with a sterile mortar and pestle, and the powder was vortexed in 1.2 ml TRIzol Reagent (Sigma-Aldrich, USA). The homogenized samples were incubated for 5 min at room temperature and 240 µl chloroform (Merck, Darmstadt, Germany) was added to promote the complete dissociation of the nucleoprotein complexes. The samples were mixed vigorously and centrifuged for 20 min at 12,000 x g and 4ºC. Centrifugation separated the sample into a lower red phenol-chloroform phase and an upper colorless aqueous phase. The RNA was precipitated from the aqueous phase by mixing with 600 µl isopropanol (Sigma-Aldrich, USA). The samples were incubated at room temperature for 10 min and centrifuged for 10 min at 12,000 x g and 4ºC. The supernatant was removed and the RNA pellet was washed with 75% ethanol for 25 min on ice. The samples were centrifuged for 5 min at 12,000 x g and 4ºC and then DNA contamination was removed on-column using RNase-free DNase (QIAGEN, Hilden, Germany). The RNA concentration was determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.4.6 Relative quantification of gene expression by qRT-PCR in different tissues

First-strand cDNA synthesis with 2 µg of total RNA was carried out using the Omniscript® Reverse Transcription kit (QIAGEN). Quantitative real-time PCR (qRT-PCR) was carried out using a BioRad CFX96^{TM} system. Each 25-µl reaction comprised 5 ng cDNA, 1x iQ SYBR green supermix (BioRad, Hercules, CA, USA) and 5 µM of the forward and reverse primers designed using expressed sequence tags (ESTs) from the maize genome database (**Table 2**). The primers were designed using Genamics Expression software (Genamics, Hamilton, New Zealand). Relative expression levels were calculated on the basis of serial dilutions of cDNA (100–0.16 ng) which were used to generate standard curves for each gene. Triplicate amplifications were carried out in 96-well optical reaction plates by first heating to 95°C for 5 min followed by 40 cycles at 95°C for 30 s, 59.4°C for 30 s and 72°C for 30s. Amplification 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 with BioRad CFX96^{TM} software. Values

represent the mean of three replicates ± standard error. Each qRT-PCR for each gene was carried out two times. Amplification efficiencies were compared by plotting the ΔCt values of different primer combinations of serial dilutions against the log of starting template concentrations using the CFX96^{TM} software. The Ct values were adjusted to the standard curves and were normalized against the levels of *actin* mRNA.

Gene	Forward primer sequence	Reverse primer sequence
gme1	5'-GCGATGAGATGGTGAGCATG-3'	5'-CAGCCCATCCTTGAGCTTCAT-3'
gme2	5'-ATTGGAAGCGACGAAATGG-3'	5'-CTCCTTGATGAGCGTGTTGTC-3'
ggp	5'-TTTTCCTGTTCCCCCAGTG-3'	5'-TAGTCCATCCTCCGTTTCAGC-3'
gpp	5'-ACGGATGTGTGGATCATTGG-3'	5'-GCTGGGGTCAAAAACAAGGC-3'
gdh	5'-TGATGGAAGGGGACTGTTGG-3'	5'-CCGTATGGGAAAGGCTTATTC-3'
galldh	5'-CCAAGAAGAAGACCGTCACG-3'	5'-ATGTTGCACCAGTGCCATG-3'
actin	5'-CGATTGAGCATGGCATTGTCA-3'	5'-CCCACTAGCGTACAACGAA-3'

Table 2. Primer sequences used for quantitative real-time PCR analysis, including the maize *actin* reference gene and endogenous genes from the maize L-galactose biosynthesis pathway.

2.4.7 Statistical analysis

Five B73 maize plants were used for each experiment, and one sample from each of the five plants was used for RNA extraction for the different tissues that were analyzed. The qRT-PCR data represent the mean of six replicates ± standard errors. Five biological replicates were used for each AsA measurement at each time point (n = 5). The AsA content was calculated by comparison with values on the standard curve. Values represent mean ± standard error. The data were analyzed by two-way ANOVA using the residual mean square as the estimate variability.

2.5 Results

2.5.1 *In silico* characterization of the L-galactose pathway in maize

L-galactose pathway ESTs identified from the databases as discussed above are listed in **Table 3**. Two maize *gme* cDNA sequences were found in GenBank with *gme1* cDNA (EU960153) showing 70% identity to *gme2* cDNA (EU964829).

Genes	Accession number of maize sequence	Accession number of the query sequence (species)
ggp	DT943063	NM_118819.2 (*Arabidopsis thaliana*)
gpp	DV506736; DR828759	EU700060.1 (*Nicotiana tabacum*)
gdh	BQ527895; CD436358	NM_119525.5 (*Arabidopsis thaliana*)
galldh	DT943591; DR820300; DV542570; EC868512	NM_114662.2 (*Arabidopsis thaliana*)

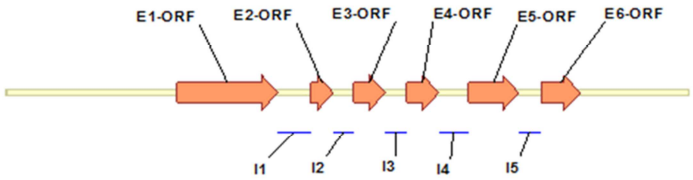
Table 3. Maize L-galactose pathway EST sequences from the maize genomic database corresponding to query genes from other species.

The corresponding rice genes (*Oryza sativa* L. ssp. *japonica*) were also recovered from GenBank in order to compare rice and maize and establish a framework connecting different grass species (**Fig. 2**). This comparison also provided integrated data concerning gene locations in cereal species, allowing the physical and genetic maps of maize to be aligned with the rice genome sequence.

This analysis revealed two putative loci for the maize *gme* gene on chromosomes 1 and 4 **(Fig. 2A, 2B**), but only one each for *ggp*, *gpp*, *gdh* and *galldh* **(Fig. 2C–F)**. Maize *ggp, gdh* and *galldh* were located on chromosomes 6, 10 and 2, respectively (**Fig. 2C, 2E, 2F**). Although gaps were found in the maize *gpp* sequence and its locus could not be confirmed, its rice ortholog was localized on chromosome 3 (**Fig. 2D**). The rice *gme1* and *gme2* genes were located on chromosomes 10 and 11, respectively. The exon-intron structures of *gme1* and *gme2* were similar in maize and rice (**Fig. 2A, 2B**). Rice *ggp* and *galldh* could not been found in the rice database due to sequence gaps, but rice *gdh* was located on chromosome 12 (**Fig. 2E**). Interestingly, the complete maize *galldh* gene was located on chromosome 2 (**Fig. 2F**), but the distal part of the gene also matched sequences on chromosomes 1 and 4 suggesting the presence of truncated genes.

Fig. 2A

1



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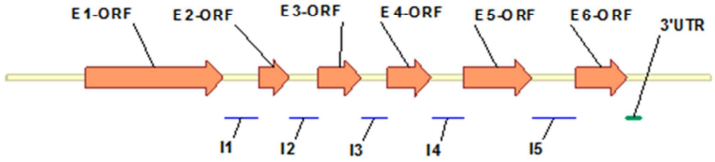
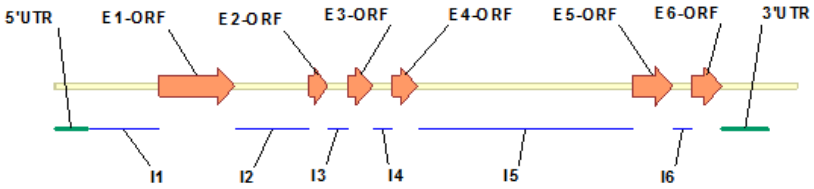


3

Species	Gene	cDNA (Acc. Num.)	Genomic DNA (Acc.Num.)	Chromosome
Zea mays	*gme1*	EU960153	AC211378	1
Oryza sativa	*gme1*	AB193582	AC016780	10

Fig. 2B

1



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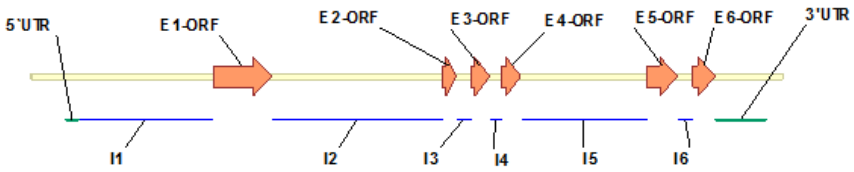
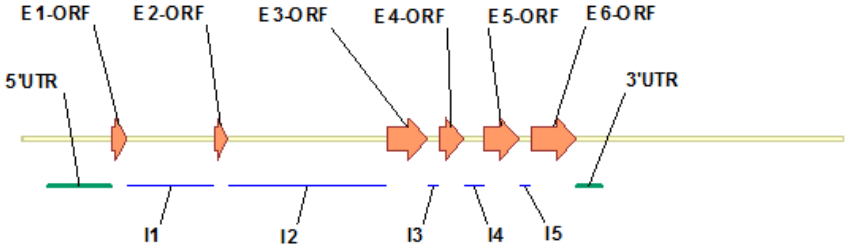


3

Species	Gene	cDNA (Acc. Num.)	Genomic DNA (Acc.Num.)	Chromosome
Zea mays	*gme2*	EU964829	AC194666	4
Oryza sativa	*gme2*	NM001074715	AC146334	11

Fig. 2C

1

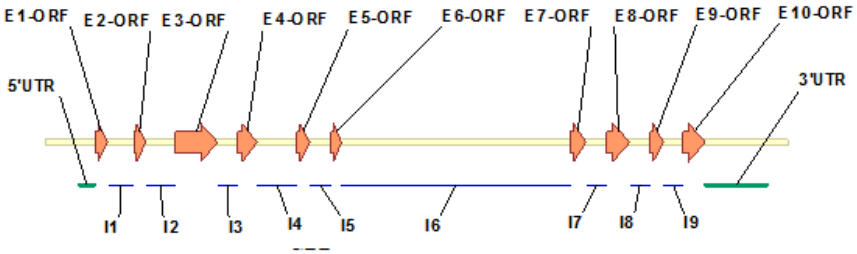


3

Species	Gene	cDNA (Acc. Num.)	Genomic DNA (Acc.Num.)	Chromosome
Zea mays	*ggp*	NM001156750	AC194394	6

Fig. 2D

2

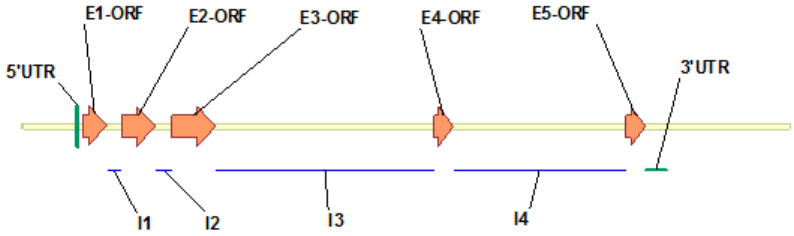


3

Species	Gene	cDNA (Acc. Num.)	Genomic DNA (Acc.Num.)	Chromosome
Oryza sativa	*gpp*	NM001057109	AC133003	3

Fig. 2E

1



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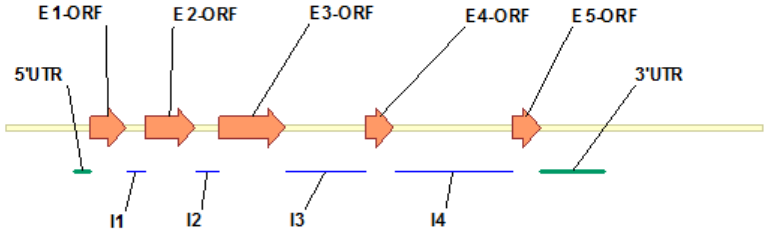
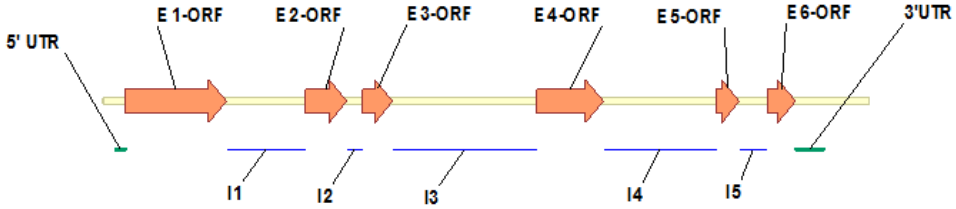


3

Species	Gene	cDNA (Acc. Num.)	Genomic DNA (Acc.Num.)	Chromosome
Zea mays	*gdh*	DQ456873	AC205424	10
Oryza sativa	*gdh*	DQ456875	AL731783	12

Fig. 2F

1



3

Species	Gene	cDNA (Acc. Num.)	Genomic DNA (Acc.Num.)	Chromosome
Zea mays	*galldh*	BT061119.1	AC210690	2

Figure 2 Genomic characterization and localization of the maize and rice genes presenting the L-galactose biosynthesis pathway. (A) GDP-mannose-3',5'-epimerase (*gme*) 1; (B) *gme2*; (C) GDP-L-galactose phosphorylase (*ggp*); (D) L-galactose-1-phosphate phosphatase (*gpp*); (E) L-galactose dehydrogenase (*gdh*); (F) L-galactono-1,4-lactone dehydrogenase (*galldh*). Genomic structures represent maize (1) and rice (2) and the accession numbers of cDNA and genomic sequences are listed (3).

2.5.2 Copy number of endogenous maize genes

DNA blots of genomic DNA from maize cultivar B73 leaves were used to determine the copy number of genes representing the L-galactose biosynthesis pathway. Genomic DNA was digested with different individual restriction enzymes (*Bam*HI (B), *Eco*RI (E), *Hin*dIII (H) and *Xba*I (X)) and hybridized with specific probes for *gme, ggp, gpp, gdh* and *galldh*. A single band was detected in all digests for *ggp, gpp* and *gdh*, suggesting a single copy was present in the maize genome (**Fig. 3**). However, *galldh* hybridized to different bands in each digest, probably because the primers for g*alldh* probe synthesis were based on the end of the cDNA, which matched the full sequence present on chromosome 2, and the truncated sequences present on chromosomes 1 and 4 (Section 2.5.1). The g*me* probe was designed to hybridize to both *gme1* (EU960153) and *gme2* (EU964829) in the consensus region, although the primer was designed based on the *gme2* sequence (**Fig. 4**). DNA blot analysis with the *gme* probe revealed two bands in the *Hin*dIII digest (**Fig. 3**), suggesting the presence of two gene copies.

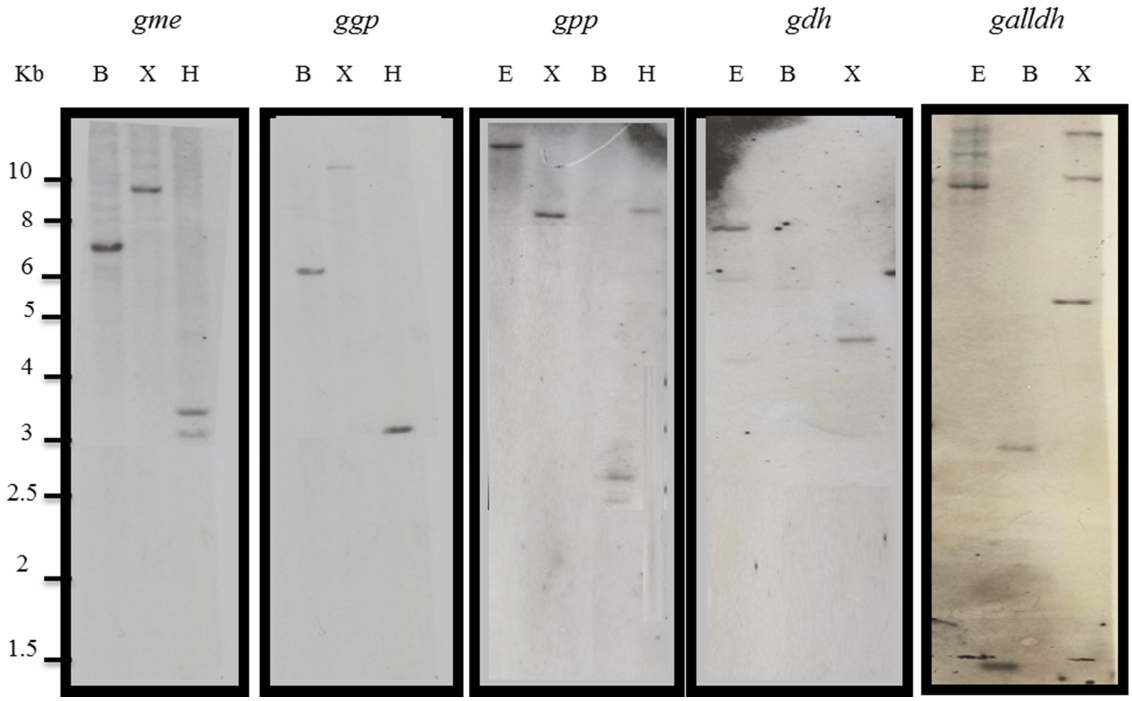


Figure 3. Genomic DNA blot analysis of maize cultivar B73 probed with sequences representing the L-galactose biosynthesis pathway. Genomic DNA isolated from leaf tissue was digested overnight with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H) and *Xba*I (X) and was probed with DIG-labeled probes specific for *gme*, *ggp*, *gpp*, *gdh* and *galldh*. Band sizes were compared to the 1-kb DNA ladder (Promega, Spain). Abbreviations: GDP-mannose-3',5'-epimerase, *gme*; GDP-L-galactose phosphorylase, *ggp*; L-galactose 1-phosphate phosphatase, *gpp*; L-galactose dehydrogenase, *gdh*; L-galactono-1,4-lactone dehydrogenase, *galldh*.

Figure 4. DNA sequence alignment of the *gme* probe, *Zm.gme1* cDNA (EU960153) and *Zm.gme2* cDNA (EU964829). The *gme* probe was designed to hybridize to both genes in a conserved region although the primers were based on the *gme2* sequence. Underlined sequences represent the primers. Highlighted bases are those that differ between the two genes.

2.5.3 Ascorbic acid content in different maize tissues

The total AsA content was measured in the endosperm at two stages (20 and 30 dap), young leaves (1 month old), mature leaves (4 months old), and roots (at 4 days and 10 days after germination). The results showed that the AsA content varied among the different tissues (**Fig. 5**). Leaves contained the highest total AsA content, at ~400 nmol g^{-1} fresh weight (FW). Endosperm contained the lowest amount (~145 nmol g^{-1} FW). The total AsA content of the roots was greater than the endosperm but declined during development (220 ± 12.1 nmol g^{-1} FW at 4 days and 168 ± 9.9 nmol g^{-1} FW at 10 days after germination) (**Fig. 5**). Generally, young tissue such as endosperm at 20 dap, young leaves and roots at 4 days after germination contained more total AsA than older tissues such as endosperm at 30 dap, mature leaves and roots at 10 days after germination. Interestingly, the predominant form of AsA in roots and endosperm was DHA. During leaf development, a slight reduction in the total AsA content was observed (405.08 ± 9.8 nmol g^{-1} FW for young leaves and 400.1 ± 8.2 nmol g^{-1} FW for mature leaves), primarily reflecting the loss of AsA rather than DHA (360.6 ± 18.3 nmol g^{-1} FW for young leaves and 326.3 ± 21.2 nmol g^{-1} FW for mature leaves). DHA levels increased only slightly during leaf development (45.2 ± 14.05 nmol g^{-1} FW in young leaves and 73.8 ± 14.7 nmol g^{-1} FW in mature leaves). The total AsA content declined during endosperm development (167.6 ± 9.9 nmol g^{-1} FW at 20 dap and 143 ± 10.1 nmol g^{-1} FW at 30 dap) mainly due to the loss of AsA, whereas DHA levels remained constant. In contrast, the total AsA content of the roots declined due to the loss of both AsA and DHA (**Fig. 5**).

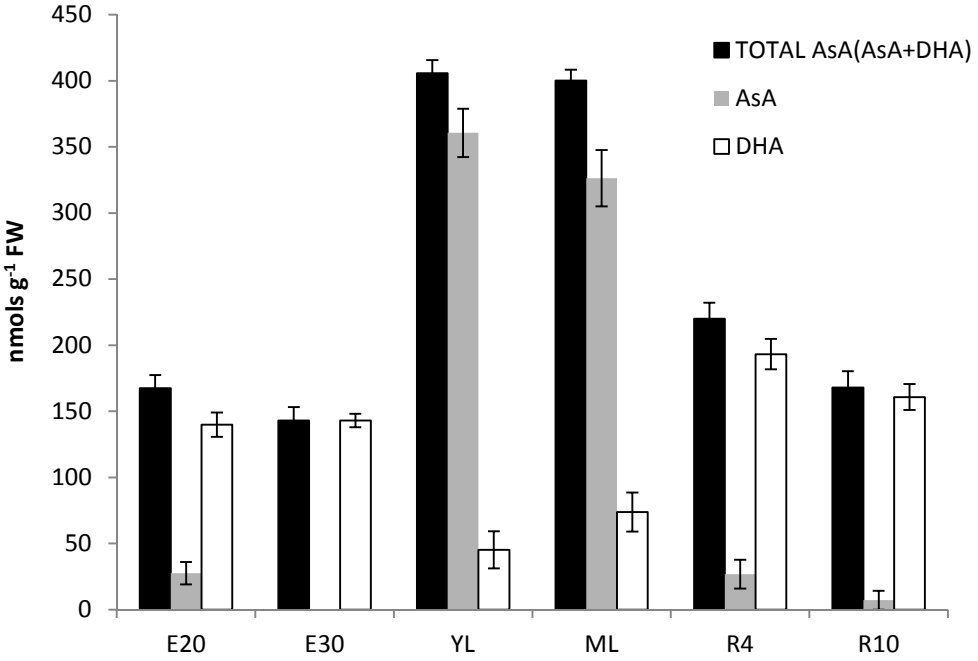


Figure 5. Total ascorbic acid (AsA plus DHA), reduced ascorbic acid (AsA) and dehydroascorbate (DHA) levels in different maize tissues (cultivar B73) in nmol g^{-1} fresh weight (FW). Endosperm at 20 dap (E20) and 30 dap (E30); young leaves (YL) and mature leaves (ML) and root at 4 days (R4) and 10 days after germination (R10). Data represent the mean ± standard error (n = 5).

2.5.4 Endogenous expression profile of genes representing the L-galactose biosynthesis pathway in different maize tissues

Quantitative real-time PCR was used to determine the levels of endogenous transcripts *gme1, gme2, ggp, gpp, gdh* and *galldh* in three different maize tissues at different developmental stages: endosperm at 20, 25 and 30 dap (E20, E25, E30), young leaves (YL), mature leaves (ML) and roots at 10 days after germination (R10). The *actin* gene was used as an internal standard. The results showed that mRNA expression levels were highest in young leaves (except *gme2*) and lowest in the endosperm (**Fig. 6**). The expression levels of *gme1, gme2* and *galldh* were lower in mature leaves compared to roots at 10 days after germination (**Fig. 6A, 6B, 6F**). The expression levels of *gme1, gme2* and *ggp* during endosperm development and *ggp* in roots were only just above the detection threshold (**Fig. 6A, 6B, 6C**). Interestingly, *gdh* was expressed at high levels in all tissues compared to the other transcripts and was expressed at high levels in young leaves (**Fig. 6E**), followed in order by *ggp*, *gpp*, *gme1*, *galldh* and finally *gme2* (**Fig. 6**).

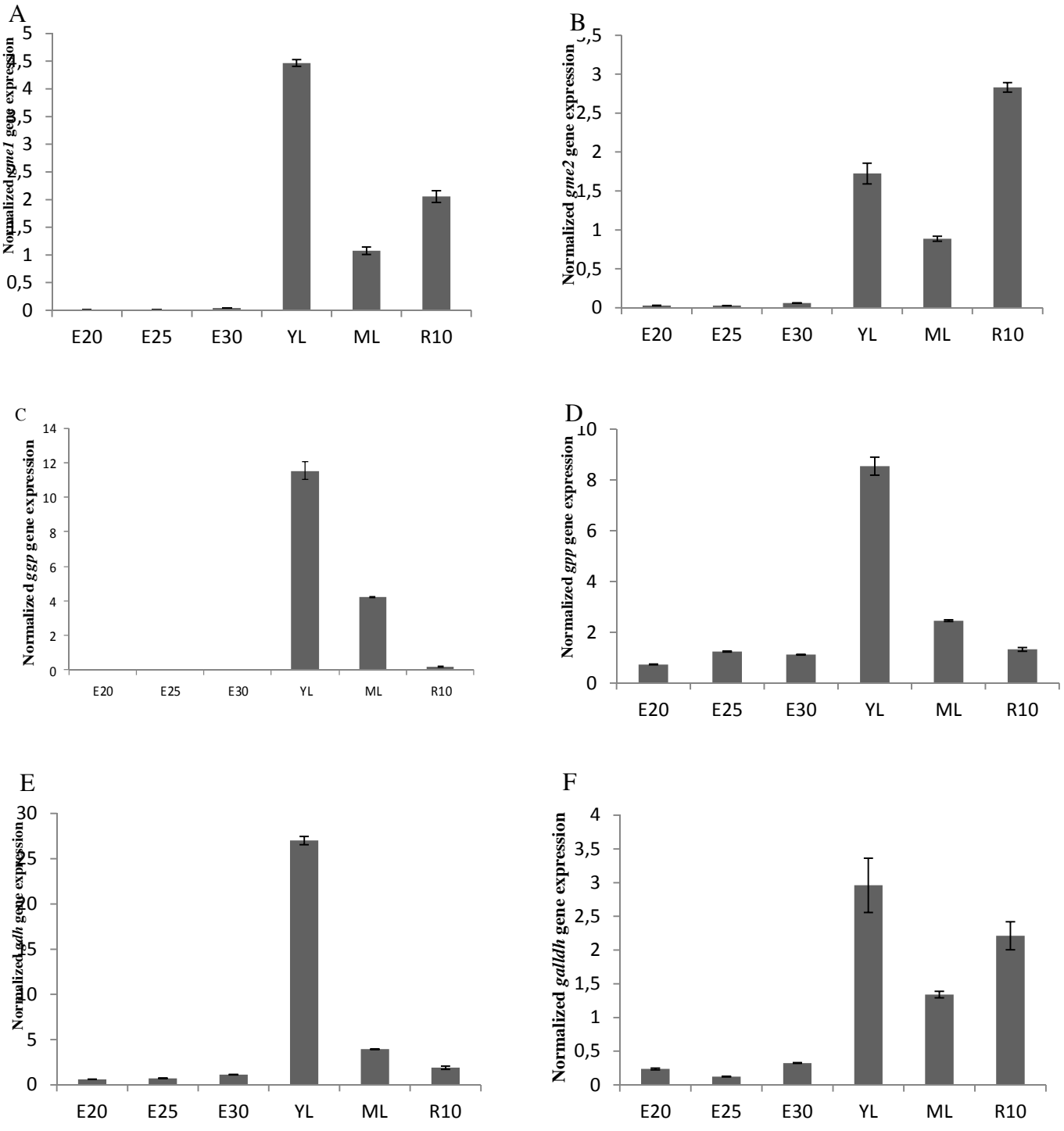


Figure 6. Transcript levels of endogenous genes representing the L-galactose biosynthesis pathway in maize (cultivar B73) tissues. Data were normalized against *actin* mRNA and represent the mean of six replicates ± standard error. Abbreviations: endosperm at 20 dap (E20), 25 dap (E25) and 30 dap (E30); young leaves (YL); mature leaves (ML); and roots at 10 days after germination (R10). Transcripts: (A) GDP-mannose-3',5'-epimerase 1 (*gme1*); (B) *gme2*; (C) GDP-L-galactose phosphorylase (*ggp*); (D) L-galactose 1-phosphate phosphatase (*gpp*); (E) L-galactose dehydrogenase (*gdh*); and (F) L-galactono-1,4-lactone dehydrogenase (*galldh*).

2.6 Discussion

2.6.1 Identification of maize genes involved in the L-galactose pathway

In order to study the regulation of AsA metabolism in maize tissues, the first step was to determine the copy number of genes involved in the L-galactose pathway using a combination of bioinformatics and genome analysis. The selected genes encompass one part of the three processes that could determine AsA levels in maize: biosynthesis, turnover and transport. Genes from alternative biosynthesis pathways were discounted because there were no maize orthologs in the maize EST database although maize genes from the L-galactose pathway were identified (**Table 3**). *In silico* analysis revealed one copy each of *ggp, gpp, gdh* and *galldh*, which mapped to different loci, as confirmed by DNA gel blot analysis (**Fig. 2C, 2D, 2E, 2F**). There are also single copies of *gdh* in Arabidopsis and spinach (Gatzek et al., 2002; Mieda et al., 2004), *galldh* in lotus and legumes such as pea and bean (Matamoros et al., 2006) and *ggp* in kiwifruit (Bulley et al., 2009). In contrast, two *ggp* genes are found in Arabidopsis: *VTC2* and *VTC5* (Dowdle et al., 2007). We identified two maize *gme* genes (**Fig. 2A, 2B**) as previously reported in tomato and rice, although only one copy is found in Arabidopsis (Wolucka and Van Montagu, 2003; Watanabe et al., 2006; Du et al., 2006; Zhang et al, 2011). Our *in silico* data resolved rice *gme1* to chromosome 10 and rice *gme2* to chromosome 11 (**Fig. 2A, 2B**).

2.6.2 Ascorbic acid content in maize tissues

AsA tends to accumulate at higher levels in photosynthetic tissues compared to non-photosynthetic tissues such as seeds, roots and tubers (Davey et al., 2000). The AsA content in maize was measured in endosperm, leaves and roots at different developmental time points: endosperm at 20 and 30 dap, young and mature leaves, and roots at 4 and 10 days after germination. Our results showed that maize leaves had a higher total AsA content than roots and endosperm, consistent with reports describing the AsA content of different tomato tissues: highest in leaves followed by flowers, fruits and roots (Zhang et al., 2011). In contrast, the highest AsA content in Arabidopsis was reported in flowers, and was 2–3 times greater than the content in leaves (Conklin et al., 2000; Lorence et al., 2004).

AsA is involved in developmental processes such as cell division and expansion (Smirnoff, 2000b; Conklin, 2001; Arrigoni and De Tullio, 20002), and accordingly we found that AsA levels were higher in young tissues (endosperm at 20 dap, young leaves and roots at 4 days after germination) than in its older tissues, reflecting the higher rates of cell expansion and cell division in the former (Smirnoff et al., 2001; Arrigoni and De Tullio, 2002). The Arabidopsis mutants *vtc1* and *vtc2* have constitutively low AsA levels compared to wild type plants and significantly shorter shoots which accumulate less biomass (Olmos et al., 2006). The low levels of AsA in *vtc2* mutants also affect the growth of the primary root (Olmos et al., 2006).

During the development of maize leaves, the AsA content decreased but the DHA content increased. The accumulation of AsA in young maize leaves reflected the higher expression levels of all the L-galactose pathway genes (*gme1, gme2, ggp, gpp, gdh* and *galldh*). Similar results have been reported in apple leaves, with the exception of *ggp* and *gpp* (Li et al., 2010b). The capacity to synthesize AsA in young leaves is therefore greater than in mature leaves, and the rate of AsA biosynthesis exceeds the rate of loss by oxidation (DHA degradation) whereas the opposite occurs in mature leaves.

During maize endosperm development, the AsA content decreased whereas the DHA content remained constant. Previous studies have shown that mature maize kernels contain little or no AsA but small amounts of DHA (Tommasi et al., 1999) and that the gradual physiological dehydration that accompanies the onset of desiccation tolerance induces the ascorbate peroxidase system and the degradation of AsA (Kermode et al., 1990). No correlation was found between the expression profiles of the four biosynthetic genes and the corresponding AsA profile during maize endosperm development, suggesting that the regulation of AsA accumulation in maize is more complex and may also involve AsA transport from leaves to sink tissues or alternative pathways. For example, AsA levels in strawberry correlated with the induction of genes encoding the enzymes GalUR and MYOX representing alternative synthesis pathways (Cruz-Rus et al., 2011) (**Fig. 1**). Further studies have confirmed AsA transport via the phloem from source leaves to sink tissues such as root tips, shoots and floral organs in Arabidopsis, *Medicago sativa* and potato (Franceschi and Tarlyn, 2002; Tedone et al., 2004).

The regulation of AsA accumulation in root meristems appears to involve turnover though the activity of AO (Kerk and Feldman, 1994; 1995). A model for the control of root development has been proposed that interconnects AO, auxins and AsA (Kerk et al., 2000). In this model, the accumulation of auxins at the root tip leads to an increase in AO activity and a subsequent reduction in AsA content, thus inhibiting cell division (Kerk et al., 2000). The low levels of AsA also influence root morphology (Olmos et al., 2006). The primary roots of Arabidopsis *vtc2* mutants generally show poor gravitropic responses and a greater number of lateral roots (Olmos et al., 2006). This suggests that low levels of AsA promote lateral root formation. AsA levels decline during early maize root development (4–10 days after germination) which may also reflect the formation of lateral roots.

2.6.3 Regulation of L-galactose pathway genes in maize

Although all the L-galactose pathway genes were expressed at high levels in young leaves (**Fig. 6**), *gdh* was much more active than the other genes. GDH is a later-acting enzyme in the pathway, catalyzing the conversion of L-galactose to L-galactono-1,4-lactone. GDH activity was shown to vary from 50 pmol min^{-1} mg^{-1} protein in *Brassica* species to 200 pmol min^{-1} mg^{-1} in barley and pea, with maize achieving an intermediate value of 200 pmol min^{-1} mg^{-1} (Gatzek et al., 2002). Like the other genes, we found that *gdh* was expressed at higher levels in young leaves than mature leaves. Similar results were observed in apple, were younger leaves (1 month old) contained more AsA and more abundant *gdh* transcripts than mature leaves at 5 months old (Li et al., 2010b). In spinach, GDH is competitively inhibited by AsA (Mieda et al., 2004), explaining the reported end-product feedback inhibition of AsA biosynthesis (Pallanca and Smirnoff, 2000). This may explain why the *gdh* transcript in mature maize leaves declined when the AsA content reached a higher level in young leaves (**Fig. 5 and 6E**). However, AsA was shown to accumulate after L-galactose feeding (Wheeler et al., 1998) and the overexpression of Arabidopsis GDH had no effect on the AsA content of tobacco (Gatzek et al., 2002). Further investigation is therefore necessary to determine the role of GDH in the control of AsA synthesis via the L-galactose pathway.

The *gpp* gene was initially identified in the AsA-deficient Arabidopsis mutant *vtc4* (Conklin et al., 2006). The corresponding enzyme catalyzes conversion of L-galactose-

1-phosphate to L-galactose, but it can also hydrolyze L-galactose 1-phosphate and *myo*-inositol 1-phosphate (**Fig. 1**). Both substrates are involved in two AsA biosynthesis pathways but L-galactose 1-phosphate is used more efficiently (Laing et al., 2004). GPP could play an important role in the control of AsA biosynthesis in leaves because it is upregulated at the mature stage (Li et al., 2010b) and is known to regulate AsA accumulation during tomato fruit development and ripening (Ioannidi et al., 2009). Maize *gpp* was expressed in all tissues, suggesting that it may not be controlled by light intensity.

The *ggp* gene may play a major role in the regulation of AsA biosynthesis (Dowdle et al., 2007; Linster and Clarke, 2008; Bulley et al., 2009 and 2012). Arabidopsis GGP catalyzes the conversion of GDP-L-galactose to L-galactose 1-phosphate (Linster et al., 2007; Laing et al., 2007; Dowdle et al., 2007). There are two paralogs (VTC2 and VTC5) with similar properties in Arabidopsis (Dowdle et al., 2007). Double *vtc2*/*vtc5* mutants are unable to grow unless supplemented with AsA, showing that the D-mannose pathway is the only physiologically significant source of AsA in Arabidopsis, and that AsA is essential for seedling growth (Dowdle et al., 2007). Maize *ggp* is expressed strongly in leaves but the transcripts are barely detectable in roots or endosperm, suggesting that maize *ggp* could be regulated by light. The Arabidopsis *VTC2* gene is induced by a high light intensity and the expression of both *VTC2* and *VTC5* peaks at the beginning of the light cycle, suggesting that this enzyme might play a significant role in the light-dependent regulation of AsA biosynthesis (Dowdle et al., 2007).

GME converts GDP-D-mannose to GDP-L-galactose and GDP-L-gulose (Wolucka and Van Montagu, 2003). In tomato plants, GME is an important control point between cell-wall and AsA metabolism (Gilbert et al., 2009). Maize has two GME genes (*gme1* and *gme2*) like rice and tomato (Ying et al., 2006; Watanabe et al., 2006; Gilbert et al., 2009). In tomato, *gme2* is expressed more strongly in leaves than fruit whereas the expression of tomato *gme1* is similar in both tissues (Gilbert et al., 2009). Although tomato *gme1* and *gme2* are constitutively expressed in all tissues, notable differences were observed in fruits. The overexpression of tomato *gme2* in transgenic tomato also induced *gme1*, suggesting the genes are co-regulated (Gilbert et al., 2009). In maize, *gme1* was expressed more strongly in leaves than roots, whereas *gme2* was strongly expressed in roots, and neither gene was expressed at high levels in the endosperm (**Fig.**

6A, 6B). This suggests that maize *gme1* may have a specific role in leaves and *gme2* in roots, but they may also be co-regulated. Similar results were observed in rice, i.e. both transcripts were abundant in vegetative tissues including leaves, shoots, stems and roots, but expression levels were relatively low in developing rice seeds (Ying et al., 2006).

Finally, GalLDH catalyzes the oxidation of L-galactono-1,4-lactone to AsA. In maize, *galldh* was expressed at higher levels in young leaves than mature leaves, expression in the root was also higher than in mature leaves, and only low expression levels were observed in the endosperm (**Fig. 6F**). In melon, *galldh* was expressed at higher levels in AsA-rich photosynthetic tissues such as leaves and stems compared to tissues with low AsA levels such as roots and seeds (Pateraki et al., 2004). However, the Arabidopsis *galldh* gene was expressed at a much higher level in roots than that in mature leaves, although as in maize the AsA level in the roots was lower than in mature leaves (Tamaoki et al., 2003). In contrast to the transcript levels, GalLDH activity in Arabidopsis roots was lower than in the mature leaves (Tamaoki et al., 2003). It is possible that the activity of GalLDH is post-transcriptionally regulated because mRNA levels were higher than the corresponding enzymatic activity. However, in maize leaves and roots, the expression of *galldh* mRNA correlated well with the AsA concentration. In young Arabidopsis rosette leaves, the expression profile of *galldh* mRNA and GalLDH enzyme activity were parallel to the diurnal change in AsA levels (Tamaoki et al., 2003). The *galldh* transcript levels in melon fruits also correlated with the increase in AsA levels during ripening (Pateraki et al., 2004). GalLDH activity was similar in the roots and leaves of alfalfa, pea and lotus plants, whereas bean GalLDH activity was low in roots but not in leaves (Matamoros et al., 2006). The *galldh* mRNA was present at similar levels in maize leaves and roots, but the AsA content was higher in the leaves (**Fig. 5 and 6F**), suggesting tissue-specific differences in the transport, degradation or utilization of AsA. The expression of *galldh* in leaves may also depend on light intensity, or may be inhibited by AsA feedback, as suggested in tobacco suspension cells (Tabata et al., 2002). There may also be alternative routes for AsA biosynthesis (Lorence et al., 2004).

2.7 Conclusions

The AsA content of plant cells is regulated by developmental processes that may differ according to genotype, tissue or cell type. AsA biosynthesis appears to be the major source of AsA in plant tissues, although the precise level of AsA is determined by the balance between synthesis, oxidation and loss of DHA. AsA accumulates when the rate of synthesis exceeds the loss of DHA by oxidation, which appears to be restricted to early developmental stages, e.g. in young leaves with high capacity for AsA biosynthesis. Further work is required to investigate AsA biosynthesis via alternative pathways, transport and degradation to define additional factors regulating the AsA content of plants.

2.8 Future prospects and recommendations

The results presented in this chapter provide insight into the regulation of AsA metabolism in different maize tissues. However, further experiments focusing on other AsA biosynthesis pathways, transport and degradation are required to understand the regulation of AsA accumulation in maize in more detail.

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CHAPTER 3

ASCORBIC ACID SYNTHESIS AND METABOLISM IN MAIZE ARE SUBJECT TO COMPLEX GENOTYPE-DEPENDENT FEEDBACK REGULATION DURING ENDOSPERM DEVELOPMENT

Chapter 3: Ascorbic acid synthesis and metabolism in maize are subject to complex and genotype-dependent feedback regulation during endosperm development

3.1 Abstract

L-ascorbic acid (vitamin C) is an antioxidant and electron donor whose metabolism in plants is under strict feedback control. The factors that influence its accumulation in staple crops are only partially understood. One way to gain insight into the regulation of L-ascorbic acid metabolism in maize is to investigate the endogenous metabolic pathways in different genetic backgrounds, and how they interact with imported pathways in transgenic plants. It is important to focus on the endosperm because this can be used as a source of vitamin C in nutritionally-enhanced crops. We began by investigating the developmental profile of L-ascorbic acid accumulation in the endosperm of three diverse maize genotypes and a transgenic line expressing rice dehydroascorbate reductase, which enhances L-ascorbic acid recycling. We determined the transcript levels of all the key genes as well as the specific levels of ascorbic acid and dehydroascorbate. L-ascorbic acid levels were high 20 days after pollination and declined thereafter. We found significant genotype-dependent variations in the transcript levels of some genes, with particular complexity in the ascorbic acid recycling pathway. Our data will help to elucidate the complex mechanisms underlying the regulation of L-ascorbic acid metabolism in plants, particularly the impact of genetic background on the strict regulation of ascorbic acid metabolism in endosperm cells.

3.2 Introduction

L-ascorbic acid (AsA) or vitamin C is a weak sugar acid that acts as an antioxidant and an electron donor for redox enzymes in animals and plants (Cruz-Rus et al., 2011). In animals, it is essential for the efficient synthesis of collagen, carnitine and various neurotransmitters, and the synthesis and breakdown of tyrosine (Davey et al., 2000). Humans and some other mammals must acquire AsA in their diets because they lack L-gulono-1,4-lactone oxidase, the final enzyme in the pathway (Linster and Schaftingen, 2007). Although fruits and vegetables are excellent sources of AsA (Hancock and Viola, 2005), most staple crops only produce low levels. The ability to increase AsA levels would therefore enhance the nutritional value of staple crops by providing additional vitamin C (Chen et al., 2003).

Most AsA in plants is produced via the L-galactose pathway (**Fig. 1**) in which D-mannose is converted into AsA via a series of L-galactose intermediates (Wheeler et al., 1998; Ishikawa et al., 2006). Alternative pathways can produce AsA via GDP-L-gulose, D-galacturonic acid and *myo*-inositol (Wolucka and van Montagu, 2003; Agius et al., 2003; Lorence et al., 2004). These pathways complement the major L-galactose pathway in specific tissues or under different physiological conditions (Ishikawa et al., 2006).

The AsA pool is fed not only by de novo synthesis but also through the AsA recycling pathway, which involves the enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Halliwell and Gutteridge, 1989; Noctor and Foyer, 1998) (**Fig. 1**). AsA is oxidized to monodehydroascorbate (MDHA) by APX and ascorbate oxidase (AO).). MDHA is an unstable radical that can spontaneously undergo disproportionation to AsA and DHA, or it can be reduced back to AsA by MDHAR, which requires NAD(P)H as a reductant. Dehydroascorbate (DHA) can be reduced back to AsA by DHAR, using reduced glutathione (GSH) as the reductant (**Fig. 1**). The oxidized glutathione (GSSG) is then reduced by glutathione reductase (GR) (Loewus, 1988; Smirnoff, 1995). AsA recycling is particularly active in the chloroplasts to help prevent oxidative stress by removing the large amounts of hydrogen peroxide generated by photosynthesis (Loewus and Loewus, 1987; Smirnoff et al., 2001). The principal recycling enzymes are often found as

multiple isoforms localized in different compartments including the cytosol, mitochondria, peroxisomes and microbodies (Obara et al., 2002; Teixeira et al., 2004).

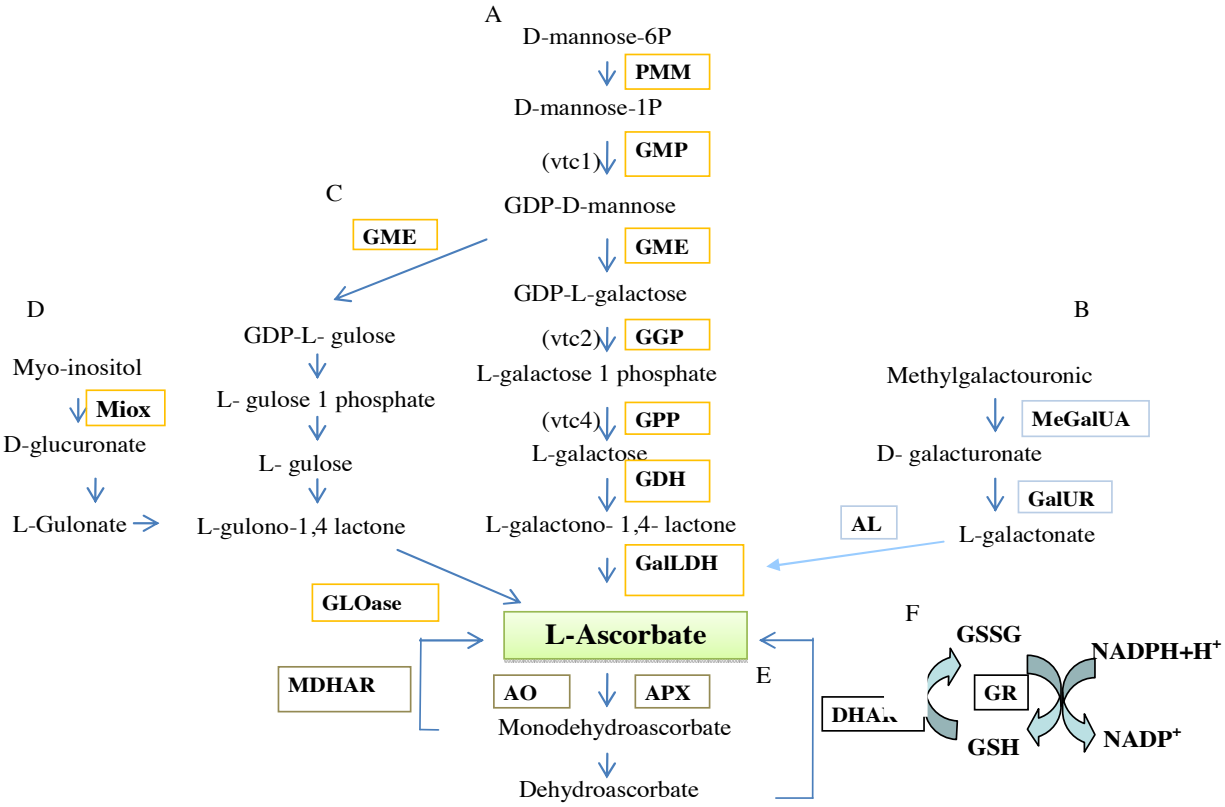


Figure 1: Postulated ascorbic acid biosynthesis and recycling pathways in plants (Ishikawa et al., 2006). Entries in parentheses indicate the names of ascorbic acid-deficient (VTC) *Arabidopsis thaliana* mutants. (A) L-galactose pathway enzymes: phosphomannomutase (PMM), GDP-mannose pyrophosphorylase (GMP), GDP-mannose-3',5'-epimerase (GME), GDP-L-galactose phosphorylase (GGP), L-galactose-1-phosphate phosphatase (GPP), L-galactose dehydrogenase (GDH), L-galactono-1,4-lactone dehydrogenase (GalLDH). (B) D-galacturonic pathway enzymes: methylesterase (MeGalUA), D-galacturonate reductase (GalUR), aldonolactonase (AL). (C) L-gulose pathway and (D) *myo*-inositol pathway enzymes: *myo*-inositol oxygenase (MIOX), L-gulonolactone oxidase (GLOase). (E) Ascorbic acid recycling pathway enzymes: ascorbate oxidase (AO), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR).

The AsA pool in plants is under complex regulation, reflecting the combination of multiple biosynthesis and recycling pathways, specific long-distance AsA transport systems (Franceschi and Tarlyn, 2002; Tedone et al., 2004), the requirement for AsA during diverse physiological processes, the tissue-specific distribution of enzymes, and

the regulation of the corresponding enzymes by light, oxidative stress, wounding and pathogens (Noctor and Foyer, 1998; Davey et al., 2000; Valpuesta and Botella, 2004; Smirnoff, 2000a). Most AsA is found in photosynthetic tissues (Davey et al., 2000) although it is also required in sink tissues for processes such as cell cycle regulation and phytohormone signaling (Smirnoff, 2000b). With this complex variety of factors influencing AsA levels in plants, it is difficult to gain insight into the key components of the regulatory network and determine the ideal intervention points for vitamin C fortification (Zhu et al., 2007).

The efficient engineering of vitamin C levels in crops therefore requires more fundamental knowledge concerning the regulation of pathways involved in AsA biosynthesis, recycling and catabolism. To address this challenge, we investigated AsA accumulation in developing maize endosperm as a function of the genetic background, both in terms of the differences between genotypes and also the influence of a transgene encoding an enzyme from the AsA recycling pathway.

3.3 Aim

The aim of the work described in this chapter was to understand in depth the regulation of ascorbic acid metabolism in maize endosperm. We therefore investigated the L-galactose and AsA recycling pathways at the mRNA and ascorbic acid/dehydroascorbate levels in the endosperm of wild-type maize (three different genetic backgrounds) and a transgenic line with enhanced ascorbic acid recycling in order to gain insight into the key components of the ascorbic acid regulatory network.

3.4 Materials and methods

3.4.1 Plant material

We selected three genetically-diverse genotypes of maize (*Zea mays* L.), namely A632 and EP42 (yellow endosperm, provided by Dr Ana Burton, CSIC, Pontevedra, Spain) and M37W (white endosperm, provided by the CSIR, Pretoria, South Africa). We also included a transgenic M37W line (L3) expressing the rice *dhar* gene in an endosperm-specific manner (Zhu et al., 2008). All plants were grown at 28/20°C (day/night) temperature with a 10-h photoperiod and 60–90% humidity for the first 50 d, then at 21/18°C with a 16-h photoperiod thereafter. Endosperm tissue was excised from immature seeds at 20, 25, 30 and 40 days after pollination (dap), frozen in liquid nitrogen and stored at –80ºC.

3.4.2 DNA sequence analysis and database searching

The maize cDNA sequences encoding GDP-L-galactose phosphorylase (GGP), L-galactose-1-phosphate phosphatase (GPP), L-galactose dehydrogenase (GDH), L-galactono-1,4-lactone dehydrogenase (GalLDH), APX, MDHAR and DHAR were identified by searching GenBank (www.ncbi.nlm.nih.gov) and the maize EST database MaizeGDB (www.maizedgb.org) with BLAST using orthologous genes as query sequences (see results Section 3.5.1). We found one sequence each for the genes *ggp, gpp, gdh, galldh* (de novo synthesis) and *dhar*, as well as four paralogs of *mdhar* and six of *apx*.

3.4.3 Isolation of total RNA and mRNA blot analysis

Total RNA was isolated from the endosperm of immature maize seeds from each of the four genotypes at three developmental stages (20, 25 and 30 dap) as described in detail in Section 2.4.5. Three seeds from each of the 12 samples were pooled for RNA preparation. DNA was removed on-column using RNase-free DNase (QIAGEN, Hilden, Germany). The mRNA concentration was determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Total RNA (30 µg) was fractionated on a denaturing 1.2% (w/v) agarose gel containing formaldehyde prior to blotting. The membrane was probed with digoxigenin-labeled

partial cDNAs prepared using the PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany), with hybridization carried out at 50°C overnight using DIG Easy Hyb. The membrane was washed twice for 5 min in 2x SSC, 0.1% SDS at room temperature, twice for 20 min in 0.2x SSC, 0.1% SDS at 68°C, and then twice for 10 min in 0.1x SSC, 0.1% SDS at 68°C. After immunological detection with anti-DIG-AP (Fab-Fragments Diagnostics GmbH, Germany) chemiluminescence generated by disodium 3-(4-methoxyspiro (1,2-dioxetane-3,2'-(5'-chloro)tricyclo(3.3.1.13,7)decan)-4-yl) phenyl phosphate (CSPD) (Roche, Mannheim, Germany) was detected on Kodak BioMax light film (Sigma-Aldrich, USA) according to the manufacturer's instructions. The rice *dhar* probe (600 bp) was generated using forward primer 5'-ATG GGC GTG GAG GTG TGC GTC AAG G-3' and reverse primer 5'-GCT CTT ACG CAT TCA CTT TTG GTG-3'.

3.4.4 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was carried out as described in detail in Section 2.4.6. The primers for each gene were designed using EST sequences found in the maize genome database (http://www.maizegdb.org) and are listed in **Table 1**. The primers were designed using Genamics Expression software (Genamics, Hamilton, New Zealand).

3.4.5 Measurement of ascorbic acid and dehydroascorbate levels

The total ascorbic acid content (AsA + DHA), reduced ascorbic acid content (AsA) and dehydroascorbate content (DHA) were determined in endosperm extracts from nine individual seeds for each of 16 samples (four genotypes, four developmental stages: 20, 25, 30 and 40 dap) as described in Section 2.4.4.

Gene	Forward primer	Reverse primer
ggp	5'-TTTTCCTGTTCCCCCAGTG-3'	5'-TAGTCCATCCTCCGTTTCAGC-3'
gpp	5'-ACGGATGTGTGGATCATTGG-3'	5'-GCTGGGGTCAAAAACAAGGC-3'
gdh	5'-TGATGGAAGGGGACTGTTGG-3'	5'-CCGTATGGGAAAGGCTTATTC-3'
galldh	5'-CCAAGAAGAAGACCGTCACG-3'	5'-ATGTTGCACCAGTGCCATG-3'
dhar	5'-TGTCAGCGACTGATCTTAGCC-3'	5'-TCACGGCTGAAAAGAGCCTT-3'
mdhar1	5'-TGGTGTTTCCTGAACCTTGG-3'	5'-ATGGCATCAGCATCAAACCAC-3'
mdhar2	5'-GACTTGGCGTGGCAGTTCTA-3'	5'-TCGAGAAACACGCTGACGA-3'
mdhar3	5'-GATCATGCTCGGAAGTCTGC-3'	5'-TTCGACTGCTTCGCCGACAT-3'
mdhar4	5'-TCCTGGTGGAAATGCTGTTG-3'	5'-CATGCAGTGTTTCTCAGGGAA-3'
apx1	5'-GCTTTTGTCGGGAGAGAAGG-3'	5'-GCTTCGGTGTAGTCAGCAAA-3'
apx3	5'-GTGCCTGGACTACGAACCCTT-3'	5'-CACAAGAGGGCGGAAGACAG-3'
apx6	5'-CTCCCAACTGACAAGGCACTG-3'	5'-TACCCCAAAGGCACTCTGTG-3'
actin	5'-CGATTGAGCATGGCATTGTCA-3'	5'-CCCACTAGCGTACAACGAA-3'

Table. 1 Primer sequences for quantitative real time PCR, including maize *actin* (reference gene) and endogenous maize genes encoding enzymes involved in ascorbic acid metabolism.

3.4.6 Statistical analysis

Three wild-type plants from each variety and three transgenic plants were used for each experiment, and one seed from each of the three plants was used for endosperm RNA extraction. The qRT-PCR data represent the mean of six replicates ± standard errors. The data were analyzed by two-way analysis of variance followed by Student's t-test using the residual mean square in the ANOVA as the estimate of variability. Nine endosperm biological replicates, three from each plant, were used for each AsA measurement at each time point (n = 9). The AsA content was calculated by comparison with values obtained from a standard curve. Values represent means ± standard errors. The data were analyzed by two-way ANOVA as described above.

3.5 Results

3.5.1 Selection of genes for qRT-PCR analysis

We selected seven genes to represent the AsA biosynthesis and recycling pathways: four genes from the L-galactose pathway (*ggp, gpp, gdh* and *galldh*) and three from the recycling pathway (*apx, mdhar* and *dhar*). Single maize ESTs representing *ggp, gpp, gdh* and *galldh* were found in MaizeGDB, suggesting these genes were unique in the maize genome (**Table 2**). In contrast, four Arabidopsis *mdhar* paralogs were available as query sequences (NM_115148.3, NM_120444.4, D84417.2 and NM_113698.3) and each aligned with a different maize sequence (*mdhar1, mdhar2, mdhar3* and *mdhar4*). Similarly, six maize *apx* ESTs were identified. However, *apx2, apx3* and *apx4* showed >90% identity so only *apx3* was used in our qRT-PCR experiments. In contrast, *apx1* showed 54% identity to *apx5*, 60% identity to *apx6* and 77% identity to *apx2/3/4*, whereas *apx5* showed 49% identity to *apx6* and 58% identity to *apx2/3/4*. Finally, there was 58% identity between *apx6* and *apx2/3/4*. We were unable to identify suitable primers for *apx5*. Therefore we analyzed *apx1*, *apx3* (representing *apx2/3/4*) and *apx6* separately. As for *mdhar*, there were several Arabidopsis *dhar* cDNA sequences available as queries (NM_121676.3, NM_101814.4 and NM_106182.3) although these all aligned with a single maize *dhar* cDNA (**Table 2**).

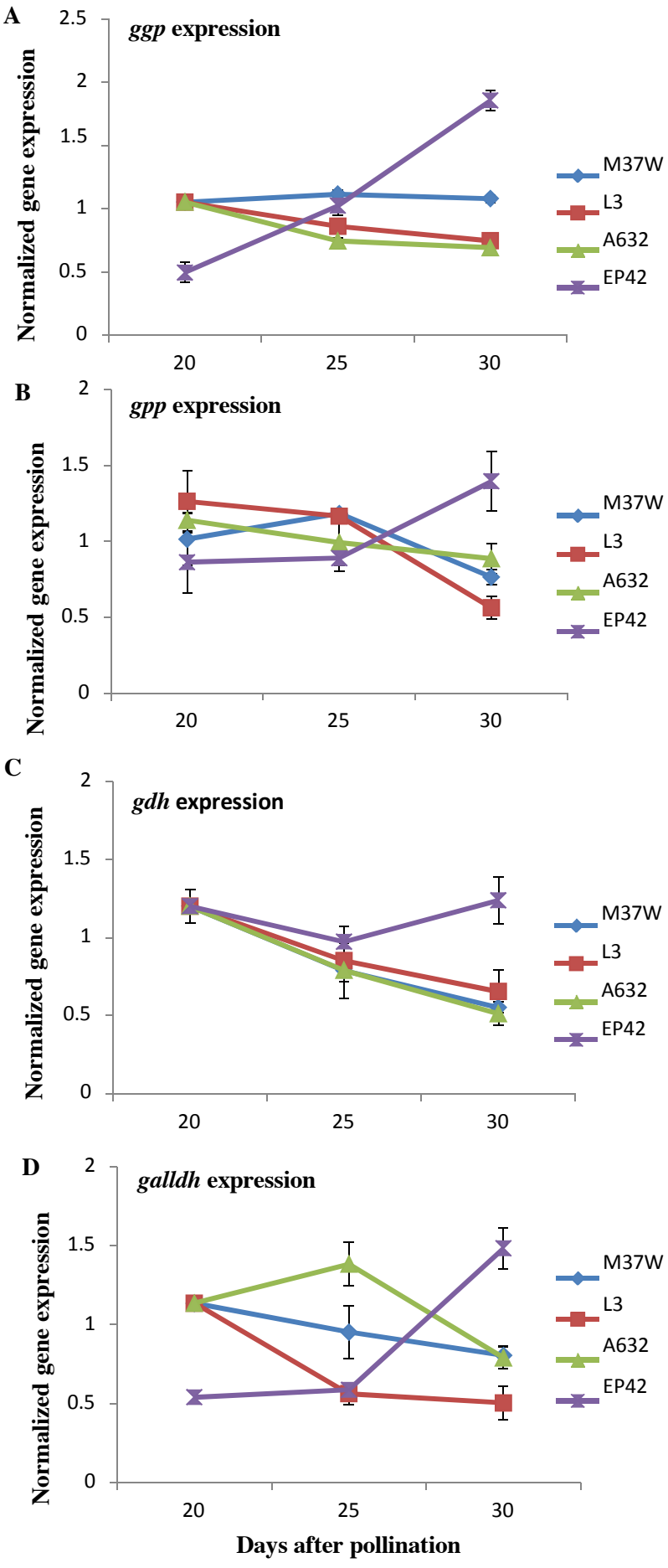
Genes	**Accession number of maize sequence**	**Accession number of the query sequence (Species)**
ggp	DT943063 (EST)	NM_118819.2 (*Arabidopsis thaliana*)
gpp	DV506736; DR828759 (EST)	EU700060.1 (*Nicotiana tabacum*)
gdh	BQ527895; CD436358 (EST)	NM_119525.5 (*Arabidopsis thaliana*)
galldh	DT943591; DR820300; DV542570; EC868512 (EST)	NM_114662.2 (*Arabidopsis thaliana*)
apx1	NM_001177011.1 (cDNA)	NM_001177011.1 (*Zea mays*)
apx2	NM_001156720.1 (cDNA)	NM_001156720.1 (*Zea mays*); localized in cytosol
apx3	NM_001159274.1 (cDNA)	NM_001159274.1 (*Zea mays*)
apx4	NM_001158777.1 (cDNA)	NM_001158777.1 (*Zea mays*); localized in cytosol
apx5	NM_001147633.1 (cDNA)	NM_001147633.1 (*Zea mays*)
apx6	NM_001139033.1 (cDNA)	NM_001139033.1 (*Zea mays*)
mdhar1	CO461725 (EST)	NM_115148.3 (*Arabidopsis thaliana*)
mdhar2	EC878222; CK787312; CO440054 (EST)	NM_120444.4 (*Arabidopsis thaliana*); localized in cytosol
mdhar3	FL028349; CO448964 (EST)	D84417.2 (*Arabidopsis thaliana*); localized in chloroplasts
mdhar4	EE020823 (EST)	NM_113698.3 (*Arabidopsis thaliana*); localized in peroxisoma
dhar	DR807318; CO523991; BU197695; CA828222 (EST)	NM_121676.3 (*Arabidopsis thaliana*)
	No EST found	NM_101814.4 (*Arabidopsis thaliana*)
	No EST found	NM_106182.3 (*Arabidopsis thaliana*)

Table 2. Maize sequences corresponding to enzymes involved in ascorbic acid metabolism.

3.5.2 Endogenous gene expression in the L-galactose pathway declines in the developing endosperm

We measured the abundance of four endogenous transcripts representing de novo AsA biosynthesis via the L-galactose pathway, namely *ggp*, *gpp*, *gdh* and *galldh* (**Fig. 1**). The transcript levels were measured at 20, 25 and 30 dap in all three genotypes and transgenic line L3 (**Fig. 2**). The expression of all four genes declined during endosperm development in all genotypes except EP42, where there was a significant increase in expression ($P < 0.01$) at 30 dap (**Fig. 2**). Transgenic line L3 showed a similar expression profile to its wild-type parent (M37W) and to genotype A632.

Figure 2. Quantitative real time PCR analysis showing relative mRNA levels for endogenous ascorbic acid biosynthesis genes at different stages of endosperm development (20, 25 and 30 dap) in four genetic backgrounds. Data were normalized against *actin* mRNA and represent the means of six replicates ± standard errors. Transcripts: (A) GDP-L-galactose phosphorylase (*ggp*); (B) L-galactose-1-phosphate phosphatase (*gpp*); (C) L-galactose dehydrogenase (*gdh*); and (D) L-galactono-1,4-lactone dehydrogenase (*galldh*).


A
ggp expression
Normalized gene expression
2.5
2
1.5
1
0.5
0
20
25
30
M37W
L3
A632
EP42
B
gpp expression
Normalized gene expression
2
1.5
1
0.5
0
20
25
30
M37W
L3
A632
EP42
C
gdh expression
Normalized gene expression
2
1.5
1
0.5
0
20
25
30
M37W
L3
A632
EP42
D
galldh expression
Normalized gene expression
2
1.5
1
0.5
0
20
25
30
Days after pollination
M37W
L3
A632
EP42

3.5.3 The expression of *apx1* and *apx3* declines significantly at 25 dap in transgenic line L3

We also monitored the endogenous *apx* transcripts at different developmental time points: 20, 25 and 30 dap. The *apx1*, *apx3* and *apx6* transcripts showed a similar developmental profile in the endosperm of all three wild-type genotypes (**Fig. 3**) but there was a significant reduction at 25 dap ($P < 0.01$) for *apx1* and *apx3* in the transgenic line L3 (**Fig. 3A, 3B**). There was no corresponding reduction in the expression of *apx6* in the transgenic line ($P > 0.05$; **Fig. 3C**).

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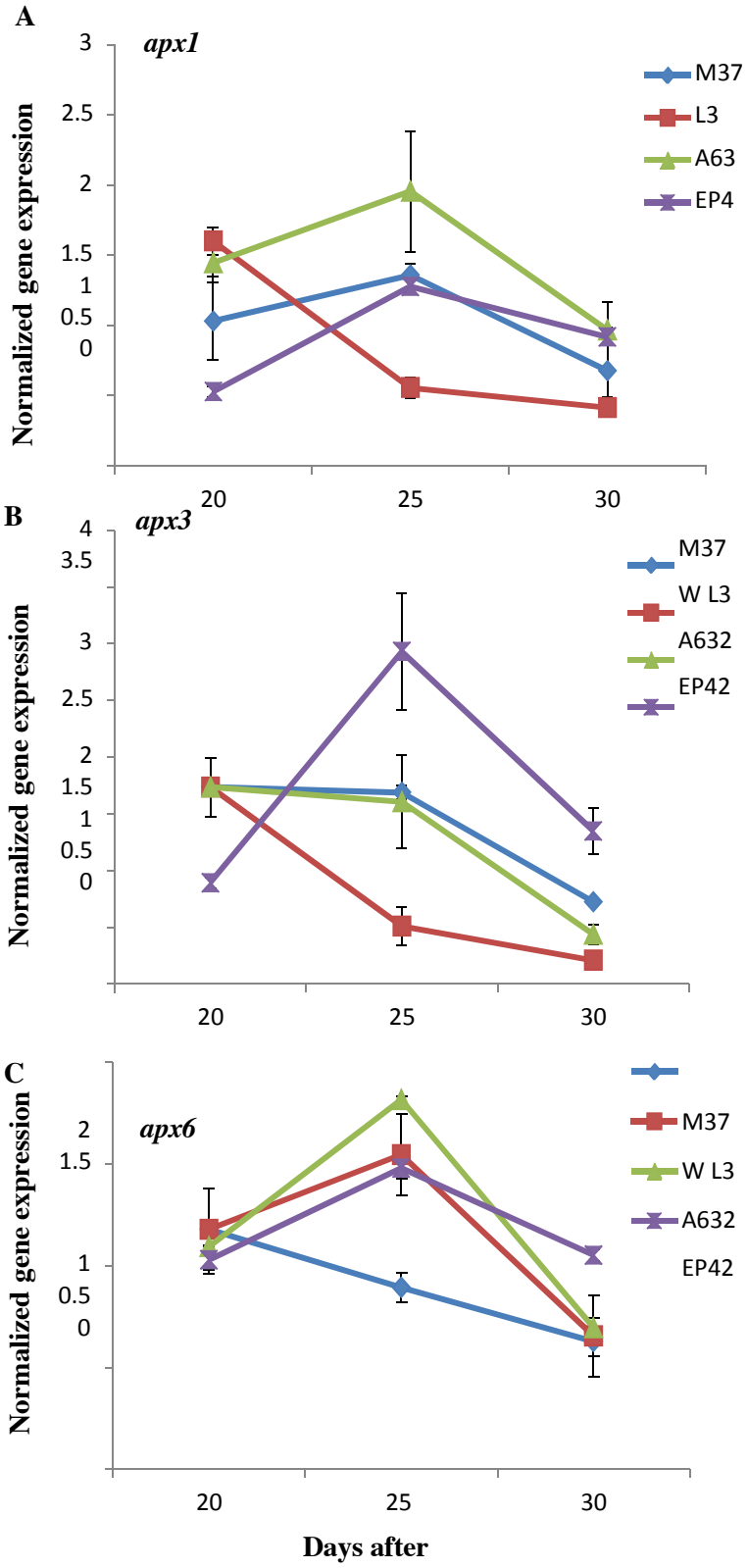
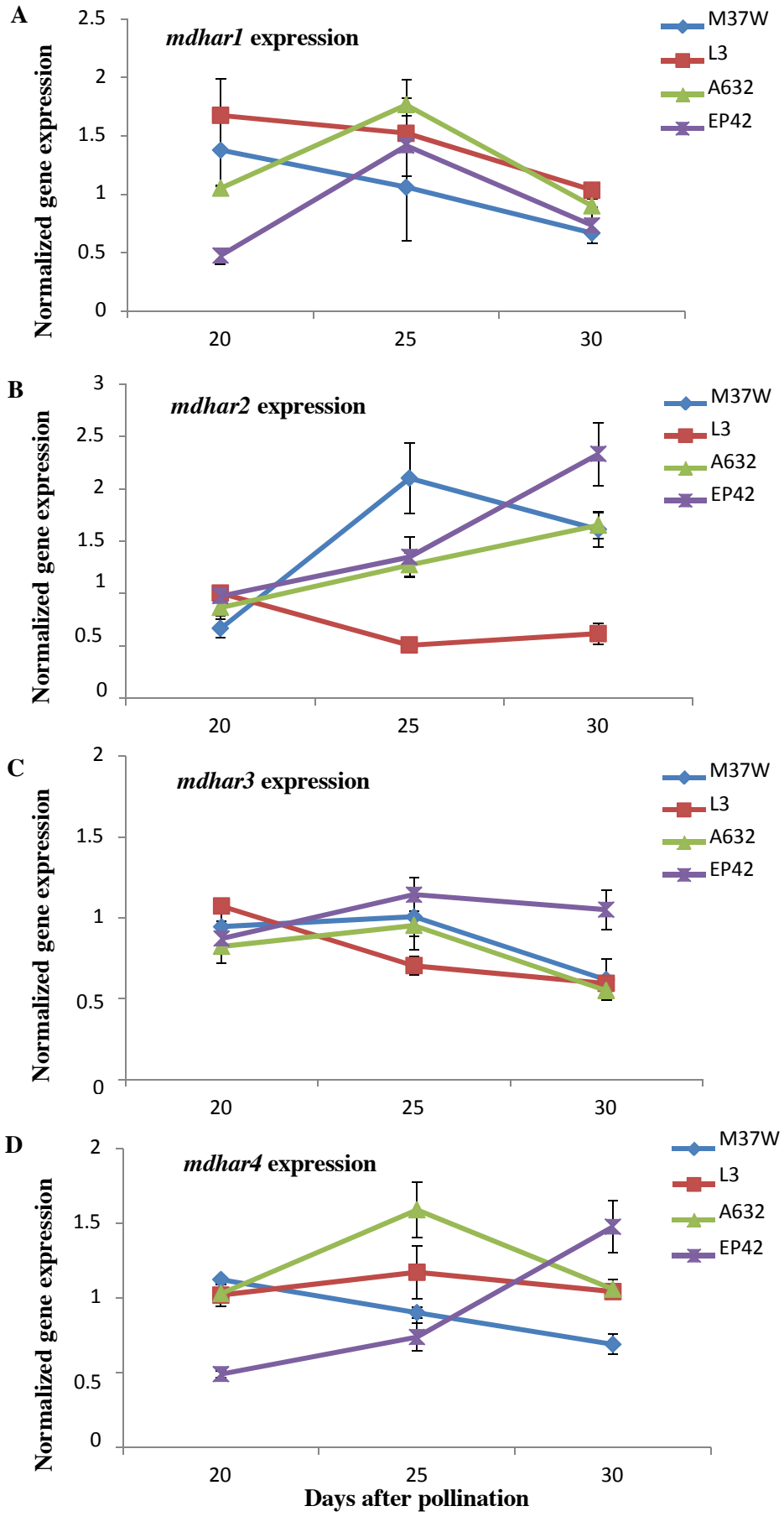


Figure 3. Quantitative real time PCR analysis showing relative mRNA levels for endogenous ascorbate peroxidase (*apx*) genes at different stages of endosperm development (20, 25 and 30 dap) in four genetic backgrounds. Data were normalized against *actin* mRNA and represent the means of six replicates ± standard errors. Transcripts: (A) *apx1*; (B) *apx3*; and (C) *apx6*

3.5.4 The expression of *mdhar2* and *mdhar3* declines significantly at 25 dap in transgenic line L3

We also monitored the endogenous *mdhar* transcripts at different developmental time points: 20, 25 and 30 dap. There was no significant difference ($P > 0.05$) in the expression profiles of *mdhar1, mdhar2, mdhar3* and *mdhar4* between the wild type genotypes M37W and A632 (**Fig. 4**) but there was a significant increase ($P < 0.01$) in the expression of *mdhar2* and *mdhar4* after 25 dap specifically in genotype EP42 (**Fig. 4B, 4D**). In contrast, there was a significant decline ($P < 0.01$) in the expression of *mdhar2* at 25 and 30 dap (**Fig. 4B**) and of *mdhar3* at 25 dap in transgenic line L3 (**Fig. 4C**) compared to the other lines.

Figure 4. Quantitative real time PCR analysis showing relative mRNA levels for endogenous monodehydroascorbate reductase (*mdhar*) genes at different stages of endosperm development (20, 25 and 30 dap) in four genetic backgrounds. Data were normalized against *actin* mRNA and represent the means of six replicates ± standard errors. Transcripts: (A) *mdhar1*; (B) *mdhar2*; (C) *mdhar3*; and (D) *mdhar4*.


A
mdhar1 expression
Normalized gene expression
2.5
2
1.5
1
0.5
0
20
25
30
M37W
L3
A632
EP42
B
mdhar2 expression
Normalized gene expression
3
2.5
2
1.5
1
0.5
0
20
25
30
M37W
L3
A632
EP42
C
mdhar3 expression
Normalized gene expression
2
1.5
1
0.5
0
20
25
30
M37W
L3
A632
EP42
D
mdhar4 expression
Normalized gene expression
2
1.5
1
0.5
0
20
25
30
Days after pollination
M37W
L3
A632
EP42

3.5.5 Expression profiling of the endogenous and heterologous dehydroascorbate reductase genes

We investigated a single endogenous *dhar* transcript and found no significant variation in expression ($P > 0.05$) between the three wild-type varieties and transgenic line L3 (**Fig. 5A**) despite the confirmed expression of the *dhar* transgene in this line, as verified by mRNA gel blot analysis (**Fig. 5B**).

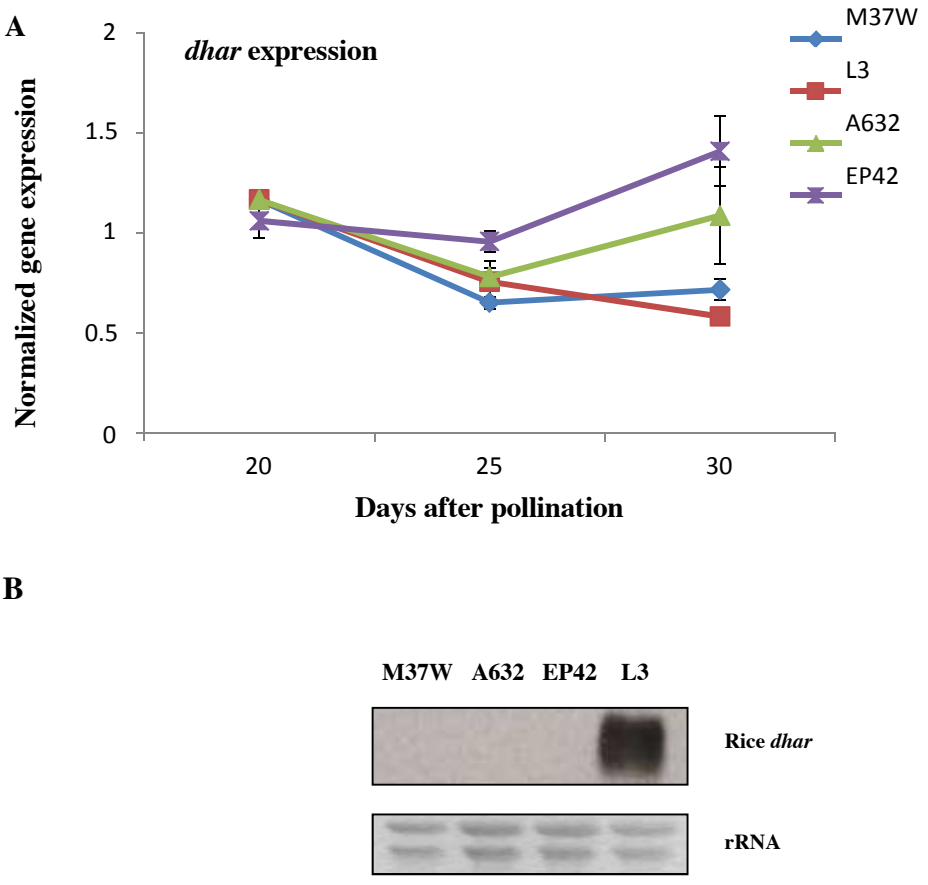


Figure 5. (A) Quantitative real time PCR analysis showing relative mRNA levels for the endogenous dehydroascorbate reductase (*dhar*) gene at different stages of endosperm development (20, 25 and 30 dap) in four genetic backgrounds. Data were normalized against *actin* mRNA and represent the means of six replicates ± standard errors. (B) An mRNA blot showing rice *dhar* transgene expression only in the endosperm of transgenic maize line L3. The loading control is rRNA stained with ethidium bromide.

3.5.6 Total ascorbic acid levels in maize endosperm reflect the different genetic backgrounds

We determined the levels of total AsA, reduced AsA and (by deduction) DHA in replicate endosperm samples, representing four developmental stages (20, 25, 30 and 40 dap) in each of the three maize genotypes and transgenic line L3.

The total AsA levels reached a maximum at 20 dap in all four genotypes, with the highest levels overall in line L3: 323 ± 9.52 nmol g^{-1} fresh weight (FW). This was followed by EP42 (284 ± 17.78 nmol g^{-1} FW), M37W (230 ± 16.26 nmol g^{-1} FW) and finally A632 (199 ± 7.88 nmol g^{-1} FW). The total AsA levels declined significantly ($P < 0.01$) after 20 dap in all four lines (**Fig. 6A**) but the DHA levels remained constant (**Fig. 6B**) indicating that the decline predominantly reflected the loss of reduced AsA (**Fig. 6C**). The decline was not steady but was characterized by a rapid fall in total AsA levels between 20 and 25 dap (reflecting the near elimination of reduced AsA) followed by a more gradual decline between 25 and 40 dap. In genotypes A632 and EP42, the progressive decline in total AsA levels between 25 and 40 dap included a significant reduction at 30 dap ($P < 0.01$), mirroring the profile of DHA during the same period (**Fig. 6B**).

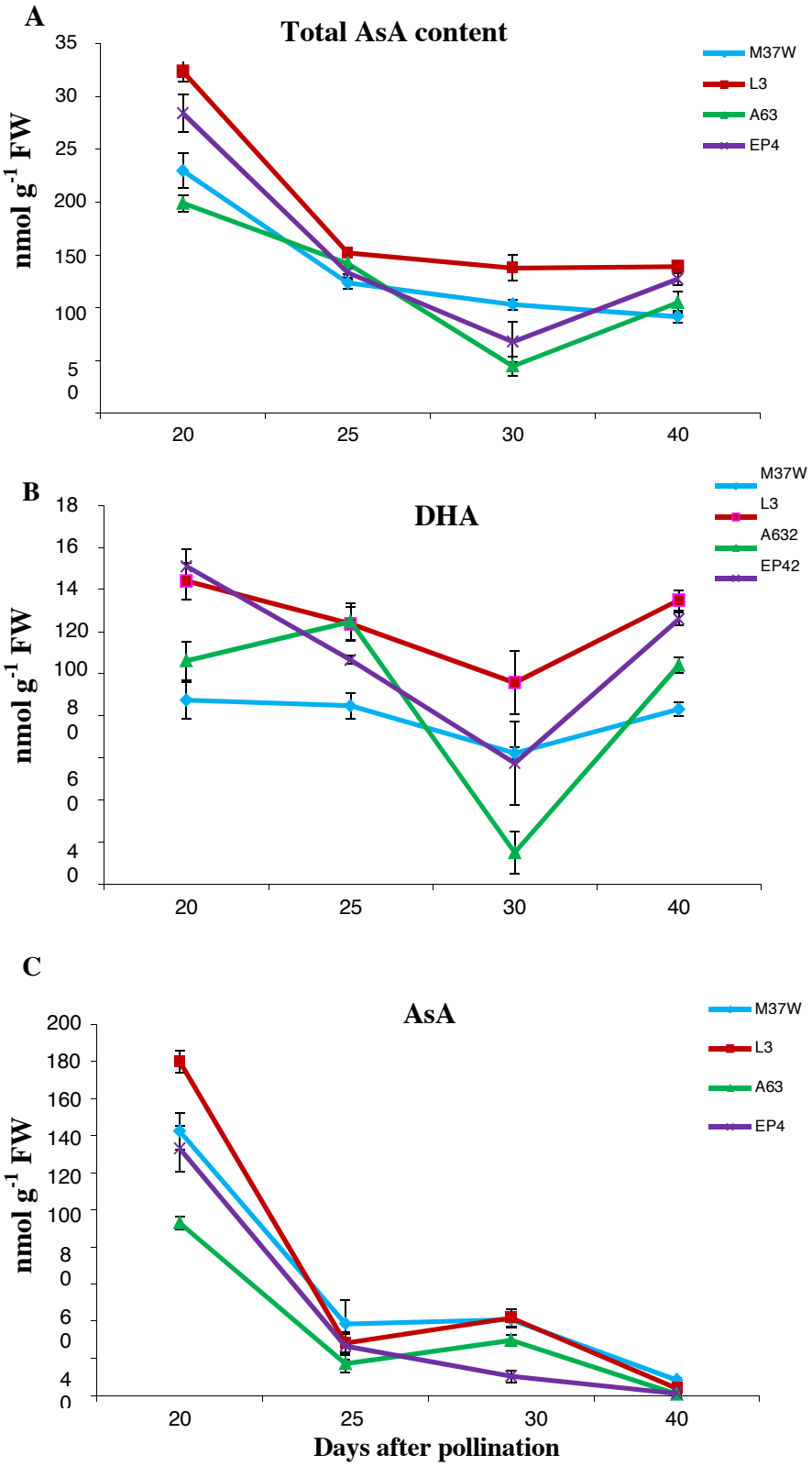


Figure 6. Ascorbic acid levels presented as nmol g^{-1} fresh weight (FW) in the endosperm tissue of four maize genotypes at 20, 25, 30 and 40 dap. (A) Total ascorbic acid levels, i.e. ascorbic acid plus dehydroascorbate (AsA plus DHA); (B) Dehydroascorbate (DHA) levels; (C) Ascorbic acid (AsA) levels.

3.6. Discussion

3.6.1 L-galactose pathway gene expression declines during endosperm development

The L-galactose biosynthesis pathway has been fully elucidated using biochemical, genetic and transgenic approaches (Wheeler et al., 1998) confirming that it is the main pathway for AsA synthesis in Arabidopsis (Conklin et al., 1999; Gatzek et al., 2002; Conklin et al., 2006; Linster and Clarke, 2008). Little is known about the regulation of AsA synthesis in maize endosperm but seeds acquire the enzymatic machinery necessary for AsA synthesis just prior to desiccation, which may indicate a role in the tolerance of oxidative stress triggered by desiccation and/or germination (Arrigoni et al., 1992; Hendry, 1993).

We investigated the contribution of the L-galactose pathway to AsA synthesis in developing maize endosperm by measuring the mRNA levels of four relevant genes in three different maize genotypes. We found that the mRNA levels of all four genes declined between 20 and 30 dap in two of the genotypes (M37W and A632) and in the transgenic line L3 derived from M37W, but increased over the same period in genotype EP42 (**Fig. 2**). This may indicate that the loss of capacity for AsA synthesis occurs later in the development of EP42 endosperm, perhaps because the enzymatic machinery is activated later in development in this variety compared to the other genotypes. It is clear that EP42 does not accumulate any more or less AsA in total than other genotypes, suggesting the overall capacity for AsA accumulation is maintained (**Fig. 6**).

The expression of genes required for AsA synthesis also declines during the development of strawberry, kiwifruit and apple fruits (Cruz-Rus et al., 2011; Bulley et al., 2009; Li et al., 2011) and genotype-dependent differences in expression profiles have also been observed. For example, *ggp* transcripts in *Actinidia eriantha* (a relative of the kiwifruit) were almost four times more abundant than in other genotypes (Bulley et al., 2009).

3.6.2 The *mdhar* and *dhar* transcripts show complementary expression profiles during maize endosperm development

The AsA recycling pathway plays an important role in the adaptation of plants to oxidative stress (Stevens et al., 2008) and also contributes to the regulation of AsA levels along with de novo synthesis, degradation and transport (Li et al., 2010). Oxidized AsA can be regenerated by the enzymes MDHAR and DHAR (**Fig. 1**) and in maize endosperm the transcripts representing these enzymes showed complementary expression profiles during development.

Transcripts representing three of the four maize *mdhar* paralogs (*mdhar1*, *mdhar3* and *mdhar4*) declined significantly in abundance during development, whereas the levels of *dhar* (and *mdhar2*) mRNA increased over the same period. Furthermore we found that *mdhar2* and *mdhar4* mRNA levels increased significantly after 25 dap specifically in the EP42 genotype (**Fig. 4B, 4D**). These differences could reflect the different rates of seed maturation among the maize genotypes. Interestingly, *mdhar* expression in red strawberry fruit was more than 17-fold higher than in immature green fruit, whereas *dhar* expression was higher in green fruits than in white and red fruits suggesting that MDHAR was the main contributor to the AsA pool in ripe strawberries and DHAR was the main contributor in green fruits (Cruz-Rus et al., 2011). These observations are consistent with previous studies in tomato (Ioannidi et al., 2009), kiwifruit (Bulley et al., 2009) and acerola (Eltelib et al., 2011), where there is a similar complementarity between MDHAR and DHAR during ripening, depending on the species and ripening phase.

In maize endosperm, *mdhar2* and *dhar* could be responsible for maintaining the AsA pool until the seeds are mature, since DHA is the most abundant form of vitamin C. MDHAR and DHAR also both appear to be active in maturing broad bean seeds (*Vicia faba*), with MDHAR activity dominating during early development but declining to about 15% of its initial level at the end of desiccation, and DHAR remaining constant throughout development and then declining to negligible levels at the end (Arrigoni et al., 1992). Interestingly, our data showed that the endogenous *dhar* transcript was present at the lowest relative levels in the L3 transgenic line, perhaps due to feedback regulation induced by the expression of the *dhar* transgene (**Fig. 5A**). The system was brought into balance by the simultaneous regulation of the *mdhar* and *apx* transcripts,

which have opposite effects on the redox state of AsA and suggest that the redox status itself may be the target for feedback regulation to maintain the AsA pool. Transgenic maize seeds overexpressing *dhar* showed no significant changes in MDHAR activity, although it was slightly higher than in wild-type plants (Chen et al., 2003).

The ascorbate-glutathione cycle scavenges reactive oxygen species (ROS) directly and indirectly in plants (Noctor and Foyer, 1998; Foyer and Noctor, 2003) as a response to many forms of abiotic stress including desiccation (Allen et al., 1997; Foyer et al., 1997). APX is an important component of this system because it is present in multiple subcellular compartments and has a high affinity for hydrogen peroxide. The electron donor is AsA, which is regenerated by MDHAR and DHAR in the presence of GSH and NADPH. GSH is oxidized to GSSG and reduced back to GSH by GR. The expression of several *apx* transcripts declined during endosperm development in the three wild-type maize lines we studied, similar to the profile observed in broad bean seeds, *Pinus pinea* and *Avena sativa*, where APX activity remained high during seed growth but declined during desiccation to negligible levels (Arrigoni et al., 1992; Tommasi et al., 1999). Interestingly, the levels of *apx1* and *apx3* mRNA were downregulated at 25 dap specifically in the transgenic line L3, whereas apx6 was unaffected, suggesting feedback regulation of *apx1* and *apx3* by the redox status of the AsA pool.

3.6.3 Expression of the rice *dhar* transgene influences the expression of genes in the ascorbic acid recycling pathway

A comparison of endogenous gene expression profiles in M37W and its derivative transgenic line L3 confirmed that the L-galactose pathway genes were not affected by the expression of the *dhar* transgene (**Fig. 2**). However, the endogenous *mdhar2* and *mdhar3* genes were significantly repressed in the transgenic line at 25 dap, and also at 30 dap for *mdhar2* (**Fig. 4B, 4C**). The *apx1* and *apx3* genes were also repressed in the transgenic line (**Fig. 3A, 3B**). Despite the downregulation of at least four genes involved in AsA recycling, there were no correlated changes in the AsA/DHA levels, which remained consistently higher throughout endosperm development in line L3 compared to the wild-type parent. In an earlier study, there was no observed change in endogenous MDHAR and APX activities in transgenic maize lines expressing wheat

dhar cDNA, although the endogenous transcripts were not measured along with the enzyme activities (Chen et al., 2003).

The modulation of DHA levels in the transgenic seeds from line L3 may therefore exert negative feedback on redox-sensitive genes such as *apx1*, *apx3*, *mdhar2* and *mdhar3* (**Fig. 7**), but this is not translated into enzyme activities perhaps because the effect is masked by the more dramatic loss of enzyme activity during seed development, as seen for MDHAR, DHAR and APX in broad bean (Arrigoni et al., 1992) and AO in pea (Matamoros et al., 2010). DHA is generated from AsA by APX and AO, and is then converted to MDHA. This can be converted back to AsA by MDHAR or it can break down naturally into DHA, which is either hydrolyzed to form diketogulonic acid or converted back to AsA by DHAR using GSH as the reductant (**Fig. 7**). If DHA exceeds a critical threshold, the data in this chapter suggest that genes involved in AsA oxidation and recycling are tightly regulated to ensure the AsA pool is maintained (**Fig. 7**).

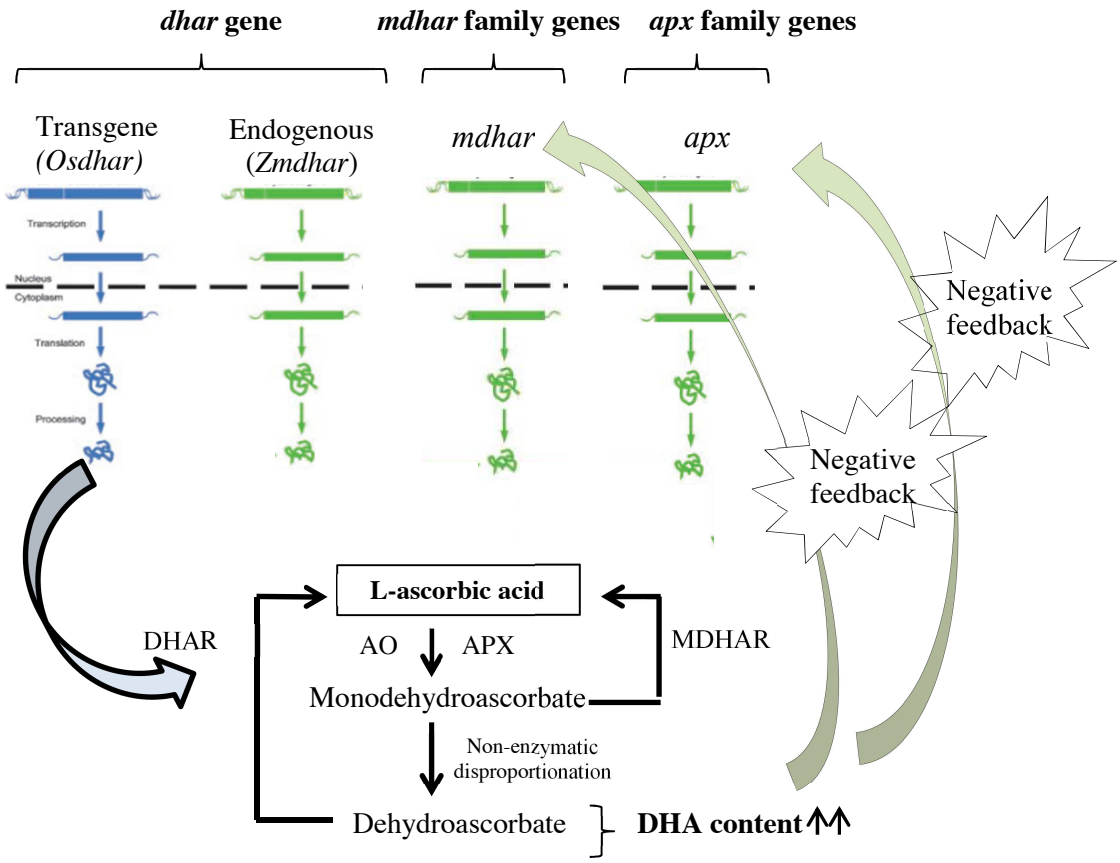


Figure 7. Proposed model accounting for the tight regulation of the AsA pool in maize. If DHA exceeds a critical upper threshold, it inhibits AsA recycling genes at the level of transcription, but if it falls below a critical lower threshold, these genes are induced. Abbreviations: ascorbate oxidase, AO; dehydroascorbate reductase, DHAR; monodehydroascorbate reductase, MDHAR; ascorbate peroxidase, APX; *Zea mays*, Zm; *Oryza sativa*, Os.

3.7 Conclusions

AsA biosynthesis via the L-galactose pathway declines during endosperm development and causes the total AsA content of seeds to decline. The loss of total AsA after 20 dap predominantly reflects the loss of reduced AsA, because DHA levels remain steady during the development and maturation of seeds. AsA levels are under strict yet complex and genotype-dependent feedback regulation during endosperm development which appears to be controlled predominantly by APX and MDHAR. These enzymes may in turn be regulated at the level of transcription by the abundance of DHA, suggesting a novel strategy by which plants control the redox state in their seeds.

3.8 Future prospects and recommendations

The results presented in this chapter confirm that the AsA pool in maize endosperm is subject to complex and genotype-dependent feedback regulation during development. The capacity to synthesize AsA via the L-galactose pathway declines during endosperm development and the AsA content declines. Further work is required to investigate AsA transport from source tissues to maize seeds and also additional AsA biosynthesis pathways that might feed into the AsA pool during seed development.

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CHAPTER 4

TOWARDS THE CREATION OF TRANSGENIC MAIZE GERMPLASM CONTAINING GENES FOR ASCORBIC ACID BIOSYNTHESIS

Chapter 4: Towards the creation of transgenic maize germplasm containing genes for ascorbic acid biosynthesis

4.1 Abstract

Biofortification is a promising strategy for the development of staple crops producing substantially higher levels of ascorbic acid (vitamin C). Combinatorial genetic transformation creates a population of plants containing random combinations of multiple transgenes integrated at the same locus, preventing segregation in subsequent generations. The main objective of the experiments discussed in this chapter was to establish a combinatorial transgenic maize population comprising different combinations of genes related to ascorbic acid metabolism. The analysis of such plants should provide insight into the interactions among ascorbic acid pathways and help in the development of strategies to boost the accumulation of ascorbic acid in the endosperm. The library of transgenic maize plants expressed multiple transgenes in the endosperm and was suitable for preliminary molecular and biochemical analysis, but many additional plants will need to be regenerated and characterized for a more comprehensive understanding of ascorbic acid metabolism in maize.

4.2 Introduction

Ascorbic acid (AsA) is an antioxidant organic compound that acts as an electron donor in many different enzymatic reactions in animals. Humans and some other mammals cannot synthesize this molecule because they lack L-gulono-1,4-lactone oxidase, the final enzyme in the biosynthesis pathway, and must instead acquire it as an essential nutrient (vitamin C) in the diet. Plants are the main dietary source of AsA for humans so boosting its accumulation in edible plant organs is an important target for the nutritional enhancement of staple crops. However, AsA metabolism in higher plants is regulated in a complex manner, reflecting the combination of multiple biosynthesis and recycling pathways as well as long-distance AsA transport between organs, and more information is therefore required to unravel these mechanisms before the impact of genetic engineering on AsA levels can be predicted reliably (Ishikawa et al., 2006; Franceschi and Tarlyn, 2002; Tedone et al., 2004).

4.2.1 Ascorbic acid in human health

AsA plays many important roles in human metabolism, including the synthesis and stabilization of neurotransmitters and the reduction of iron compounds, enhancing the gastrointestinal absorption of dietary non-heme iron (Basu and Dickerson, 1996). It is also required for collagen and carnitine synthesis (Davidson et al., 1997; Rebouche, 1991), thus insufficient vitamin C in the diet causes scurvy, which results in the breakdown of connective tissue fibers and muscular weakness (Steward and Guthrie, 1953; Bartholomew, 2002). Plants are the most important source of AsA in the human diet due to the accumulation of AsA in chloroplasts (Smirnoff, 1996). AsA in hydrated storage organs such as fruits and vegetables is a better source than dry seeds, which tend to contain only minimal levels (Hancock and Viola, 2005). The recommended dietary intake (RDI) for AsA is 90 mg/day for adults. However, only 46 mg/day is necessary to prevent scurvy (Carr and Frei, 1999). A number of studies have shown that increasing vitamin C intake up to 120 mg/day can reduce the risk of chronic diseases such as cancer, cardiovascular disease and cataracts, probably by boosting antioxidant capacity (Carr and Frei, 1999).

4.2.2 Ascorbic acid in biofortified crops

Metabolic engineering has been used to increase the AsA content of model species such as Arabidopsis and tobacco as well as important crops (**Table 1**). The principal strategy is to upregulate the expression of the genes encoding enzymes in the AsA biosynthesis and recycling pathways (**Fig. 1**). However all previous studies have involved the expression of single genes (Ishikawa et al., 2006). These efforts have been moderately successful, in some cases elevating the AsA content of different tissues, but in other cases the increase has been negligible.

The expression of the acerola phosphomannomutase (*pmm*) and GDP-D-mannose pyrophosphorylase (*gmp*) genes in tobacco achieved a 2-fold increase in AsA levels in the leaves (Badejo et al., 2008; 2009). The corresponding enzymes catalyze early steps in the L-galactose pathway. The overexpression of GDP-mannose-3'5'-epimerase (*gme*) in tomato fruits and leaves increased the amount of AsA by only 1.19–1.60-fold (Zhang et al., 2011a). The expression of kiwifruit GDP-L-galactose phosphorylase (*ggp*) in Arabidopsis increased the AsA content in leaves almost 4-fold, whereas the transient co-expression of kiwifruit *ggp* and *gme* increased the AsA content of tobacco leaves by up to 12-fold (Bulley et al., 2009). The stable overexpression of *ggp* in tomato, strawberry and potato increased fruit and tuber AsA levels by 6-fold, 2-fold and 3-fold, respectively (**Table 1**) (Bulley et al., 2012). However, in the transgenic tomato fruits, the higher AsA levels were associated with the loss of seeds (Bulley et al., 2012). The overexpression of L-galactono-1,4-lactone dehydrogenase (*galldh*) in transgenic tobacco cells increased the AsA content by 2-fold (Tokunaga et al., 2005).

The overexpression of strawberry D-galacturonate reductase (*GalUR*) from the D-galacturonic pathway doubled AsA levels in Arabidopsis leaves and tripled them in potato tubers (Agius et al., 2003; Hemavathi et al., 2009). The expression of a rat L-gulono-1,4-lactone oxidase (*GLOase*) increased the AsA content of lettuce approximately 7-fold (Jain and Nessler, 2000) (**Table 1**).

Genes involved in AsA recycling can also be used to increase AsA levels. The expression of wheat dehydroascorbate reductase (*dhar*) increased the AsA content by 2.4-fold in tobacco leaves and by 2-fold in maize seeds (Chen et al., 2003) but only by 1.6-fold in potato tubers and mature leaves (Qin et al., 2011). However, the expression of *dhar* increased AsA levels by up to 6-fold in maize endosperm (Naqvi et al., 2009)

(**Table 1**). The suppression of ascorbate oxidase (*ao*) in tomato fruit doubled the AsA content (Zhang et al., 2011b). The expression of monodehydroascorbate reductase (*mdhar*) increased the AsA content to a lesser extent in tobacco (Eltayeb et al., 2007; Yin et al., 2010) and in tomato (Haroldsen et al., 2011).

The studies described above indicate that the AsA content in plants must be tightly regulated, therefore the expression of multiple enzymes may help to relieve bottlenecks and increase the total AsA content. In this chapter, I describe experiments in which three different combinations of transgenes were tested in an attempt to generate plants with higher levels of AsA in the endosperm and to gain insight into the regulatory network controlling AsA metabolism in maize.

4.3 Aim

The aim of the experiments described in this chapter was to select a set of metabolic genes suitable for boosting AsA levels in maize endosperm, to construct independent transformation vectors containing these genes under the control of endosperm-specific promoters, and to generate transgenic maize plants by combinatorial transformation for further analysis.

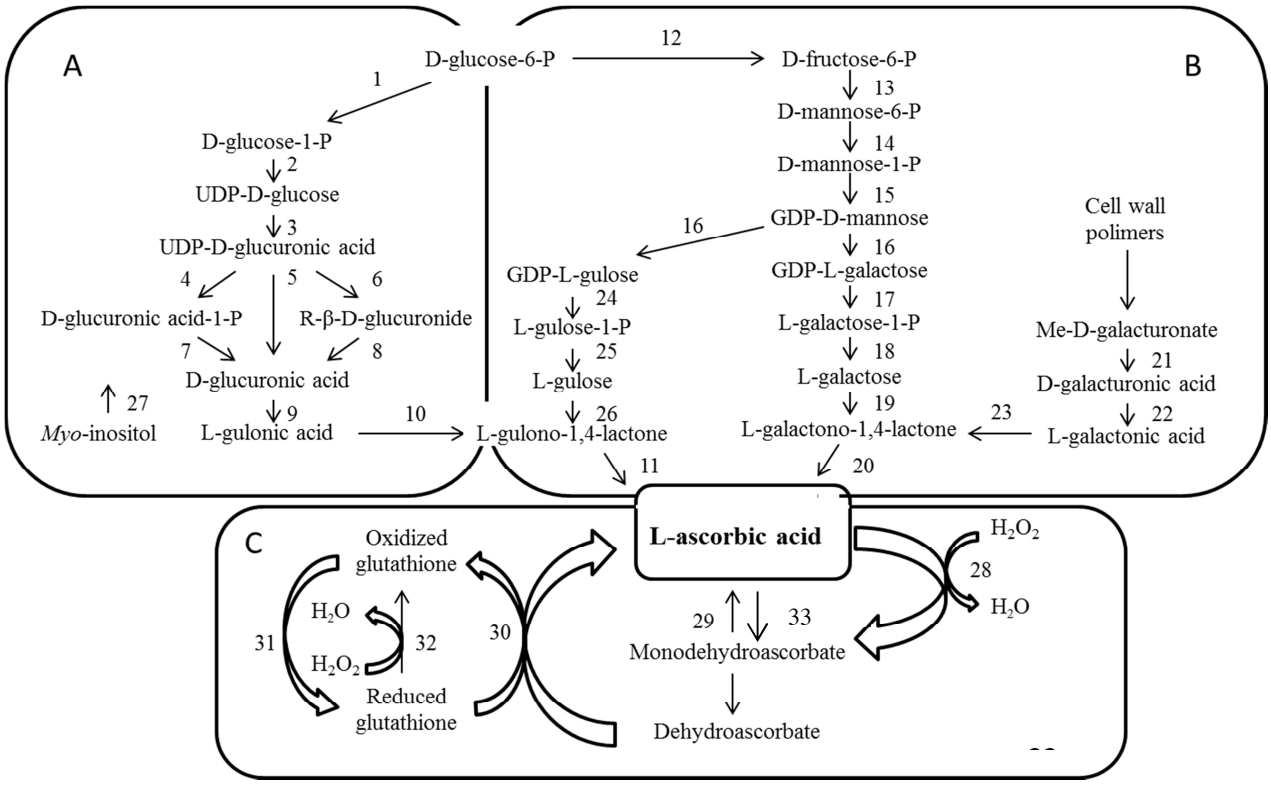

Figure 1. Proposed biosynthesis pathways for AsA in (**A**) animals (reactions 1–11), (**B**) plants (reactions 12–27) and (**C**) the recycling pathway (reactions 28–33), adapted from Cruz-Rus et al. (2012): L-galactose pathway (reactions 12–20); L-gulose pathway (reactions 16–11); D-galacturonic pathway (reactions 21–20); and *myo*-inositol pathway (1–10, 27). The enzymes catalyzing these reactions are: 1, phosphoglucomutase; 2, UDP-D-glucose pyrophosphorylase; 3, UDP-D-glucose dehydrogenase; 4, D-glucuronate-1-phosphate uridylyltransferase; 5, UDP-glucuronidase; 6, UDP-glucuronosyltransferase; 7, D-glucuronokinase; 8, D-glucuronidase; 9, D-glucuronate reductase; 10, aldono-lactonase; 11, L-gulono-1,4-lactone oxidase; 12, D-glucose-6-phosphate isomerase; 13, D-mannose-6-phosphate isomerase; 14, phosphomannomutase; 15, GDP-mannose pyrophosphorylase; 16, GDP-mannose-3',5'-epimerase; 17, GDP-L-galactose phosphorylase; 18, L-galactose-1-phosphate phosphatase; 19, L-galactose dehydrogenase; 20, L-galactono-1,4-lactone dehydrogenase; 21, methylesterase; 22, D-galacturonate reductase; 23, aldono-lactonase; 24, phosphodiesterase; 25, sugar phosphatase; 26, L-gulose dehydrogenase; 27, *myo*-inositol oxygenase; 28, ascorbate peroxidase; 29, monodehydroascorbate reductase; 30, dehydroascorbate reductase; 31, glutathione reductase; 32, glutathione peroxidase; 33, ascorbate oxidase.

Plant species	Gene (source)	Promoter	Vitamin C level	Reference
Maize (*Zea mays*)	*dhar* (wheat; *Triticum aestivum*)	Maize Ubiquitin-1 (constitutive)	39.6 µg/g FW (225 nmol/g FW) in kernel (**2-fold**)	Chen et al., 2003.
	dhar (rice; *Oryza sativa*)	Barley D- hordein (endosperm specific)	26.4 µg/g FW (150 nmol/g FW) in endosperm * (**6-fold**)	Naqvi et al., 2009 .
Tomato (*Solanum lycopersicum*)	*gme* (tomato; *Solanum lycopersicum)*	CaMV 35S (constitutive)	387.5 µg/g FW in fruit (**1.6-fold**)	Zhang et al., 2011a.
	ggp (kiwifruit; *Actinidia chinensis*)	CaMV 35S	1109.5 µg/g FW in fruit (**6-fold**)	Bulley et al., 2012.
	dhar (tomato)	FMV34S (constitutive)	1159 µg/g FW in fruit (**1.6-fold**)	Haroldsen et al., 2011.
Lettuce (*Lactuca sativa*)	GLOase (rat; *Rattus norvegicus*)	CaMV 35S	102 µg/g FW (**7-fold**)	Jain and Nessler, 2000.
Potato *(Solanum tuberosum*)	*dhar* (potato)	CaMV 35S	915.8 µg/g FW in tuber (**1-fold**)	Qin et al., 2011.
	ggp (kiwifruit)	Polyubiquitin (PAT)	352.2 µg/g FW in tuber (**3-fold**)	Bulley et al., 2012.
	GalUR (strawberry)	CaMV35S	528.4 µg /g FW in tuber in tuber (**3-fold**)	Hemavathi et al., 2009.
	dhar (sesame; *Sesamum indicum*)	CaMV35S	370 µg/g FW in tuber (**1.6-fold**)	Goo et al., 2008.
Strawberry (*Fragaria × ananassa*)	*ggp* (kiwifruit)	CaMV 35S	1303.3 µg/g FW in fruit (**2-fold**)	Bulley et al., 2012.
Tobacco (*Nicotiana tabacum*)	*GLOase* (rat)	CaMV 35S	84.5 µg /g FW (**7-fold**)	Jain and Nessler, 2000.
	dhar (wheat)	CaMV 35S	493 µg/g FW (**2.4-fold**)	Chen et al., 2003.
	galldh (tabacco)	CaMV 35S	(**2-fold**)	Tokunaga et al., 2005.
	gmp (acerola; *Malphighia glabra)*	CaMV 35S and Mg.GMP promoter	176 µg/g FW (**2-fold**)	Badejo et al., 2008.
	pmm (acerola)	CaMV 35S	700 µg/g FW (**2-fold**)	Badejo et al., 2009**.**
Arabidopsis thaliana	*ggp* (kiwifruit)	CaMV 35S	2160 µg/g FW (**4-fold**)	Bulley et al., 2009.
	GalUR (strawberry)	CaMV 35S	1232 µg/g FW (**3-fold**)	Agius et al., 2003.
	miox4 (*Arabidopsis thaliana*)	CaMV 35S	880 µg/g FW (**3-fold**)	Lorence et al., 2004.

Table 1. Vitamin C enhancement by genetic engineering in plants. Gene abbreviations: dehydroascorbate reductase, *dhar*; GDP-D-mannose pyrophosphorylase, *gmp;* GDP-L-mannose-3',5'-epimerase, *gme*; GDP-L-galactose phosphorylase, *ggp*; L-galactono-1,4-lactone dehydrogenase, *galldh*; L-gulono-1,4-lactone oxidase, *GLOase*; phosphomannomutase, *pmm*; D-galacturonate reductase, GalUR; *myo*-inositol oygenase, miox. Dry weight, DW; fresh weight, FW. *Assuming 76% water content in maize grain (http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/SR24/reports/sr24fg11.pdf).

4.4 Materials and methods

4.4.1 Gene cloning

Genes from the AsA biosynthesis and recycling pathways were cloned by PCR using sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/), except *ggp* which was synthesized based on the kiwifruit (*Actinidia* spp.) sequence (accession number GU339036) by Eurofins MWG Operon Company (Ebersberg, Germany), adjusting for maize codon usage. The *ao* RNAi sequence (EC871426.1) was found by searching the maize EST database (www.maizegdb.org) with the *Arabidopsis thaliana* (Arabidopsis) *AO* cDNA (NM_122118.4). The genes, sources and accession numbers are listed in **Table 2**.

Gene	Source	Accession Number	Promoter	Terminator
gme	Kiwifruit (*Actinidia* spp.)	GU339037	Maize γ-zein	Nopaline synthase
ggp	Kiwifruit (synthesized)	Adapted from GU339036 utilizing maize codon usage	Wheat low molecular Weight glutenin	Nopaline synthase
galldh	Tobacco (*Nicotiana tabacum*)	AB048530.1	Barley D-hordein	ADPGPP
ao RNAi	Maize (*Zea mays*)	Consensus maize sequence (EC871426.1)	Barley D-hordein	ADPGPP
dhar	Rice (*Oryza sativa*)	AY074786	Barley D-hordein	ADPGPP

Table 2. Summary of the genes used for maize transformation, including the source of the genes, GenBank accession number (http://www.ncbi.nlm.nih.gov/) and the endosperm-specific promoter and terminator. The *Oryza sativa dhar* gene was cloned from rice line EYI105 by Naqvi et al. (2009) and the *bar* gene encoding phosphinothricin acetyltransferase, which detoxifies phosphinothricin (PPT), was used as a selectable marker.

4.4.2 RNA isolation, RT-PCR and vector construction

Total RNA was isolated from kiwifruit flesh, tobacco leaves and maize leaves as described in Section 2.4.5. The kiwifruit *gme* cDNA, tobacco *galldh* cDNA and maize *ao* RNAi sequence were cloned by RT-PCR using specific forward and reverse primers, containing terminal restriction sites to facilitate vector construction (**Table 3**). Each RT-PCR comprised 2 μg template RNA for first-strand cDNA synthesis with the Omniscript® Reverse Transcription Kit (QIAGEN, Hilden, Germany), and amplification was carried out, after a 3-min 94°C hold step, by 35 cycles at 94°C for 45 s, 60°C for 45 s and 72°C for 3 min, and a final extension at 72°C for 10 min.

Gene	Position	Restriction site	Primer sequence
gme	Forward	*Bam*HI	5'-GCGGGAATTCGATTAGAATGGGAAG-3'
	Reverse	*Kpn*I	5'-CTAGTGATTCTTCATTCTTTGCC-3'
ggp	Forward	*Bam*HI	5'-ATGCTGAAGATAAAGCGGGTTCCG-3'
	Reverse	*Eco*RI	5'-TCAGTGCTGGACCAAGCATTCCT-3'
galldh	Forward	*Not* I	5'-CGACGACGTTCAAATGCTTCGTTC-3'
	Reverse	*Sac*II	5'-CCAGTTACACAGCCTCAGATGAAGAGC-3'
ao RNAi	Forward	*Eco*RI-*Xba*I	5'- CACATCGACGGCTACGCCTTC-3'
	Reverse	*Spe*I- *Bam*HI	5'- GTACTCGTCCCTGAGCGACC-3'

Table 3. Primers used to amplify full-length AsA-related genes.

The RT-PCR products were cleaned using the Geneclean II Kit (MP Biomedicals, LLC, France), inserted into pGEM®-T easy (Promega, Madison, Wisconsin, USA) and transferred to competent *Escherichia coli* cells, which were plated and incubated at 37°C overnight under ampicillin selection. Plasmid DNA was isolated from white colonies using the Miniprep Kit (Promega, Madison, Wisconsin, USA) and positive samples were sequenced in house (Servei de Seqüenciació, UdL). Cassettes were isolated using the appropriate restriction enzymes and ligated into the transformation vectors selected for each gene construct. Transformation vectors GZ63, pHor and pHor-GUS contain the endosperm-specific maize γ-zein and barley D-hordein promoters, paired with the nopaline synthase terminator for GZ63 and the rice ADPGPP terminator for pHor and pHor-GUS (Torrent et al., 1997; Sørensen et al., 1996). The *Oryza sativa dhar* gene was cloned from rice inbred line EYI105 by Naqvi et al. (2009). The *ggp* gene was amplified by PCR and transferred directly to plasmid p326, containing the

wheat low-molecular-weight (LMW) glutenin promoter (Colot et al., 1987; Stoger et al., 1999) and nopaline synthase terminator.

4.4.3 Maize combinatorial transformation

Maize plants (*Zea mays* L., cv. M37W) were grown in the greenhouse and growth room 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. M37W immature zygotic embryos were excised at 10–14 days after pollination (dap) and cultured on N6 medium. After 5 days, the embryos were transferred to N6 medium containing high osmoticum (0.2 M mannitol, 0.2 M sorbitol) for 5–6 h prior to bombardment, then bombarded with 10 mg of coated gold particles and incubated as above for 10–16 h thereafter. The gold particles were coated at a molar ratio of 3:1 against the selectable *bar* marker plasmid derived from pAHC20 (Christensen and Quail, 1996; Christou et al., 1991) making adjustments for the size of each construct. Bombarded callus was selected on phosphinothricin-supplemented medium as previously described (Drakakaki et al., 2005). Transgenic plantlets were regenerated and hardened off in soil. Independent putative transgenic events were identified and characterized by DNA and mRNA blot analysis. Positive transgenic plants were self-pollinated to produce T1 seeds. **Figure 2** shows the process described above and **Table 4** shows the different media used for each step.

4.4.4 DNA analysis of putative transgenic plants

Genomic DNA was extracted from the leaves of transformed plants as described in Section 2.4.3. Putative transgenic events were characterized by PCR using three different sets of primers designed to cover the entire cassette for each transgene in order to confirm their integrity (**Fig. 3**). **Table 5** lists the primer sequences. Primer set 1 was used for the promoter and transgene (forward primer located in the promoter and reverse primer in the transgene); primer set 2 was used for the transgene only (both primers in the transgene); and primer set 3 was used for the transgene and terminator (forward primer in transgene and reverse primer located in the terminator) (**Fig. 3**). The corresponding plasmids were used as positive controls. PCR amplifications were carried out under standard conditions using 250 ng of genomic DNA and 0.5 units of GoTaq®

DNA Polymerase, heating to 95ºC for 3 min, followed by 30 cycles of 94ºC for 45 s, 60ºC for 45 s, 72ºC for 90 s, and a final extension at 72ºC for 10 min.

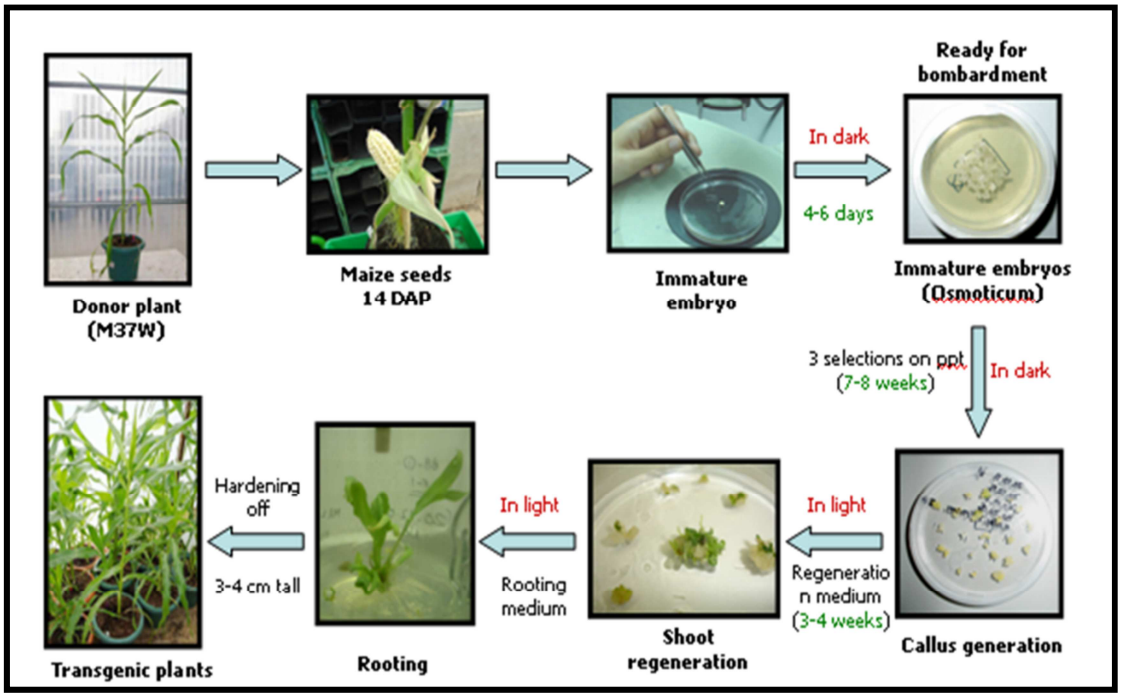

Figure 2. Maize transformation by direct DNA transfer, showing the protocol for the preparation of maize embryos prior to bombardment and the regeneration of transgenic plants.

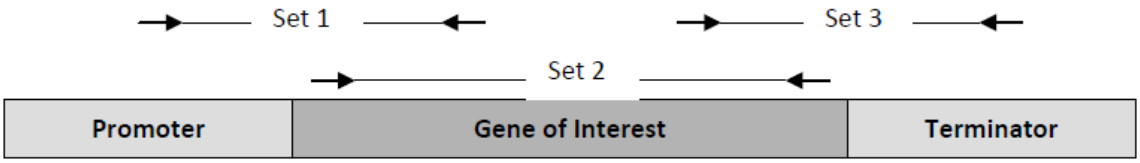

Figure 3. Sets of primers used to detect the cassette for each integrated transgene and confirm transgene integrity.

Compounds	**N6**	**N6 osmoticum**	**N6/PPT**	**MR1/PPT (Shoot development)**	**MR2/PPT (Root development)**
N6 macronutrients	50 ml	50 ml	50 ml	---	---
N6 micronutrients	5 ml	5 ml	5 ml	---	---
N6 Fe-EDTA source	5 ml	5 ml	5 ml	5 ml	5 ml
Sucrose	20 g	20 g	20 g	30 g	30 g
Casein hydrolase	0.1 g	0.1 g	0.1 g	---	---
L-proline	2.8 g	2.8 g	2.8 g	---	---
2,4-D	1 mg	1 mg	1 mg	0.025 mg	---
D-mannitol	---	36.4 g	---	---	---
D-sorbitol	---	36.4 g	---	---	---
Gelrite	4 g	---	4g	4 g	4 g
Agarose	---	4g	---	---	---
N6 vitamins	5 ml	5 ml	5 ml	5 ml	5 ml
$AgNO_3$	10 mg	10 mg	10 mg	0.85 mg	0.85 mg
PPT	---	---	3 mg	3 mg	3 mg
MS salts	---	---	---	4.4 g	4.4 g
Benzylaminopurine (BAP)	---	---	---	10 mg	---

Table 4. Medium composition (amounts listed allow the preparation of 1 liter of medium). All media were adjusted to pH 5.8 with 1 M KOH.

Transgene	**Primer set 1**	**Primer set 2**	**Primer set 3**
gme	F: 5´-AGACCATTAGCTTTAT CTACTCCAG-3´ R: 5'-CGTCCACGGACACCCTCTGGG-3'	F: 5'-GCCTCGCACATTGCAAGGCGACTG -3' R: 5'-CGTCCACGGACACCCTCTGGG-3'	F: 5'-GCCTCGCACATTGCAAGGCGACTG -3' R: 5´-GTGCCTTGAACTGCTTTTATTCTT-3´
ggp	F: 5´-AAGTACGCTTGTAGCTAGTGCA-3´ R: 5'-CAAGCAAATGGAGGAGTCGGATACCGC-3'	F: 5'-GCTCCAGTCGCCTTTCTTGACTCTCTC-3' R: 5'-CAAGCAAATGGAGGAGTCGGATACCGC-3'	F: 5'-GCTCCAGTCGCCTTTCTTGACTCTCTC-3' R: 5´-CTTCTTTACTCCACCATCTCGTC-3´
galldh	F: 5´-CTAACTAACACAGCCGTGCACAT-3´ R: 5'-CAGTTCAGTGAATGATAGTTCATC-3'	F: 5'-CGCTCCGTTACCTGATGATCTTCA-3' Reverse: 5'-CAGTTCAGTGAATGATAGTTCATC-3'	F: 5'-CGCTCCGTTACCTGATGATCTTCA-3' R: 5´-GTGCCTTGAACTGCTTTTATTCTT-3´
ao RNAi	F: 5´- CTAACTAACACAGCCGTGCACAT-3´ R: 5´-GTGAATTCACTAGTCGAGCATCTCTTCAGCGTAAGGGTA-3´	F: 5´- GGTCTAGAGGATCCGCACCTCTGGCAACCGGGTGAAGGT-3´ R: 5´-GTGAATTCACTAGTCGAGCATCTCTTCAGCGTAAGGGTA-3´	F: 5´- GGTCTAGAGGATCCGCACCTCTGGCAACCGGGTGAAGGT-3´ R: 5´-GTGCCTTGAACTGCTTTTATTCTT-3´
dhar	F: 5´-CTAACTAACACAGCCGTGCACAT -3´ R: 5´-GCTCTTACGCATTCACTTTTGGTG-3´	F: 5´-ATGGGCGTGGAGGTGTGCGTCAAGG-3` R: 5´-GCTCTTACGCATTCACTTTTGGTG -3´	F: 5´-ATGGGCGTGGAGGTGTGCGTCAAGG -3´ R: 5´-GTGCCTTGAACTGCTTTTATTCTT -3´

Table 5. Forward (F) and reverse (R) primer sequences to identify putative transgenic plants by genetic screening. Primer set 1 spans the promoter and transgene (forward primer located in the promoter and reverse primer in the transgene); primer set 2 spans the transgene only (both primers in the transgene); primer set 3 spans the transgene and terminator (forward primer in transgene and reverse primer located in the terminator).

4.4.5 mRNA analysis

Total RNA was isolated from endosperm at 25 dap as described in Section 2.4.5, and 30-µg aliquots were used for mRNA blot analysis as described in detail in Section 3.4.3. Probes were synthesized using genomic DNA PCR primer set 2 (**Table 5**).

4.4.6 Ascorbic acid measurements

The total ascorbic acid content (AsA + DHA), reduced ascorbic acid content (AsA) and dehydroascorbate content (DHA) were determined in T2 endosperm tissue at 20 dap as described in Section 2.4.4. Ten independent biological replicates of each sample were used for AsA measurements.

4.4.7 Statistical analysis

Ten biological replicates were used for each AsA measurement at each time point sample (n = 10). The AsA content was calculated by comparing with values obtained from a standard curve. Values represent mean ± standard error. The data were analyzed by two-way analysis of variance followed by a t-test using the residual mean square in the ANOVA to estimate variability.

4.5 Results

4.5.1 Gene cloning and vector construction

We selected five representative genes involved in AsA biosynthesis and recycling. The g*me*, *galldh* and *ao* RNAi genes were cloned from total RNA extracted from kiwifruit flesh, tobacco leaves and maize leaves, respectively, whereas the *ggp* gene was synthetized based on the kiwifruit sequence but adjusting for maize codon usage.

The individual transgenes were transferred to separate vectors containing different endosperm-specific promoters. The *gme* gene was transferred to vector GZ63, which contains the maize γ-zein promoter. The *ggp* gene was transferred to vector p326, which contains the wheat low molecular weight (LMW) glutenin promoter. The *galldh* gene was transferred to vector pHor, which contains the barley D-hordein promoter. The RNAi construct was transferred to vector pHor-GUS, which contains the barley D-hordein promoter and part of the *gus*A gene, which allows the formation of hairpin *ao* RNA. The rice *dhar* gene was already available cloned in vector pHor.

4.5.2 Combinatorial transformation

Three different combinations of transgenes were chosen in order to investigate the complexity of AsA metabolism and to boost the accumulation of vitamin C in maize endosperm tissue.

The transgene combination selected for experiment 1 was *gme, ggp, galldh* and *dhar*, which was chosen to achieve the highest vitamin C levels based on previous reports in the literature. The transgene combination for experiment 2 was *gme* and *galldh*, because these genes are involved in different AsA biosynthesis pathways (*gme* in the L-galactose and L-gulose pathways and *galldh* in the L-galactose and D-galacturonic pathways, as shown in **Fig. 1**) and their co-expression allowed us to investigate how these pathways interact. The transgene combination for experiment 3 was *galldh* and *ao* RNAi. This combination was selected in order to boost the levels of reduced AsA rather than DHA. **Table 6** summarizes the different experiments and gene combinations.

The genes were introduced into 14-day-old M37W zygotic embryos as described in Section 4.4.3, using the *bar* gene as a selectable marker under the control of the maize ubiquitin 1

(*Ubi-1*) promoter and first intron. Independent transgenic plants were regenerated for each combination of transgenes, and all plants were morphologically similar to wild-type plants.

Experiments	**Gene combination**	**Objective**
1	*gme* + *ggp* + *galldh* + *dhar*	Boost vitamin C levels in maize endosperm
2	*gme* + *galldh*	Study interactions between different AsA pathways
3	*galldh* + *ao* RNAi	Accumulate higher levels of reduced AsA than DHA

Table 6. Summary of the three experiments with different combinations of transgenes.

4.5.3 Molecular characterization of the transgenic maize plants

The putative T0 transgenic plants were screened by genomic DNA PCR using three sets of primers for each transgene (except *bar*), and the confirmed transgenic plants were grown to allow further analysis of gene expression in the T1 endosperm tissue by mRNA blot.

Total RNA was immobilized on nylon membranes and sequentially tested with the different gene-specific probes. These mRNA blots confirmed the accumulation of full-length transcripts of the size expected for each of the transgenes (*gme* = 1.2 kb, *ggp* = 1.3 kb, *galldh* = 1.7 kb, *dhar* = 670 bp and *ao* RNAi = 820 bp) as shown in **Fig. 4**. Transgenic plant 1 (from experiment 1) expressed all four transgenes: *gme, ggp, galldh* and *dhar*. Two independent transgenic plants were recovered in experiment 2, one of which expressed neither transgene (plant 2) whereas the other expressed both *gme* and *galldh* (plant 3). Experiment 3 (*galldh* and *ao* RNAi) generated four different transgenic plants, two of which (plants 4 and 5) showed no expression of either transgene, whereas plant 6 expressed *galldh* alone, and plant 7 expressed both transgenes (**Fig. 4**).

The mRNA blot analysis revealed some heterogeneity in the level of transgene expression and showed that transgenic plant 1 from experiment 1 achieved the highest level of *galldh* expression (**Fig. 4**).

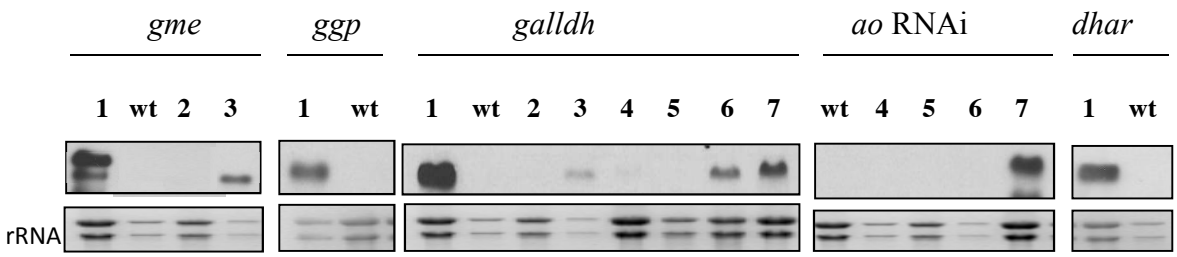

Figure 4. mRNA blot analysis showing endosperm-specific transgene expression in the different transgenic plants. Transgenic plant 1 from experiment 1 (*gme, ggp, galldh* and *dhar*); transgenic plants 2 and 3 from experiment 2 (*gme* and *galldh*) and transgenic plants 4–7 from experiment 3 (*galldh* and *ao* RNAi). Wild-type (wt) RNA was used as a control. The transgenes were: GDP-mannose-3'5'-epimerase (*gme*), GDP-L-galactose phosphorylase (*ggp*), L-galactono-1,4-lactone dehydrogenase (*galldh*), dehydroascorbate reductase (*dhar*) and ascorbate oxidase (*ao*) RNAi. The loading control was rRNA stained with ethidium bromide.

4.5.4 Ascorbic acid measurements

We analyzed the AsA content of the endosperm in the T2 seed generation from transgenic plant 1 because insufficient numbers of T1 seeds were available. The other transgenic plants will be analyzed when T2 seeds become available. The total AsA content in plant 1 T2 endosperm was 236.65 nmol/g FW (41.67 μg/g FW), comprising 115 nmol/g FW AsA and 121 nmol/g FW DHA (**Fig. 5**). Wild-type plants contained 91 nmol/g FW (16 μg/g FW) total AsA comprising 21.4 nmol/g FW AsA and 69.65 nmol/g FW DHA (**Fig. 5**). Transgenic maize plant 1 therefore showed a significant ($P < 0.001$) increase in the total AsA content compared to wild-type plants.

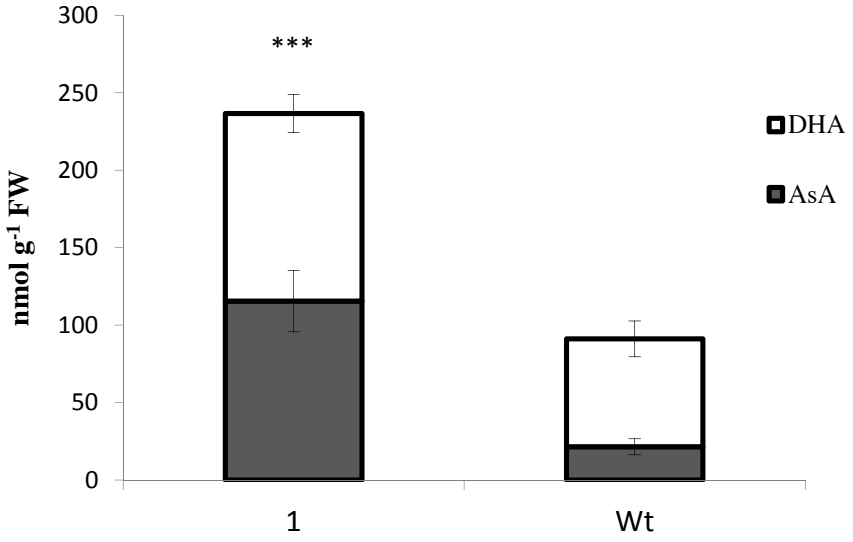

Figure 5 The ascorbic acid content (grey bars) and dehydroascorbate content (white bars) of T2 generation endosperm tissue from transgenic plant 1 at 20 dap, measured in nmol g^{-1} fresh weight (FW). Wild type (Wt) maize was used as a control. Data represent the mean of ten replicates ± standard error (n = 10). *** $P \leq 0.001$).

Gene	Promoter	Vitamin C level	Reference
dhar	Constitutive	**39.6 µg/g FW** (225 nmol/g FW) in kernel (**2-fold**)	Chen et al., 2003.
dhar	Endosperm-specific	**26.4 µg/g FW** (150 nmol/g FW) in endosperm * (**6-fold**)	Naqvi et al., 2009.
gme, ggp, galldh, dhar	Endosperm-specific	**41.67 µg/g FW** (236.65 nmol/g FW) in endosperm (**2.5-fold**)	This study

Table 7. Vitamin C enhancement in maize seeds by genetic engineering. Abbreviations: dehydroascorbate reductase, *dhar*; GDP-L-mannose-3',5'-epimerase, *gme*; GDP-L-galactose phosphorylase, *ggp*; L-galactono-1,4-lactone dehydrogenase, *galldh*; fresh weight, FW.
*Assuming 76% water content in maize grain (http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/SR24/reports/sr24fg11.pdf).

4.6 Discussion

AsA (vitamin C) is an essential nutrient in humans and many other animals, and plants provide the primary dietary source of this molecule. In addition to its nutritional role in humans, which has the greatest impact in undernourished and special-care populations, AsA in plants is associated with resistance to different forms of stress and better postharvest qualities such as extended shelf life (Cruz-Rus et al., 2012). The investigation of AsA biosynthesis, recycling, catabolism and transport pathways is the first step towards achieving higher AsA levels in crops. However, AsA also plays a key metabolic role in plants, and there are many homeostatic mechanisms in place to maintain its concentration and redox status within a certain range. These regulatory mechanisms, which are still largely uncharacterized, limit the ability of single-gene approaches in specific tissues to increase the AsA content of edible plant tissues. Therefore, only limited success has been achieved by single-gene genetic engineering (**Table 1**) and it will be necessary to improve our basic knowledge of all the pathways involved in AsA biosynthesis, recycling and catabolism in order to develop more sophisticated multigene engineering strategies.

We therefore selected three different strategies targeting multiple steps in the maize AsA biosynthesis and recycling pathways in an attempt to increase the concentration of AsA in the endosperm and to understand the regulation of AsA metabolism in this tissue. AsA biofortification in maize would be beneficial because this is the staple crop for up to one third of the world's population and in many developing countries it is the only source of calories and nutrients (Naqvi et al., 2011).

One of the three strategies was the co-transformation of maize with *gme, ggp, galldh* and *dhar* (combinatorial experiment 1), in order to achieve optimal AsA synthesis and accumulation. Another strategy was the co-transformation of maize with *gme* and *galldh*, which represent different pathways and could therefore provide insight into the prevalence of these pathways and their potential interactions at different endosperm developmental stages. The final strategy was the co-transformation of maize with *galldh* and *ao* RNAi, aiming to increase the levels of reduced AsA at the expense of DHA and simultaneously to minimize AsA catabolism. Focusing on reduced AsA could boost AsA accumulation despite the presence of feedback mechanisms because DHA may be responsible for inhibiting the transcription of AsA metabolic genes in maize endosperm (see Chapter 3).

Combinatorial genetic transformation was used to generate transgenic plants with the combinations of transgenes listed above. This method has previously been used for the biofortification of maize with vitamin A (Zhu et al., 2008) and also with a combination of vitamins A, C and folic acid (Naqvi et al., 2009). We generated one transgenic plant in experiment 1 (plant 1) which expressed the *gme, ggp, galldh* and *dhar* transgenes; two transgenic plants in experiment 2 (plants 2 and 3, although only plant 3 expressed both the *gme* and *galldh* transgenes); and four transgenic plants in experiment 3 (plants 4–7), although plant 6 expressed *galldh* alone and plant 7 expressed both the *galldh* and *ao* RNAi transgenes whereas the others expressed neither transgene. The differential transgene expression among the plants in each experiment was likely to reflect the independent transformation events, because the integration site and transgene organization has a significant impact on expression (Kohli et al., 2006).

Although a small number of transgenic plants were regenerated, only transgenic plant 1 produced enough T2 to measure the AsA content in the endosperm. The total AsA content was 2.5-fold higher than wild-type endosperm (**Table 7**), consistent with previous reports in maize in which 2–6-fold increases in AsA levels were achieved (Naqvi et al., 2009; Chen et al., 2003). The remaining transgenic lines have yet to produce enough T2 seeds for analysis and therefore await further investigation.

The absolute content of vitamin C in transgenic maize endosperm was previously boosted to 26.4 μg/g FW (Naqvi et al., 2009) and 39.6 μg/g FW (Chen et al., 2003) whereas transgenic plant 1 in our experiment achieved a concentration of 41.67 μg/g FW. The recommended range of vitamin C intake in humans is 45–90 mg per day, therefore 1–2 kg of transgenic plant 1 maize would be needed daily to reach satisfactory intake levels. Much greater enhancement is therefore required before transgenic maize can provide a substitute for other interventions such as conventional fortification and vitamin C supplements.

4.7 Conclusions

We selected four genes involved in AsA metabolism based on our current knowledge of the rate limiting steps in the AsA pathway in plants and also the relative importance of the recycling pathway as a major contributor to the AsA content in plant tissues. We then proposed different transgene combinations that would provide insight into the complex regulation of AsA metabolism and potentially achieve higher AsA levels in maize endosperm. We generated transgenic maize plants expressing these different combinations of transgenes, including the first stable transgenic plants expressing multiple genes involved in AsA metabolism. However, more transgenic seeds are required for the comprehensive analysis of AsA levels before firm conclusion can be drawn from these experiments.

4.8 Future prospects and recommendations

Further experiments are required to obtain a larger population of transgenic plants representing each combination of transgenes, which will allow the thorough evaluation of transcript and AsA levels in the seeds thus providing greater insight into the endogenous regulatory mechanisms. Further experiments with the transgenic plants discussed in this chapter will also be carried out to quantify the metabolic intermediates in the AsA pathways in order to determine the relative importance of the introduced transgenes and the different biosynthesis and recycling pathways, and potential interactions between them.

More transformation experiments are also required in which the genes are controlled by constitutive as well as endosperm-specific promoters because this will allow the recovery of transgenic plants in which distinct components of each pathway are simultaneously expressed in vegetative and storage tissues, therefore not only improving nutritional properties also providing insight into the role of AsA in biotic and abiotic stress responses and the long-distance transport of AsA between source and sink tissues.

4.9 References

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CHAPTER 5

A QUESTION OF BALANCE – ACHIEVING APPROPRIATE NUTRIENT LEVELS IN BIOFORTIFIED STAPLE CROPS

Chapter 5: A question of balance – achieving appropriate nutrient levels in biofortified staple crops

5.1 Abstract

The biofortification of staple crops with vitamins is an attractive strategy to increase the nutritional quality of human food, particularly in areas where the population subsists on a cereal-based diet. Unlike other approaches, biofortification is sustainable and does not require anything more than a standard food-distribution infrastructure. The health-promoting effects of vitamins depend on overall intake and bioavailability, the latter influenced by food processing, absorption efficiency and the utilization or retention of the vitamin in the body. The bioavailability of vitamins in nutritionally-enriched foods should ideally be adjusted to achieve the dietary reference intake in a reasonable portion while avoiding hypervitaminosis caused by the accumulation of fat-soluble vitamins. Current vitamin biofortification programs focus on the fat-soluble vitamins A and E, and the water-soluble vitamins C and B_9 (folic acid), but the control of dosage and bioavailability has been largely overlooked. In this chapter, I discuss the vitamin content of nutritionally-enhanced foods developed by conventional breeding and genetic engineering, focusing on dosage and bioavailability. Although the biofortification of staple crops could potentially address micronutrient deficiency on a global scale, further research is required to develop effective strategies that match the bioavailability of vitamins to the requirements of the human diet.

5.2 Introduction

Up to one third of the world's population is currently at risk from vitamin deficiency disorders, which can have a severe impact on metabolism resulting in cumulative damage to health (Farré et al., 2011; Yuan et al., 2011). Strategies to address vitamin deficiency include the provision of supplements, the fortification of processed foods and the biofortification of crops to increase the vitamin content at source (Zhu et al., 2007; Goméz-Galera et al., 2010). Health authorities in the developed world have established dietary reference intakes (DRIs) for vitamins, which are based on recommended daily allowances (RDAs) and tolerable upper levels (ULs). The RDA is defined as the daily dietary intake level of a nutrient considered sufficient to meet the requirements of 97.5% of healthy individuals in each life-stage and gender group, and the UL is defined as the highest daily consumption that current data have shown to cause no side effects in humans (IOM, 2000; 2002). For example, the RDA for vitamin A is 900 μg/day and the UL is 3000 g/day. Strategies that address vitamin deficiency must therefore strive to achieve the DRI for each vitamin without exceeding the UL.

Most strategies developed to address vitamin deficiency diseases focus on the total amount of vitamin provided rather than its bioavailability. The latter is important because many different factors affect the efficiency with which vitamins are taken up from food, including the nature of the food matrix, the manner in which the food is prepared and the efficiency with which available free vitamin molecules are absorbed in the human gut. Furthermore, some vitamins are absorbed as pro-vitamins which are converted into their final form in the human body. For example, vitamin A is generally absorbed either as retinol from meat and dairy sources or as β-carotene from plant sources, whereas the functional forms of vitamin A are retinal and retinoic acid. The US Institute of Medicine has therefore recommended that the DRI for vitamin A is expressed as retinol activity equivalents (RAEs) which take bioavailability and metabolic conversion into account (IOM, 2001; 2011). Each RAE corresponds to 1 µg of pure retinol in oil, 2 µg of β-carotene in oil, 12 µg of dietary β -carotene (accounting for the impact of the food matrix on bioavailability) or 24 µg of dietary α-carotene, γ-carotene or β-cryptoxanthin, since these carotenoids carry only one active retinol group whereas β-carotene carries two (Bai et al., 2011).

The DRI must also take into account the requirements of different life stages and gender groups. As stated above, the DRI for vitamin A is 900 μg/day or 900 RAEs. However, this is

the value for adult males. The DRI for females is 700 RAEs, rising to 770 RAEs in pregnancy and 1300 RAEs when lactating, and the DRI for children is 400–500 RAE depending on age (IOM, 2001). The DRI values for the four vitamins discussed in this chapter are listed in **Table 1**, providing a breakdown by gender and life-stage where appropriate. In some cases, the requirements are highly specific, e.g. folic acid requirements increase from 400 μg/day for adult females to 600 μg/day during the first 12 weeks of pregnancy to reduce the risk of neural tube defects (Shaw et al., 1995; Berry and Li, 2002).

A balanced and varied diet including fresh fruit, vegetables, fish and meat provides the DRI for all vitamins and other nutrients, but such diets are difficult to achieve in many developing-country settings because fresh food is either unavailable or too expensive for the majority of the population and the best that can be provided is a predominantly cereal-based diet (Zhu et al., 2007; Gómez-Galera et al., 2010). Although fruits and vegetables are good sources of vitamins, cereals (particularly milled grains) are poor sources of many nutrients, despite providing adequate amounts of carbohydrate and protein. Micronutrient deficiency can be addressed by providing supplements or by fortifying processed foods such as flour with vitamins, but these approaches are generally unsustainable in developing countries because they require continuous investment, efficient government-level infrastructure for production and distribution, and local compliance to achieve adequate doses (Pérez-Massot et al., 2013). An alternative is the biofortification of cereal crops at source by enhancing their intrinsic levels of vitamins, which requires investment only at the development stage and can thereafter rely on existing food processing and distribution networks without specific strategies to ensure local compliance (Christou and Twyman, 2004; Farré et al., 2010a; Zhu et al., 2013).

There has been significant recent progress in the biofortification of staple crops with vitamins, including the key examples of Golden Rice producing high levels of -carotene (Paine et al., 2005) and Multivitamin Corn simultaneously producing high levels of β-carotene, ascorbic acid (vitamin C) and folic acid (Naqvi et al., 2009). However, a large majority of the biofortification studies that have been published thus far have measured absolute levels of the enhanced vitamins without considering bioavailability, which will be critical for the success of biofortification programs. Any biofortified crop deployed in the field will need to provide the DRI of each nutritional component in a reasonable portion, i.e. consistent with a standard daily food intake of 130–150 g/day of fruits and vegetables, up to

1.6 g/day of oil and up to 350 g/day of rice (Dapcich et al., 2004). However, the dosage must also take into account the impact of food processing, cooking and co-dietary factors such as the consumption of nutritional promoters and inhibitors. The dose should be adjusted to provide adequate levels of nutrients without the risk of hypervitaminosis (Section 5.3) and one final significant challenge is that these ideal dosage levels must be achieved for different vitamins simultaneously to avoid merely shifting the challenge of micronutrient deficiency from one set of nutrients to another (Naqvi et al., 2009; Yuan et al., 2011).

5.3 Hypervitaminosis

Tolerable upper limits have been established for most vitamins, including the four discussed in this chapter (**Table 1**).

Water-soluble vitamins must be ingested daily because they cannot be stored in the body. Therefore, although tolerable upper limits have been proposed for ascorbic acid and folic acid, it is unusual to see clinical effects unless there is contraindication with metabolically-related drugs. For example, the tolerable upper limit for ascorbic acid is 2000 mg/d, but an intake of >100 mg results in rapid excretion in the urine without harmful effects (Cho et al., 2000; Levine et al., 1996). Similarly the tolerable upper limit for folic acid is 1 mg/d for adults, but there is no consensus about what blood concentrations of folic acid might cause harm (Smith et al., 2008). In contrast, fat-soluble vitamins are stored in the body and overdosing can cause harmful effects. In adults, the tolerable upper limit for vitamin A is 3 mg/d, and clinical studies have shown that acute doses above this limit or chronic doses of 7.5–15 mg/d can induce anorexia, headaches, nausea and muscle weakness in the short term (Hathcock et al., 1990) and osteoporosis in the long term (Penniston and Tanumihardjo, 2006). The tolerable upper limit for vitamin E in adults is 1 g/d, and although no harmful effects have been observed from acute doses of up to 3.2 g/day overdosing can exacerbate the blood coagulation defect of vitamin K deficiency caused by malabsorption or anticoagulant therapy. High levels of vitamin E are therefore contraindicated in these subjects (Bramley et al., 2000; Kappus and Diplock, 1992).

Age	Female /Male		Pregnancy		Lactation	
Vitamin A[a] (mg/day)						
DRI/UL	**DRI**	**UL**	**DRI**	**UL**	**DRI**	**UL**
0-6 months	0.4	0.6	-	-	-	-
7-12 months	0.5	0.6	-	-	-	-
1-3 years	0.3	0.6	-	-	-	-
4-8 years	0.4	0.9	-	-	-	-
9-13 years	0.6	1.7	-	-	-	-
14 + years	0.7/0.9*	3	0.77	3	1.3	3
Folic acid[b] (mg/day)						
0-12 months	0.065-0.08	ND	-	-	-	-
1-3 years	0.15	0.3	-	-	-	-
4-8 years	0.2	0.4	-	-	-	-
9-13 years	0.3	0.6	-	-	-	-
14 + years	0.4*	1	0.6	1	0.5	1
Vitamin E[c] (mg/day)						
0-12 months	4-5	ND	-	-	-	-
1-3 years	6	200	-	-	-	-
4-8 years	7	300	-	-	-	-
9-13 years	11	600	-	-	-	-
14+ years	15*	1000	15	1000	19	1000
Vitamin C (mg/day)						
0-12 months	40-50	ND	-	-	-	-
1-3 years	15	400	-	-	-	-
4-8 years	25	650	-	-	-	-
9-13 years	45	1200	-	-	-	-
14-18 years	75	1800	80	1800	115	1800
19+ years	90*	2000	85	2000	120	2000

Table 1. Dietary reference intakes (DRIs) and the tolerable upper intakes levels (UL) for the four vitamins discussed in this chapter**.** The DRI is based on the older recommended daily intake (RDI), which is the average daily intake sufficient to meet the requirements of 97.5% of healthy individuals. It is calculated from an estimated average requirement (EAR). If sufficient evidence is not available to establish the EAR, an adequate intake (AI) may be used instead, which is the mean intake for healthy breastfed infants or that sufficient to cover the needs of all healthy individuals in other life-stage and gender groups, accepting uncertainty in the percentage of individuals covered by this intake. * means the DRI for the four vitamins discussed in this chapter.
NOTE: UL is the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population. Unless otherwise specified, the UL represents total intake from food, water and supplements. In the absence of an UL, extra caution may be warranted in consuming levels above recommended intakes. Members of the general population are advised not to exceed the UL routinely. The UL is not meant to apply to individuals who are treated with the nutrient under medical supervision or to individuals with predisposing conditions that modify their sensitivity to the nutrient.
[a] As preformed vitamin A only.
[b]As dietary folic acid equivalents (DFE). 1 DFE = 1 µg food folic acid = 0.6 µg of folic acid from fortified food or as a supplement consumed with food = 0.5 µg of a supplement taken on an empty stomach.
[c]As α-tocopherol, which includes *RRR*-α-tocopherol, the only form of α-tocopherol that occurs naturally in foods, and the *2R*-stereoisomeric forms of α-tocopherol (*RRR*-, *RSR*-, *RRS*-, and *RSS*-α-tocopherol) that occur in fortified foods and supplements. It does not include the *2S*-stereoisomeric forms of α-tocopherol (*SRR*-, *SSR*-, *SRS*-, and *SSS*-α-tocopherol), also found in fortified foods and supplements.
[b,c] The ULs for vitamin E and folic acid apply to synthetic forms obtained from supplements, fortified foods, or a combination of the two.
ND = Not determinable due to lack of data of adverse effects in this age group and concern with regard to lack of ability to handle excess amounts. Source of intake should be from food only to prevent high levels of intake.
DRI taken from the DRI reports, see *www.nap.edu* (*Dietary Reference Intakes for Folate* (1998); *Dietary Reference Intakes for Vitamin C, Vitamin E* (2000)) and UL is adapted from IOM 2011.

5.4 The impact of food processing on vitamin levels and bioavailability in food

5.4.1 Fat-soluble vitamins

The stability of carotenoids differs according to the properties of the food matrix even when the same processing and storage conditions are used (Rodriguez-Amaya, 1997). However, the processing method also has a significant impact. For example, some β-carotene is lost during microwaving, steaming, boiling and sautéing, but much more substantial losses occur during deep-frying, prolonged cooking, and combinations of several preparation or processing methods such as baking and pickling (Rodriguez-Amaya, 1997). Whereas almost all cooking methods reduce the overall content of β-carotene, some simultaneously increase its bioavailability by releasing it from the food matrix (Hart and Scott, 1995). Products that are often consumed raw (e.g. carrots, tomatoes and other fruits) may therefore be less suitable sources of β-carotene than cereals, potatoes and canola oil, which are cooked before consumption, even though the overall levels are higher before cooking. For example, although microwave cooking reduces the overall amount of β-carotene it also increases tissue degradation and the amount of β-carotene available for extraction (Howard et al., 1999). These factors explain why only minor changes in β-carotene levels were observed in tomato juice during processing (Lin and Chen, 2005) whereas the levels of bioavailable β-carotene can increase or decrease in carrots and cassava depending on the cooking method (Gayathri et al., 2004; Carvalho et al., 2012).

Vitamin E is a group of related compounds known as tocochromanols, which are divided into the tocopherols and tocotrienols. Vitamin E levels in cereals are also affected by storage and cooking, but there are conflicting reports in the literature suggesting that the bioavailable levels increase, decrease or do not change (Bernhardt and Schlich, 2006; Mazzeo et al., 2011). Minimal losses were recorded when cereal grains were stored under optimal conditions in the absence of insect pests, but processing by milling resulted in significant losses (Pomeranz, 1992; Borrelli et al., 2008). Furthermore, the tocochromanol levels in wheat, barley and oat were reported to fall by 30% during extrusion cooking (Zielinski et al., 2001), whereas cooking in water was shown to increase the total tocochromanol levels in red and white rice by 32% and 37%, respectively (Finocchiaro et al., 2007). Importantly, this increase reflected the 23% loss of soluble dry matter during water cooking which resulted in the concentration of lipophilic compounds (Finocchiaro et al., 2007). Such reports

demonstrate the importance of standardized methods for measuring and recording nutritional components in foods to allow direct and meaningful comparisons.

The processing of plant oils can also reduce the availability of tocochromanols, e.g. pre-heating, cooking and screw-pressing can reduce levels by 15% in olive oil, 25% in soybean and rapeseed oils, 28.5% in high-oleic safflower oil, 32% in corn oil and 35–40% in cotton seed and peanut oil (Kanematsu et al., 1983; Ortega García et al., 2006).

5.4.2 Water-soluble vitamins

Vitamin C is also lost during cooking, but the amount depends on the temperature, the degree of leaching into the cooking medium, the surface area of the food exposed to water, oxygen levels, pH, and the presence of pro-oxidation factors (Lešková et al., 2006). Vitamin C in potatoes is rapidly degraded during boiling and frying, but is less susceptible during braising, sautéing and pressure-cooking, and only minimal losses occur during baking and microwaving (Han et al., 2004). In addition, boiling in salted water can offer partial protection from degradation (Han et al., 2004).

The levels of plasma vitamin C were measured in human subjects after the consumption of either fresh strawberries or strawberries stored in an open container at 4ºC for 4 days. Although the levels of ascorbic acid in the fruits were the same, the plasma ascorbic acid levels were higher in subjects who had consumed the stored strawberries, which was attributed to the modification of bioactive compounds that influence the bioavailability of ascorbic acid (Azzini et al., 2010).

Folic acid is also lost during cooking and food preparation, reflecting a combination of thermal degradation and leaching into the cooking water (Basset and Sammán, 2010). Leaching was found to be the major factor, e.g. boiling in water reduced the folic acid content of broccoli and peas more than blanching and microwaving, whereas minimal losses were recorded after steaming, which involves the least contact with water (Stea et al., 2006). The boiling of peeled and unpeeled potatoes resulted in folic acid losses of 52% and 37%, respectively, suggesting that the skin protects against folic acid loss (Stea et al., 2006). This also indicated the greater role of leaching compared to thermal degradation in the loss of folic acid (Dang et al., 2000; Scott et al., 2000).

5.5 Vitamin biofortification to improve nutrition

As discussed above, the biofortification of crops at source provides a sustainable approach to improve the nutritional quality of food without the continual need for a specialized distribution infrastructure or compliance monitoring (Farré et al., 2010a; Pérez-Massot et al., 2013). Two strategies have been developed to increase the vitamin levels in plants, one involving conventional breeding and the other involving genetic engineering.

Numerous studies have now shown that only genetic engineering can provide the means to produce nutritionally enhanced crops that can meet DRI values. For example, different genetic engineering strategies have yielded tomato fruits producing (in separate plants individually) up to 205 μg/g fresh weight of β-carotene (D'Ambrosio et al., 2004), 1159 μg/g fresh weight of ascorbic acid (Haroldsen et al., 2011), 10 μg/g fresh weight of folic acid (Díaz de la Garza et al., 2007) or 4.57 μg/g fresh weight of α-tocopherol (Seo et al., 2011). In contrast, conventional breeding in different tomato varieties has achieved maximum levels of 20 μg/g fresh weight of β-carotene (Lenucci et al., 2006), 180 μg/g fresh weight of ascorbic acid (Proteggente et al., 2002) or 0.29 μg/g fresh weight of folic acid (Bekaert et al., 2008). Focusing on β-carotene, the difference in achievement therefore equates to 28% of the DRI in conventionally-bred tomatoes (Lenucci et al., 2006) compared to 285% of the DRI in tomato lines produced by genetic engineering (D'Ambrosio et al., 2004). This means that the genetically-engineered tomatoes would be required as minor food additives rather than whole fruit in order to achieve the DRI and avoid hypervitaminosis (Section 5.3). We provide a breakdown of the nutritional values of other conventional and genetically-engineered crops in **Table 2**.

Most of the nutritionally-enhanced crops reported thus far produce higher levels of a single vitamin, and can therefore be regarded as targeted intervention crops suitable for populations with particular nutrient deficiencies (Pérez-Massot et al., 2013; Berman et al., 2013). However, many developing country populations suffer from multiple nutrient deficiencies simultaneously, and in the future it will be necessary to develop staple crops providing the DRI for several vitamins and other nutrients in one variety (Section 5.6). Again, this is beyond the abilities of conventional breeding, since each additional nutritional trait would require individual selection followed by introgression into existing nutritionally-enhanced locally-adapted varieties. In contrast, it is possible to introduce multiple genes directly into

local cultivars by genetic engineering in order to enhance different nutritional components simultaneously (Naqvi et al., 2009). Only one such report has been published thus far, describing a genetically-engineered corn line producing higher levels of ascorbic acid, β-carotene and folic acid (Naqvi et al., 2009). This was achieved by combinatorial nuclear transformation with genes representing the three separate metabolic pathways (Zhu et al., 2008). The adoption of staple crops producing higher levels of multiple nutrients will help to improve the health and well-being of the world's poorest people.

5.6 Multivitamin enhanced crops

The biofortification of local elite crop varieties with multiple vitamins will require a combination of breeding and genetic engineering. Breeding alone cannot be used to produce nutritionally-complete crops because the selection process is too complex (Naqvi et al., 2009; 2010; 2011). Thus far, one genetically-engineered Multivitamin Corn variety has been described (Naqvi et al., 2009) and this was simultaneously enhanced for three different vitamins (ascorbic acid, β-carotene and folic acid). **Table 3** shows the percentage of DRI provided by the best-performing transgenic crops enhanced with the vitamins discussed in this chapter. Ideally, it would be useful to combine these traits so that a single portion could meet the DRI for all vitamins e.g. a transgenic tomato providing 950 μg/g fresh weight of β-carotene, 1159 μg/g fresh weight of ascorbic acid, 11.04 μg/g fresh weight of folic acid and 4.57 μg/g fresh weight of α-tocopherol (**Table 3)**. In corn, the best-performing transgenic lines developed thus far yield 59.3 μg/g dry weight of β-carotene, 344.75 μg/g dry weight of α-tocopherol, 1.94 μg/g dry weight of folic acid and 168 μg/g dry weight of ascorbic acid.

Crop	Method *	Vitamin level	Portion required to achieve the recommended dietary intake (RDI) (grams of the transgenic plant) **	A reasonable daily portion (RDP)***	Percentage (%) RDI provided by a RDP	Reference	Food processing (Retention or losses of the vitamin after cooking process)	Percentage (%) RDI provided by a RDP after food processing	Reference
Vitamin A									
Rice (*Oryza sativa*) (12:1)	GE	31 µg/g dry weight (DW) β-carotene	348.4 g	130 g/day	37%	Paine et al., 2005	Golden Rice Boiling 30min 0% loses of β-carotene	37%	Tang et al., 2009
Canola (*Brassica napus*) (2:1 ratio)	CB	0.49 µg/g fresh weight (FW) β-carotene	3673 g	1-1.6 g/day "as oil"	0.03-0.04%	Yu et al., 2008	Fresh	0.03-0.04%	
	GE	949 µg/g FW β-carotene (316-fold)	1.89 g		53-84%	Shewmaker et al., 1999		53-84%	
	GE	857 µg/g FW β-carotene	2.1 g		48-76%	Ravanello et al., 2003		48-76%	
	GE	90.76 µg/g FW β-carotene (185.2-fold)	19.8 g		5-8%	Yu et al., 2008		5-8%	
	GE	214 µg/g FW β-carotene (1070-fold)	8.41 g		12-19%	Fujisawa et al., 2009		12-19%	
	GE	0.40 µg/g FW β-carotene (6-fold) (10% water content)	4500 g		0.02-0.03%	Wei et al., 2010		0.02-0.03%	
Tomato (*Lycopersicon esculentum*) (12:1)	CB	20 µg/g FW β-carotene	540 g	150 g/day	28%	Lenucci et al., 2006	Fresh // Baked 160º 20min 81% increase of β-carotene // Canned tomato juice, losses of 22% of β-carotene	27.7% // 50% // 21.6%	Hwang et al., 2012 // Diretz and Gould, 1986
	GE	52 µg/g FW β-carotene (1.9-fold)	207.6 g		72%	Römer et al., 2000		72% // 130% // 56%	
	GE	57 µg/g FW β-carotene (7.1-fold)	189.47 g		79%	Rosati et al., 2000		79% // 143% // 62%	
	GE	63 µg/g FW β-carotene (12-fold)	171.42 g		87%	Dharmapuri et al., 2002		87% // 157% // 68%	

	GE	82.5 µg/g FW β-carotene (1.3-fold) Assuming water content of 94%	131 g		115%	Fraser et al., 2002		115% // 208% // 90%	
	GE	205 µg/g FW β-carotene (46.6-fold)	52.7 g		285%	D'Ambrosio et al., 2004		285% // 516% // 222%	
	GE	42 µg/g FW β-carotene (1.4-fold) Assuming water content of 94%	257 g		58%	Enfissi et al., 2005		58% // 105% // 45%	
	GE	20 µg/g FW β-carotene (8-fold)	540 g		27%	Davuluri et al., 2005		27% // 49% // 21%	
	GE	10.1 µg/g FW β-carotene (1.3-fold)	1069.3 g		14%	Giliberto et al., 2005		14% // 25% // 11%	
	GE	17µg/g FW β-carotene (4-fold) Assuming water content of 94%	635 g		24%	Wurbs et al., 2007		24% // 43% // 18%	
	GE	150 µg/g FW β-carotene (1.6-fold)	72 g		208%	Simkin et al., 2007		208% // 376% // 162%	
	GE	58 µg/g FW β-carotene (5-fold) Assuming water content of 94%	185.5 g		81%	Apel and Bock, 2009		81% // 145% // 63%	
	GE	49 µg/g FW β-carotene (1.4-fold) Assuming water content of 94%	220 g		68%	Fraser et al., 2007		68% // 123% // 53%	
	GE	40 µg/g FW β-carotene (2-fold)	270 g		55%	Sun et al., 2012		55% // 99% // 43%	
Potato (*Solanum tuberosum*) (12:1)	CB	0.4 µg/g FW β-carotene (Assuming water content 80%)	27000 g	130 g/day	0.5%	Römer et al., 2002	ND		

	GE	0.6 µg/g FW β-carotene	18000 g		0.7%				
	GE	2.06 µg/g FW β-carotene	5242.7 g		2.5%	Ducreux et al., 2005			
	GE	0.017 µg/g FW β-carotene	635294 g		0.02%	Diretto et al., 2006			
	GE	9.5 µg/g FW β-carotene	1137 g		11%	Diretto et al., 2007			
	GE	0.52 µg/g FW β-carotene	20769 g		0.6%	Van Eck et al., 2007			
Corn (*Zea mays*) (12:1)	CB	13.6 µg/g DW β-carotene	794 g	130 g/day	16.4%	Harjes et al., 2008	Cooking 50% retention of β-carotene	8.2%	De la Parra et al 2007**.**
	GE	57.35µg/g DW β-carotene (410-fold)	188 g		69%	Zhu et al., 2008		34.5%	
	GE	6 µg/g DW β-carotene (3.8-fold)	1800 g		7%	Aluru et al., 2008		3.6%	
	GE	59.32 µg/g DW β-carotene (169-fold)	182 g		71%	Naqvi et al., 2009.		35.5%	
Kumquat *(Fortunella spp.)* (12:1)	CB	0.70 µg/g FW β-carotene	15428.5 g	150g/day	1%	Zhang et al., 2009	Hot water dip (2min, 50ºC) Non-significant differences	1%	Schirra et al., 2008
	GE	1.72 µg/g FW β-carotene (2.5-fold)	6279 g		2.4%			**2.4%**	
Carrot*(Daucus carota)* (12:1)	GE	39 µg/g FW β-carotene (Assuming water content 87%)	277 g	150g/day	54%	Jayaraj et al., 2008	Fresh //Cooking 60min.Losses of 59% of β-carotene // Pureeing. Losses of 56% of β-carotene. // Canning carrot juice. Retention of77% of β-carotene // pressure cooked for 10 min 27% of losses of β-carotene // Boiled for 10 min 16% of losses of β-carotene	54% // 22% // 24% // 42% // 39% // 45%	Nagra and Khan,1988 // Olunlesi and Lee, 1979 // Kim and Gerber, 1988. // Gayathri et al., 2004
	GE	31.4 µg/g FW β-carotene	344 g		43%	Maass et al., 2009		43% // 18% // 19% // 33% // 31% // 36%	
	CB	0.16 µg/g FW β-carotene	67500 g		0.2%			0.2% // 0.09% // 0.088% // 0.154% // 0.15% // 0.17%	

Cassava *(Manihot esculenta)*	GE	2.67 µg/g FW β-carotene (Assuming water content 60%)	4045 g	150g/day	3.7%	Welsch et al., 2010	Booiling the roots Retention of 55.7% total carotenoid // Drying Loss of 15% or less of total carotenoids	2% //3.1%	Chávez et al., 2007 // Bechoff et al., 2010
	CB	0.164 µg/g FW β-carotene	65854 g		0.2%			0.11% //0.17%	
Vitamin E									
Corn (*Zea mays*)	GE	> 344.57 µg /g DW in seeds (6-fold)	43.53 g	130 g/day	299%	Cahoon et al., 2003	ND		
	GE	9.5 µg/g DW γ-tocopherol (3-fold)	1579 g		8%	Naqvi et al., 2010			
Canola (*Brassica napus*)	CB	670 µg/g oil in in seeds total tocochromanol	22.4 g	1-1.6 g/day "as oil"	4%-7%	Raclaru et al., 2006	ND		
	GE	1850 µg/g oil in seeds total tocochromanol (2-fold)	8 g		12%-20%				
	GE	540 µg/g of total tocochromanols in seeds (2-fold)	28 g		4%-6%	Karunanandaa et al., 2005			
	GE	1159 µg/g of total tocochromanols in seeds (1.7-fold)	13 g		8%-12%	Kumar et al., 2005			
Soybean *(Glycine max)*	CB	306 µg/g seed total tocopherol	49 g	1-1.6 g/day (as a oil)// 150 g (as a vegetable)	2%-3% // 306%	Van Eenennaam et al., 2003	Microwave (as oil) Retention of 90% of vitamin E // Cooking soybean; 165 min, soaking 16h (as vegetable)	1.8%-2.7% (oil) // 280% (vegetable)	Yoshida et al., 1990 // Masková et al., 1996
	GE	329 µg/g seed total tocopherol (γ-tocopherol represent 75 to 85%of total tocopherols)	45.6 g		2%-3% // 329%			1.8%-2.7% (oil) // 302% (vegetable)	
	GE	505 µg/g FW of total tocopherol content in seeds	30g		3%-5% // 505%	Tavva et al., 2007		3%- 5% (oil) // 465%	

							Retention of 92% of vit E	(vegetable)	
	GE	4806 µg/g seed total tocochromanols (15-fold)	3 g		32%-51% // 4800%	Karunanandaa et al., 2005		29%- 46% (oil) // 4400 % (vegetable)	
Rice (*Oryza sativa*)	GE	Improved α/γ tocopherol ratio 3.97 (wild type = 2.35) 6.00 µg/g DW α-tocopherol in seeds (1.4-fold)	2500 g	130 g/day	5%	Farré et al., 2012	ND		
Letucce (*Lactuca sativa*)	GE	17.77 µg/g FW total tocopherol	844 g	150 g/ day	18%	Ren et al., 2011	Fresh	18%	
	GE	64.55 µg/g FW total tocopherol	232 g		64%	Li et al., 2011		64%	
	CB	7.10 µg/g FW total tocopherol	2112 g		7%			7%	
Tomato *(Lycopersicon esculentum)*	GE	4.57 µg/g FW α-tocopherol	3282 g	150 g/day	5%	Seo et al., 2011.	Fresh	5%	
	CB	2.8 µg/g FW α-tocopherol	5357 g		3%			3%	
Vitamin C									
Corn (*Zea mays*)	GE	168 µg/g DW in kernel (Assuming water content 76%)	536 g	130 g/day	24%	Chen et al., 2003	ND		
	GE	106 µg/g DW in kernel	849 g		15%	Naqvi et al., 2009			
	CB	96 µg/g DW in kernel	939 g		14%	Chen et al., 2003			
Tomato (*Solanum lycopersicum*)	GE	404.8 µg/g FW in red ripe fruit(1.60-fold in red ripe fruits)	222 g	150g/day	67%	Zhang et al., 2011	Fresh // Cooked. Losses of 89.12% of ascorbate (AA)	67% // 7%	Alvi et al., 2003
	GE	1109.5 µg/g FW in fruit (6 fold increase)	81 g * But this tomato line has no seeds.		185%	Bulley et al., 2012		185% // 20%	

			It is sterile						
	GE	1159 µg/g FW (1.6 fold increase in red-ripe fruit)	78 g		193%	Haroldsen et al., 2011		193% // 21%	
	CB	180 µg/g FW	500 g		30%	Proteggente et al., 2002		30% // 3%	
Letucce (*Lactuca sativa*)	CB	<20 µg/g FW	4500 g	150g/day	3%	Proteggente et al., 2002	Fresh	3%	
	GE	792 µg/g FW (7 fold)	113 g		133%	Jain and Nessler, 2000		133%	
Potato (*Solanum tuberosum)*	GE	915.8 µg/g FW (1 fold) in tuber	98 g	130 g/day	133%	Qin et al., 2011	Cooking potatoes. Losses of 20-40% of vit C // Cooking-keeping hot (55-60ºC,1h).losses of 40% vit C // Pressur-cooking. Losses of 32.8% vitC // Microwaving, pressure-cooking. Losses of 8-17% vit C	80%-106% //80%//90%/ 110%-122%	Golaszewska and Zalewski, 2001 // Hägg et al. 1998 // Vallejo et al., 2002 // Golaszewska and Zalewski, 2001
	GE	528.4 µg/g FW in tuber (1.41–fold)	170 g		76%	Hemavathi et al., 2010		46%-61% // 46% // 51%// 63%-70%	
	GE	366 µg/g FW	246 g		53%	Bulley et al., 2012		31%-42% // 31%// 36%// // 44%-49%	
	CB	109.2 µg/g FW	824.2 g		16%	Lee et al., 2000		10%-13% // 10% //11%// 13%-15%	
Strawberry (*Fragaria × ananassa*)	GE	1303.3 µg/g FW (2 fold increase)	69 g	150 g/day	217%	Bulley et al., 2012	Freshly	217%	
	CB	634 µg/g FW	142 g		105%	Lee et al., 2000		105%	
Folate									
Tomato (*Solanum lycopersicum*)	CB	0.29 µg/g FW total folate	1379 g	150g	10.8%	Bekaert et al., 2008	Fresh	10.8%	
	GE	6.18 µg /g FW total folate (14-fold)	65 g		231%	Waller et al., 2010		231%	

	GE	1.76 µg /g FW total folate (2-fold)	227 g		66%	Díaz de la Garza et al., 2004		66%	
	GE	10 µg /g FW total folate (25-fold)	39 g		380%	Díaz de la Garza et al., 2007		380%	
Corn (*Zea mays)*	CB	0.19 µg /g DW total folate	2105 g	130 g/day	6.1%	Bekaert et al., 2008	ND		
	GE	1.94 µg /g DW total folate(2-fold)	206 g		63%	Naqvi et al., 2009			
Rice (*Oryza sativa*)	CB	0.08 µg /g	5000 g	130 g/day	2.6%	Bekaert et al., 2008	ND		
	GE	16.9 µg /g (100-fold)	24 g		549%	Storozhenko et al., 2007b			
Lettuce (*Lactuca sativa*)	GE	1.89 µg /g FW Total folate (5.4-fold)	212 g	150g	71%	Nunes et al., 2009	Fresh	71%	
	CB	0.35 µg /g FW Total folate	1143 g		13%			13%	

Table 2 Transgenic crops enhanced with vitamin A, E, C and/or folic acid
* Method; conventional breeding (CB) or genetic engineering (GE).
**Vitamin A: Calculation for 900 retinol activity equivalents (RAEs) as a dietary reference intake (DRI) for vitamin A. Each RAE corresponds to 1 µg retinol, 2 µg of β-carotene in oil, 12 µg of dietary β-carotene, or 24 µg of the three other dietary provitamin-A carotenoids (IOM, 2001). Vitamin E: Calculation for 15 mg/day as a dietary reference intake (DRI) for vitamin E (IOM, 2001). Vitamin C: Calculation for 90 mg/day as a dietary reference intake (DRI) for vitamin C (IOM, 2001). Folate: Calculation for 400 μg/day for adults as a DRI for folate (IOM, 2001).
*** The reasonable daily portion (RDP) is of vegetable and fruits is 130 g/day and 150 g/day, respectively. In the case of oil is between 1-1.6 g/day. Source: www.nap.edu, http://www.iom.edu/Global/News%20Announcements/~/media/C5CD2DD7840544979A549EC47E56A02B.ashx and Dapcich et al., 2004.
No Data (ND)

Crop/ Vitamin	Vitamin A	Vitamin E	Vitamin C	Folic acid
Corn	35.5 % (cooked) (Naqvi et al., 2009)	299 % * (Cahoon et al., 2003)	24 % * (Chen et al., 2003)	63 % * (Naqvi et al., 2009)
Rice	37 % (boiled 30 min) (Paine et al., 2005)	5 % * (Farré et al., 2012)		549 % * (Storozhenko et al., 2007b)
Potato	11. % * (Diretto et al., 2007)		80%-106 % (cooked) (Qin et al., 2011)	
Cassava	3.1 % (dryed) (Welsch et al., 2010)			
Soybean		32-51 % (as a oil) (microwave) (Karunanandaa et al., 2005)		
		4800% (as a vegetable) (cooked) (Karunanandaa et al., 2005)		
Canola	53- 84% (fresh) (Shewmaker et al., 1999)	12- 20 % (fresh) (Raclaru et al., 2006)		
Tomato	285 % (fresh) (D'Ambrosio et al., 2004)	5% (fresh) (Seo et al., 2011)	193% (fresh) (Haroldsen et al., 2011)	380% (fresh) (Díaz de la Garza et al., 2007)
Lettuce		64 % (fresh) (Li et al., 2011)	133 % (fresh) (Jain and Nessler, 2000)	71 % (fresh) (Nunes et al., 2009)
Carrot	54 % (fresh) (Jayaraj et al., 2008)			
Strawberry			217 %(fresh) (Bulley et al., 2012)	
Kumquat	2.4 % (2min, 50ºC) (Zhang et al., 2009)			

Table 3 Percentage of Dietary Recommended Intake (DRI) provided by the best-performing transgenic crop enhanced with vitamins A, E, C and/or folic acid, considering a reasonable daily portion of the crop. The processing method is shown in parentheses, whereas unprocessed food is shown with an asterisk.

5.7 The processing of biofortified crops

Few studies have considered the bioavailability of β-carotene in genetically-engineered staple crops after processing. In the case of Golden Rice, which accumulates 31 μg/g dry weight of grain by overexpressing the enzymes PSY1 and CRTI to remove an early bottleneck in the carotenoid biosynthesis pathway (Paine et al., 2005), the total amount of β-carotene was found to be the same before and after cooking (Tang et al., 2009). In transgenic potatoes

expressing the *Orange* (*Or*) gene, which leads to the induction of chromoplast development thus creating a metabolic sink for carotenoids, the levels of β-carotene were five-fold higher than in normal potatoes at harvest, i.e. 5 μg/g β-carotene dry tuber weight (López et al., 2008), but this increased to 10-fold higher when the tubers were stored at 5°C for 6 months (Li et al., 2012).

The impact of processing on nutritionally-enhanced foods can be predicted by extrapolating the effect of processing on normal foods, i.e. the percentage loss or gain of bioavailable vitamin resulting from different processing methods. For example, baking tomatoes at 160°C for 20 min increases the bioavailable β-carotene content by 81% (Hwang et al., 2012) whereas canned tomato juice contains 20% less β-carotene than fresh tomatoes (Dietz and Gould, 1986). Therefore, if we take the best performing genetically-enhanced tomato line, which produces 205 μg/g of β-carotene in the fresh tomato fruits (D'Ambrosio et al., 2004), we can predict that a reasonable portion of tomatoes (150 g/day) would provide 285% of the DRI for vitamin A when consumed as fresh fruit, 516% when consumed after baking and 222% as processed tomato juice (**Table 2**).

Similarly, β-carotene levels in carrots fall by 16% and 59% after conventional cooking for 10 and 60 min, respectively (Gayathri et al., 2004; Nagra and Khan, 1988), and by 27% after pressure cooking for 10 min (Gayathri et al., 2004). Pureeing reduces β-carotene levels by 56% (Olunlesi and Lee, 1979) and carrot juice contains 23% less β-carotene than fresh carrots (Kim and Gerber, 1988). Therefore, the best transgenic carrot variety, which produces 39 μg/g fresh weight of β-carotene (Jayaraj et al., 2008), would achieve 54% of the DRI for vitamin A if 150 g/day were consumed as raw vegetables and 22% if cooked for 60 min, compared to conventional carrots which achieve 0.2% or 0.09% if consumed raw or cooked for 60 min, respectively (**Table 2**).

The highest level of vitamin E produced thus far in transgenic tomatoes is 4.57 μg/g fresh weight, which is equivalent to 5% of the DRI assuming a portion of 150 g/day (Seo et al., 2011). Transgenic lettuce plants have been produced yielding up to 64.55 μg/g fresh weight of vitamin E, which assuming the same portion size would be equivalent to 64% of the DRI (Li et al., 2011). Food processing has a small but significant impact on vitamin E levels, e.g. microwaving reduces overall vitamin E levels in soybean oil by 10% (Yoshida et al., 1990) and cooking for 165 min reduces vitamin E levels by 8% (Masková et al., 1996).

The impact of processing on water-soluble vitamins is more potent. Transgenic tomatoes, lettuce and strawberries have been developed containing up to 1159, 792 and 1303 μg/g fresh weight of vitamin C, respectively (Haroldsen et al., 2011; Jain and Nessler, 2000; Bulley et al., 2012). These values represent 193%, 133% and 217% of the DRI for vitamin C assuming a daily portion of 150 g (**Table 2**). However, up to 89% of the vitamin C in tomatoes is lost by cooking, reducing its nutritional value to 21% of the DRI (Alvi et al., 2003). Transgenic potatoes have been produced yielding 915 μg/g fresh weight of vitamin C, which is equivalent to 133% of the DRI for vitamin C assuming a daily portion of 130 g (Qin et al., 2011). However, vitamin C is particularly susceptible during wet cooking methods and 20–40% is lost; even dry cooking methods such as microwaving reduce vitamin C levels by 8–17% (Golaszewska and Zalewski, 2001). Therefore, if the transgenic potatoes described above were prepared by conventional cooking, pressure-cooking or microwaving, the vitamin C content of a daily portion would be reduced to 80%, 90% and 110% of the DRI, respectively (**Table 2**).

Transgenic tomato and lettuce plants have been produced yielding 10 and 1.89 μg/g fresh weight of folic acid, respectively (Díaz de la Garza et al., 2007; Nunes et al., 2009). Although, as discussed above, folic acid is lost by leaching during processing, both tomatoes and lettuce tend to be eaten fresh and portions of 150 g/day would achieve 380% and 71% of the DRI respectively (**Table 2**).

Corn is a particularly interesting example to investigate because in this case it is possible to predict the nutritional values of three different vitamins in one meal. The carotenoid content of corn is reduced by more than 50% by lime-cooking (De la Parra et al., 2007), which means that a portion of Multivitamin Corn (Naqvi et al., 2009) would provide 35% of the DRI for vitamin A when prepared in this manner. Multivitamin Corn also yields 106 μg/g dry weight of vitamin C and 1.94 μg/g dry weight of folic acid (Naqvi et al., 2009) but there are no data in the literature to show how these levels would be affected by storage, processing and cooking.

5.8 The efficiency of vitamin absorption

5.8.1 Fat-soluble vitamins

The absorption, transport and distribution of vitamins A and E within the body are linked to the intake of dietary fat. The first step is the release of vitamins from the food matrix, which is strongly influenced by different food processing techniques as discussed above. The type of food and the subcellular compartment containing the vitamin also has an impact, particularly in the case of β-carotene: plasma retinol levels are up to six times higher following the consumption of fruits compared to leafy vegetables containing the same overall amount of β-carotene, suggesting it is released more easily from chromoplasts than chloroplasts (de Pee et al., 1998).

After ingestion, vitamins A and E are solubilized as micelles in the intestinal lumen in the presence of bile salts and pancreatic enzymes (Kayden and Traber, 1993; Yonekura and Nagao, 2007). The amount of fat ingested in the meal plays a crucial role because it may promote carotenoid absorption by stimulating bile secretion and increasing the luminal concentration of bile salts, which act as surfactants in the formation of mixed micelles (Hofmann, 1999). The bioavailability of vitamin E also depends on the amount of fat ingested with the meal (Lodge, 2005). There is no specific recommended dose of fat to promote vitamin adsorption (Jeanes et al., 2004) and dietary compounds that inhibit fat absorption and facilitate its excretion from the body such as plant fibers and sterols have the opposite effect on vitamin bioavailability (Yonekura and Nagao, 2009).

In the next step, fat-soluble vitamins are absorbed by epithelial cells (enterocytes) either through simple diffusion or facilitated diffusion via class B type I scavenge receptors (**Fig. 1**). They are then packed in chylomicrons which are released from enterocytes into the lymphatic system and later the circulation en route to the liver (Yonekura and Nagao, 2007). Finally, fat-soluble vitamins become associated with fat-transport proteins such the various lipoprotein receptors, which facilitate their distribution around the body.

The molecular structure of each type of vitamin influences its absorption. Hydrocarbon-rich carotenoids such as β-carotene are less bioavailable than oxygenated carotenoids (e.g. lutein) because the latter are easily incorporated into the outer portions of lipid micelles within the gastrointestinal tract and by enterocyte membranes (van het Hof et al., 1999). In the case of tocopherols, all isomers can be absorbed equally during digestion (Traber and Sies, 1996) but

once chylomicrons get to the liver, the hepatic α-tocopherol transfer protein (α-TTP) preferentially retains α-tocopherol making it the most important isomer in terms of vitamin E activity (Traber and Arai, 1999).

5.8.2 Water-soluble vitamins

Unlike fat-soluble vitamins, water-soluble vitamins must be ingested daily as they cannot be stored in the body. Although ascorbic acid is the principal active form of vitamin C, dehydroascorbic acid also has some activity and the two can easily be interconverted so it is important to consider them both in bioavailability studies (Lee and Kader, 2000). The different forms of vitamin C are absorbed by enterocytes via sodium-dependent transporters (reflecting the differential concentration of sodium ions across the plasma membrane, maintained by Na+/K+-ATPases) or via facilitated diffusion mediated by GLUT transporters, although high intracellular glucose levels can inhibit this process (Corti et al., 2010). Within the enterocyte, dehydroascorbic acid is reduced to ascorbic acid (**Fig. 1**), which leaves the cell at the basal membrane by an unknown mechanism (Malo and Wilson, 2000). Ascorbic acid is transported by the lymphatic system and the vasculature and is taken up into target cells via specific transporters (Savini et al., 2008). Ascorbic acid can also be reabsorbed by renal tube cells (Carlsson et al., 2001).

Folic acid is generally presented in the diet as polyglutamyl folic acid, which must be deconjugated by enzymes tethered to the intestinal epithelium before absorption by passive diffusion (if present at concentrations greater than 5–10 mmol/L) or otherwise by a concentration-dependent specific transport process (Gregory, 2001) (**Fig. 1**). The ratio of monoglutamyl to polyglutamyl folic acid in the diet appears to have no influence on the efficiency of absorption, suggesting that the enzymatic deconjugation is not a rate-limiting step (McKillop et al., 2006). However, the entrapment of folic acid in food matrices can influence bioavailability by rendering them inaccessible to the epithelial enzymes (Storozhenko et al., 2007a). In this context, the consumption of a folic acid tracer with a light breakfast meal reduced bioavailability by 15% compared to the folic acid tracer alone (Pfeiffer et al., 1997). Folic acid bioavailability can be increased by enhancing the activity of γ-glutamyl hydrolase (GGH) in plants, as this would be released from the vacuole following

maceration and would promote the release of unconjugated folic acid from the food matrix before ingestion (Storozhenko et al., 2007a).

Folic acid is also absorbed in the colon, which is a site of folic acid synthesis by gut bacteria (Aufreiter et al., 2009). Absorbed folic acid is transported to the liver, which contains approximately half the folic acid pool in the body (Gregory et al., 1998) and retains 10–20% of absorbed folic acid due to the first-pass effect (Gregory, 1995). The remainder is transported through the vasculature to the rest of the body. Some liver folic acid participates in the enterohepatic circulation and is secreted into the bile (Herbert, 1987).

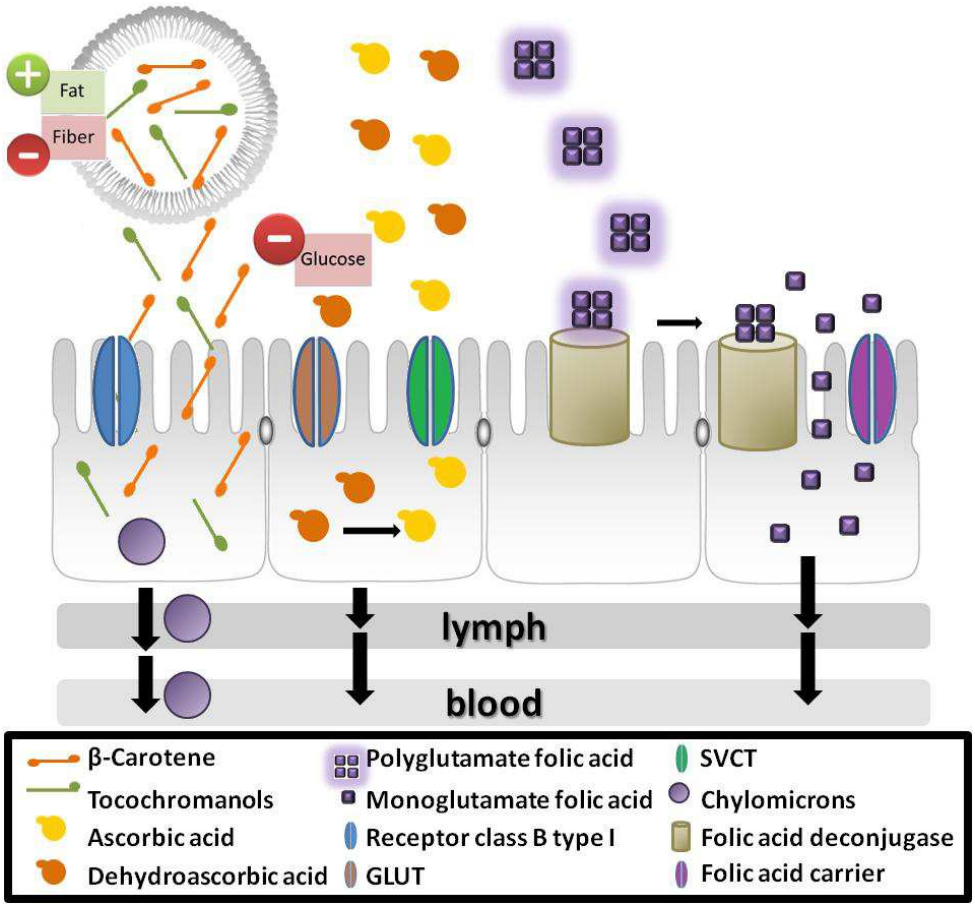

Figure 1. Mechanisms for the absorption of vitamins A, E, C and folic acid in the human body (Modified from Yonekura and Nagao, 2007). The first step is the release of vitamins from the food matrix. Fat-soluble vitamins are solubilized into micelles in the intestinal lumen and are absorbed by epithelial intestinal cells and packaged in chylomicrons. Both forms of vitamin C are absorbed by enterocytes and transported through the lymphatic system to target cells (Savini et al., 2008). Polyglutamyl folic acids in the diet must be deconjugated by enzymes tethered to the intestinal epithelium before absorption (Gregory, 2001). Abbreviations: SVCT, sodium-dependent vitamin C transporter; GLUT, facilitative glucose transporter.

5.9 Conclusions and perspectives

Vitamins are essential nutrients that play specific roles in metabolism and a DRI level has been established to promote nutritional health and reduce the likelihood of micronutrient deficiency (Lesková et al., 2006). Fruits and vegetables provide adequate sources of vitamins, but large parts of the developing world have insufficient access to such foods and rely instead on a monotonous cereal-based diet (Farré et al., 2010a). Although nutritional health can be maintained by the provision of supplements or fortified processed foods, these are expensive and unsustainable practices which require government-level intervention and continuous funding. Biofortification is an alternative strategy which is sustainable because the vitamins are produced at source by the crops, thus allowing the distribution of nutritionally-enhanced staple foods via established food distribution networks (Farré et al., 2010b; Gómez-Galera et al., 2010).

Although there has been recent progress in the development of genetically-engineered biofortified crops, most studies have focused on the total vitamin content of the crops and have overlooked the impact of bioavailability which determines whether such crops could achieve the DRI for each vitamin in a reasonable portion. Similarly, little attention has been paid to the potential dosage effects of vitamins in nutritionally-enhanced crops, which could lead to hypervitaminosis. The bioavailable amount of vitamins depends on the food matrix, the processing and cooking methods, and co-dietary factors. Different food preparation methods can reduce the total levels of vitamins by thermal degradation and/or leaching, but may also increase vitamin bioavailability by partially degrading the food matrix and therefore improving accessibility (Lešková et al., 2006).

We have considered the overall improvement in vitamin levels achieved by genetic engineering and compared these with DRI values for fresh produce and portions prepared using different cooking methods. This is an important element in the development of nutritionally-enhanced staple crops targeting specific developing country populations because the value of such products would depend on the inclusion of traditional cooking methods when calculating the impact on DRI. Our data suggest that most of the nutritionally-enhanced crops developed thus far provide an improvement over conventional crops in terms of DRI even after food processing (**Table 3;** Section 5.6) but their efficacy (biological impact under controlled conditions) and effectiveness (biological impact in real life) need to be evaluated further.

From an economic perspective, biofortified crops are likely to be more cost-effective than supplementation or conventional fortification (Gómez-Galera et al., 2010; Qaim, 2010). However, this will only be the case if such crops achieve the DRI for target vitamins in a reasonable portion, otherwise supplementation and conventional fortification will need to be implemented alongside in order to address concurrent micronutrient deficiencies. If these conditions can be met, then nutritionally-enhanced crops will provide a sustainable and economically-viable approach to address the challenge of micronutrient deficiency in developing countries (Stein et al., 2006).

Further studies will be required to determine whether biofortified crops maintain their enhanced nutritional value in local agricultural environments. The varieties must also perform well in terms of yield and pest resistance in order to perform well economically and therefore gain acceptance from farmers and consumers. The introduction of new varieties must take into account the potential impact on local markets and on the local agricultural system, because biofortified varieties may attract a market premium that could encourage farmers to adopt the biofortified variety as a marketable commodity. The development of a viable and sustainable humanitarian product must not be derailed by poor economic performance and negative public perception.

5.10 References

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CHAPTER 6

PILOT EXPERIMENTS FOR THE GENERATION OF TRANSGENIC MAIZE PLANTS ENGINEERED WITH MULTIPLE INSECTICIDAL GENES

Chapter 6: Pilot experiments for the generation of transgenic maize plants engineered with multiple insecticidal genes

6.1 Abstract

Genetically-engineered crops expressing insecticidal transgenes have been used to control pests for the last 18 years. Most of the genes are derived from the bacterium *Bacillus thuringiensis* which produces insecticidal δ-endotoxins that are highly specific to particular taxonomic groups. Several different *Bt* genes have been expressed in crops with variable levels of efficiency, and such crops can be deployed successfully in agricultural settings without harm to the environment and reducing the need for chemical pesticides. We simultaneously introduced three insecticidal genes (*cry1Ac, cry1C* and *vip3*) into the South African inbred maize variety M37W by particle bombardment, aiming to confer protection against important lepidopteran insect pests such as the European corn borer (*Ostrinia nubilalis*). We regenerated transgenic plant lines for molecular analysis at the DNA, mRNA and protein levels, finding variable expression levels but evidence that multigene transformation can efficiently introduce multiple genes of agronomic importance into the maize genome. More transgenic plants need to be regenerated for more in-depth characterization at the phenotypic level.

6.2 Introduction

The use of gene transfer technology to introduce insecticidal genes into crops provides an economic and environmentally-sustainable alternative to the use of chemical pesticides. Transgenic crops expressing *Bacillus thuringiensis* (*Bt*) δ-endotoxin genes have been grown commercially since 1996.

6.2.1 The biology of Bt toxins

The insecticidal properties of Bt toxins were discovered when dead flour moth caterpillars were found to be loaded with spores and crystals. 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 maize fields with the bacteria proved to be effective against the European corn borer (*Ostrinia nubilalis*), significantly reducing the formation of bore-holes (Husz, 1930). This eventually led to the development of Sporeine, a commercial Bt insecticide, which was used for the first time in 1938.

Later experiments showed that Bt toxins needed to be activated in the insect gut, and it was soon discovered that the critical factors 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 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 requirement for alkaline conditions, specific proteases and specific receptors explains 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).

6.2.2 Toxin structure and specificity

Many researchers have attempted to introduce taxonomic classification systems for *Bt* and its toxins using various criteria such as serotyping, phage susceptibility and plasmid profiles, and this has resulted in the classification of approximately 100 subspecies. Although there is a good correlation between *Bt* subspecies and insect host range at the family level, the relationship tends to break down at the genus and species levels because most *Bt* strains can synthesize more than one toxin, resulting in complex and overlapping host profiles. For example, most *Bt kurstaki* strains are specific for lepidopteran insects (butterflies and moths), whereas *Bt israelensis* strains are specific for dipterans (flies) and *Bt morrisoni* strains are specific for coleopterans (beetles). Other strains are not active against insects at all, e.g. *Bt* strains containing only Cry5- and Cry6-type toxins are active against nematodes.

At the genus and species levels, it is more useful to classify *Bt* strains functionally on the basis of which toxin proteins they produce, as this is a more logical way to define the host range. The toxins can be described in terms of their amino acid sequences, protein structures and modes of activity (Crickmore et al., 1998). Cry toxins interact with specific receptors located on the surface of midgut epithelial cells and are activated by host proteases following receptor binding, resulting in the formation of a pre-pore oligomeric structure that is insertion competent. In contrast, Cyt toxins directly interact with membrane lipids and are then inserted into the membrane. The known Cry and Cyt proteins now fall into 32 sets including Cyt1, Cyt2 and Cry1 to Cry67 (Crickmore et al., 2010).

Despite their sequence diversity, all Cry proteins share a similar overall tertiary structure, represented by the six structures solved thus far by X-ray crystallography (Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Ba) (**Fig. 1**). The C-terminal portion is involved in crystal formation but is not part of the mature toxin, as it is cleaved off in the insect gut. The N-terminal portion is the toxin, and comprises three domains. Domain I is a bundle of seven α-helices, six of which are amphipathic encircling the seventh hydrophobic helix, and this domain is responsible for membrane insertion and pore formation. Domain II consists of three anti-parallel β-sheets with exposed loop regions, and domain III is a β-sandwich. Both domains confer receptor binding specificity thus helping to define the host range (Boonserm et al., 2006). A current model suggests that domains II and III initially bind to primary receptors (cadherins) which cleave the toxin within domain I and induce oligomerization, which in turn promotes binding to high-affinity secondary receptors tethered to the membrane

via C-terminal glycosyl- phosphatidylinositol anchors (Soberón et al., 2009). The requirement for oligomerization has been confirmed through the isolation of dominant-negative mutations of Cry1Ab (Rodríguez-Almazán et al., 2009). An alternative model (Zhang et al., 2006) suggests that initial binding triggers a Mg^{2+}-dependent signaling cascade that causes G-protein dependent cAMP accumulation and the activation of protein kinase A. Phylogenetic analysis has established that the diversity of the Cry family evolved by the independent evolution of the three domains and by the swapping of domain III among toxins.

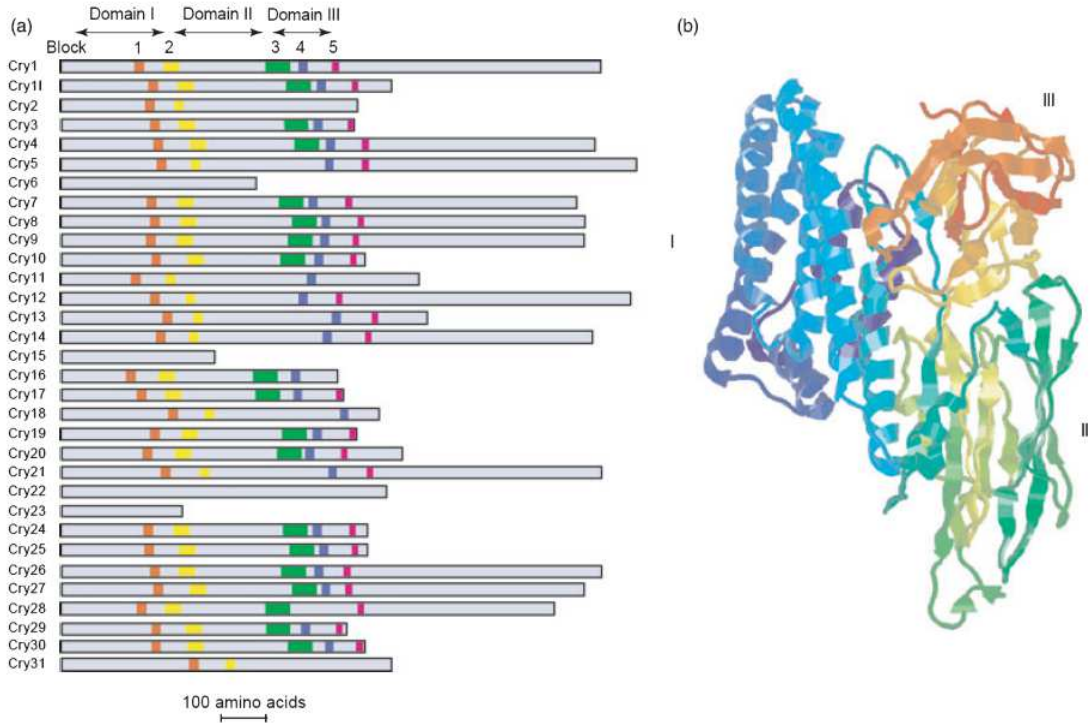

Figure 1. The structure of three-domain Cry proteins. (a) Primary structure, showing the domain organization of representative members from each Cry family. (b) Conserved tertiary structure, showing the positions of the three domains. Source: de Maagd et al. (2001).

In 1996, a new family of *Bt* insecticidal proteins was discovered which differed from Cry proteins in two major aspects (Estruch et al., 1996). Vip proteins are produced during vegetative growth whereas Cry proteins are produced during sporulation, and Vip share no sequence homology with any known Cry proteins (Estruch et al., 1996). Homologous proteins may indicate a risk that the related proteins will share binding sites on the insect midgut and are thus predisposed to cross-resistance (Tabashnik, 1994). Therefore, these differences between Cry and Vip proteins suggested the latter could provide an important

new mode of action against insect pests, and would thus be useful for mitigating the risk of Bt resistance (Jackson et al., 2007). It was later demonstrated that Vip and Cry proteins have different modes of action (Yu et al., 1997; Lee et al., 2003; Lee et al., 2006). More recently, it has been established that there are many types of Vip which can be assigned to classes based on amino acid sequence similarity with a unique system of nomenclature (Selvapandiyan et al., 2001; Bhalla et al., 2005; Crickmore et al., 2012). There are two major groups, the first comprising coleopteran-specific binary toxins related to *B. cereus* Vip1A(a) and Vip2A(a) (Carozzi and Koziel, 1997), and the second comprising the lepidopteran-specific Vip3 family, represented by Vip3a (Estruch et al., 1996).

6.2.3 The development of Bt transgenic crops

The disadvantages of topical Bt pesticides such as their short window of effectiveness have been overcome by cloning the genes encoding δ-endotoxins (Schneph and Whitley, 1981). Early experiments involving the expression of Bt transgenes in tobacco and tomato provided the first examples of genetically-modified plants with resistance to insect pests (Barton et al., 1987, Vaeck et al., 1987). However, the toxins were expressed at low levels in these plants due to the significant difference in average GC content between *Bt* and plant DNA, resulting in differences in codon preference that reduced the efficiency of protein synthesis. The bacterial genes also contained frequent ATTTA sequences, which in plants act as signals to increase the rate of mRNA turnover. Perlak et al. (1991) modified the sequence of the *cry1Ab* and *cry1Ac* genes to increase the GC content, replace sequences with four or more consecutive A/T residues and shift codon preference towards those favored in plants, increasing protein levels by up to 100-fold and achieving total yields equivalent to 0.02% total soluble protein (TSP). This was still insufficient for adequate pest control, but expression levels were increased still further using stronger promoters, more efficient polyadenylation and termination signals, and by including a heterologous intron in the expression construct. The development of synthetic *cry* genes optimized for expression in plants meant that Bt toxins were soon expressed at levels of 0.2–1% TSP (Koziel et al., 1993).

In 1995, the US Environmental Protection Agency (EPA) approved the first registration of Bt potato, maize and cotton crops, and the deployment of such 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. The cumulative reduction in pesticide use for the period 1996–2008 was approximately 356,000 tonnes (8.4%), which is equivalent to a 16.1% reduction in the associated environmental impact quotient (Sanahuja et al., 2011).

6.2.4 Strategies to prevent the emergence of Bt-tolerant pests

All insecticides create selection pressure on target populations, and the mode of action of Bt toxins (binding to a specific receptor on the surface of midgut epithelial cells) presents an opportunity for resistance to evolve via receptor mutations that abolish binding. The extensive use of insect-resistant transgenic plants expressing individual δ-endotoxin genes may therefore promote the emergence of resistant populations. Although current refugia strategies have worked better than anticipated, a number of alternative and complementary approaches have been proposed to address the possibility of emerging resistance in target pests. One of the most robust approaches is the pyramiding strategy, which maximizes the potential of gene transfer technology to introduce combinations of genes whose products disrupt different biochemical or physiological processes in the same insect, providing a multi-mechanistic defense. To overcome pyramiding resistance, insects must acquire simultaneous mutations in different genes. Because such simultaneous mutations are unlikely, pyramiding is an effective strategy for robust and lasting defense against insect pests (Gatehouse and Gatehouse, 1998).

6.3 Aim

The aim of the work described in this chapter was to construct independent transformation vectors containing the constitutively-expressed genes *cry1Ac, cry1C* and *vip3*, to create transgenic maize lines containing and expressing multiple insecticidal genes through combinatorial transformation and to identify transgenic plants for further in-depth studies.

6.4 Materials and methods

6.4.1 Gene cloning and vector construction

The *cry1C* and *vip3* genes (obtained from Dr. Raj Bhatnagar, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India) were amplified by PCR using primers containing restriction sites to facilitate cloning (**Table 1**). The PCR products were cleaned using the Geneclean II Kit (MP Biomedicals, LLC, France), inserted into pGEM®-T easy (Promega, Madison, Wisconsin, USA) and transferred to competent *Escherichia coli* cells, which were plated and incubated at 37°C overnight under ampicillin selection. Plasmid DNA was isolated from white colonies using the Miniprep Kit (Promega, Madison, Wisconsin, USA) and positive samples were sequenced. Cassettes were isolated using the appropriate restriction enzymes and ligated into the transformation vector pAL76 (Christensen and Quail, 1996), which contains the constitutive maize Ubiquitin-1 (*Ubi-1*) promoter and first intron. The *cry1Ac* gene was provided by Maqbool and Christou (1999).

Gene	Position	Restriction site	Primer
cry1C	Forward	*Bam*HI	5'-ATGGAGGAAAATAATCAAAA-3'
	Reverse	*Hin*dIII	5'-TCAGAGCTCTGGTCAATGTGA-3'
vip3	Forward	*Bam*HI	5'-ATGAACAAGAACAACACTAAG-3'
	Reverse	*Bam*HI	5'-TTACTTAATAGAGACATCGTAG-3'

Table 1. Primers used to amplify full-length insecticidal genes from *Bacillus thuringiensis*.

6.4.2 Maize transformation

Maize transformation is described in detail in Section 4.4.3.

6.4.3 Analysis of mRNA

Total RNA was isolated from leaves as described in Section 2.4.5 and mRNA blots were carried out as described in Section 3.4.3. The primer sequences used to synthesize each probe are listed in **Table 2**.

Transgene	Forward	Reverse
cry1Ac	5'-GGTCAGGGTGTCTACAGAACC-3'	5'-TCGAGTGTTGCAGTAACTGGAAT-3'
cry1C	5'-GCAGCCAACCTGCATCTCGCT-3'	5'- CTCCTGTCAATACTATAACACGTGC-3'
vip3	5'- AGGAATTCAGAGTTAACA-3'	5'- GTTGTTAGCCTTCCAGGGCTCC-3'

Table 2. Primers used to synthesize transgene-specific probes.

6.4.4 DNA analysis

Genomic DNA was extracted from leaves of T0 transformed plants as described in Section 2.4.3 and digested with *Bam*HI. DNA blot analysis was carried out as described in Section 2.4.3. The primer sequences used to synthesize each transgene probe are listed in **Table 2**.

6.4.5 Protein analysis

Proteins were extracted from fresh leaf tissue in two volumes of buffer containing 0.2 M Tris-HCl pH 7.5, 5mM EDTA and 0.1% Tween 20. The extract was vortexed for 1 h at 4°C and centrifuged for 10 min at 13,000 x g. The supernatant, containing total soluble protein, was separated under non-reducing conditions on 4–12% gradient pre-cast Bis-Tris Nu-PAGE gels (Invitrogen, Carlsbad, CA, USA) using Precision Plus protein standards (BioRad, Hercules, USA). Each sample comprised 12 µl of maize leaf extract plus 3.5 µl SDS loading buffer (0.3 M Tris HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.125% (w/v) bromophenol blue) and 2 µl β-mercaptoetanol (Sigma-Aldrich, USA). Separated proteins were blotted onto nitrocellulose membranes using the Hoefer TE70 semidry transfer system (Amersham Biosciences, Piscataway, NJ) and blocked with 2.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.47 mM KH_2PO_4). After three washes with PBS plus 0.1% Tween-20 (PBST), the Bt toxin was detected with a rabbit polyclonal anti-Cry1Ac primary antibody and a horseradish peroxidase (HRP)-conjugated polyclonal anti-rabbit IgG, diluted 1:1000 (Sigma-Aldrich, USA). After further washes, the peroxidase was detected using the ECL Plus Western Blotting Detection System (GE Healthcare, Amersham, Little Chalfont, UK).

6.5 Results

6.5.1 Vector construction and the generation of transgenic maize plants containing multiple insecticidal genes

Two genes from *Bacillus thuringiensis* (*cry1C* and *vip3*) were introduced into the pAL76 vector, downstream of the *Ubi-1* promoter and first intron. The *cry1Ac* gene was already available in pAL76 (Maqbool and Christou, 1999). The three genes were transferred simultaneously by particle bombardment into immature zygotic embryos of the South African inbred maize variety M37W along with the selectable marker *bar*. The bombarded callus was subcultured and regenerated under DL-phosphinothricin (PPT) selection. The population of regenerated transgenic maize plants was morphologically and developmentally normal.

6.5.2 Molecular characterization of transgenic maize plants

Transgene integration was studied by DNA blot analysis. Genomic DNA was digested with *Bam*HI, which recognizes a single site in each of the transforming plasmids, and was hybridized with the transgene-specific probes. This revealed a unique and complex hybridization pattern for each plant, confirming random transgene integration and that each transgenic plant had resulted from an independent transformation event (**Fig. 2**).

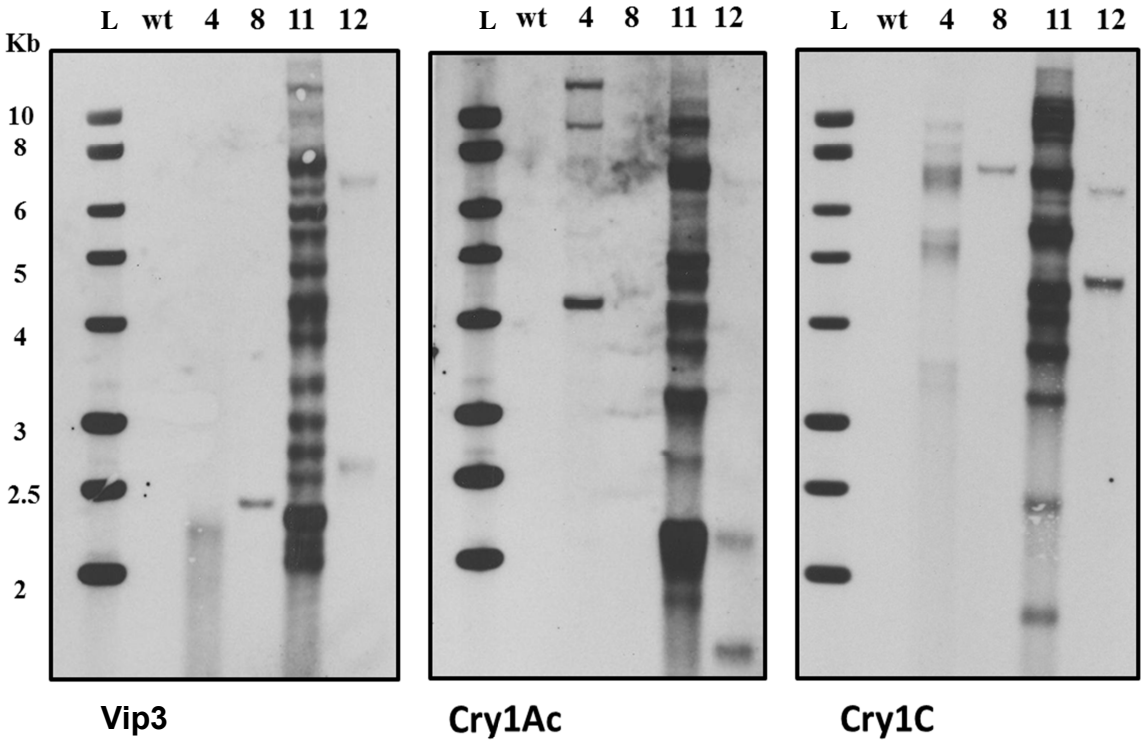

Figure 2. DNA blot analysis of maize genomic DNA from transgenic plants co-transformed with *vip3*, *cry1Ac* and *cry1C*, digested with *Bam*HI and hybridized with the corresponding transgene-specific probes. (L) 1 Kb ladder from Promega (Promega, Madison, Wisconsin, USA).Wild-type (wt) genomic DNA was used as a control (20 μg was loaded in each lane). Transgenic maize plants 4, 8, 11 and 12 were analyzed in this experiment.

Transgene expression in the regenerated plants was investigated by mRNA blot analysis. Total RNA was isolated from the leaves of T0 transgenic plants and mRNA blots were probed with the corresponding sequences (**Fig. 3**). This revealed the sizes of the transgenes (*vip3* = 2.3 kb, *cry1Ac* = 1.9 kb and *cry1C* = 1.8 kb). Transgenic plants 4 and 11 expressed all three transgenes whereas transgenic plant 8 expressed *vip3* and *cry1C* and transgenic plant 12 expressed *cry1C* alone (**Fig. 3**). Steady-state mRNA levels varied among the lines. Interestingly, *cry1C* mRNA was the only transgene expressed in all the transgenic plants and it was also expressed more strongly than *cry1Ac* and *vip3*.

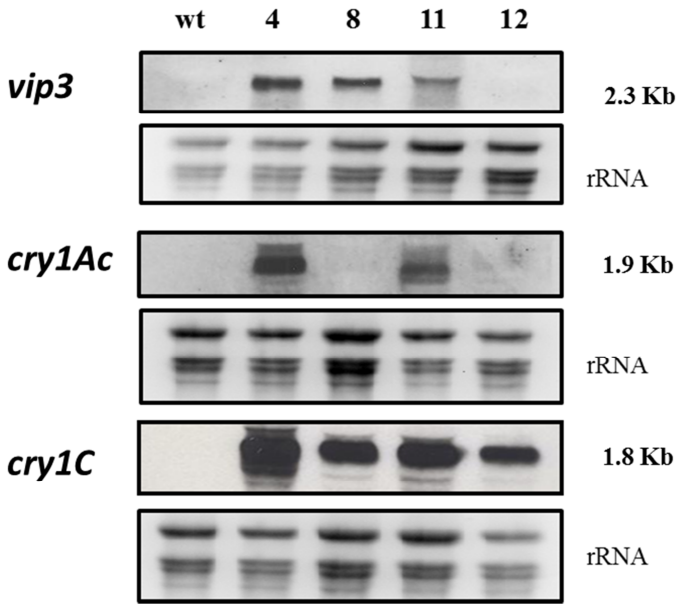

Figure 3. mRNA blot analysis of *vip3, cry1Ac* and *cry1C* in transgenic maize plants (4, 8, 11 and 12). Wild-type (wt) RNA was used as a control and 15 μg of RNA was loaded in each lane.

6.5.3 Protein accumulation in transgenic plants

Western blot analysis was carried out using extracts from the leaves of T0 transgenic plants, with wild-type leaves used as a negative control and leaves from the commercial maize variety MON810 expressing Cry1Ab as a positive control. Although our transgenic lines expressed Cry1Ac and the positive control line expressed Cry1Ab, the polyclonal antiserum used as the primary detection reagent in the experiment cross-reacted with both proteins.

The Vip3 and Cry1C proteins could not be detected because suitable primary antibodies were not available. The ~65 kDa Cry1Ac protein was only detected in transgenic plant 4, and the expression level was lower than Cry1Ab in MON810 (**Fig. 4**).

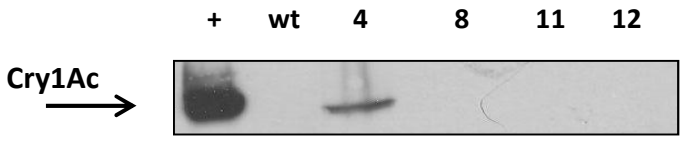

Figure 4. Western blot of total protein from the leaves of T0 transgenic maize plants 4, 8, 11 and 12 probed with antibodies specific for Cry1Ac. Total protein from wild-type (wt) leaves was used as a negative control and total protein from the leaves of MON810 plants was used as a positive control (+). The signal was detected using the horseradish peroxidase system.

6.6 Discussion

Transgenic plants expressing single insecticidal proteins from *Bt* were first commercialized in 1996 and the rate of adoption has increased annually since then, providing economic benefits to growers, reducing the use of chemical pesticides and achieving the regional suppression of some key agricultural pests (Shelton et al., 2002; Cattaneo et al., 2006; Carrière et al., 2003; Wu et al., 2008; Hutchison et al., 2010). Bt crops have emerged as a major control strategy in maize (10.2 million ha) and cotton (16.1 million ha) (James, 2010; 2011). Additional crops such as broccoli, cabbage and tobacco have also been engineered to express *Bt* genes but have yet to be grown commercially (Huesing and English, 2004).

The most effective current strategy to prevent resistance emerging in target insect populations is the use of high-dose individual-toxin plants (single mode of action) with a structured refuge (Gould, 1998; Christou et al., 2006). However, the effectiveness of this strategy is limited by its dependence on toxin dose, and it is therefore likely to fail if the toxins do not achieve their high-dose criteria or if the refuges are implemented inconsistently promoting the spread of resistance alleles. Compliance with the refuge strategy is probably responsible for the remarkable lack of resistant populations even in areas devoted to high-intensity Bt agriculture for 15 years (Christou et al., 2006). However the first generation of single-toxin Bt crops has now been superseded by strategies involving the stacking and pyramiding of insect resistance genes, providing multiple modes of action against key target pests. This approach exploits gene transfer technology to introduce multiple genes into the same plant, targeting the same pest using different mechanisms and providing a highly-effective and sustainable approach for pest control. Pyramiding can dramatically improve resistance management with smaller refuges, and reduce the dependence on high-dose criteria and rare resistance alleles (Christou et al., 2006; Sanahuja et al., 2011). In addition, theoretical models indicate that plants expressing two *Bt* genes with different modes of action are likely to delay the emergence of resistance in target insect populations more effectively than single-toxin plants (Roush, 1998). For this reason, we selected three genes (*cry1Ac, cry1C* and v*ip3*) encoding toxins with different modes of action in the insect midgut (Ballester et al., 1999; Lee et al., 2003).

6.6.1 Co- transformation

We used combinatorial genetic transformation with multiple transformation vectors to generate transgenic maize plants containing and expressing *cry1Ac, cry1C* and v*ip3*. Combinatorial transformation has previously been used for the expression of multiple insecticidal genes in rice, resulting in stable transgene transmission and expression although the expression levels of the Bt proteins varied significantly among the resulting lines (Maqbool and Christou, 1999). Nevertheless, those transgenic plants provided significant protection against three of the most important insect pests affecting rice (Maqbool and Christou, 1999; Maqbool et al., 2001).

The simultaneous introduction of multiple genes into plants by co-transformation allows the expression of multimeric proteins and reduces the time required to produce homozygous plant lines compared to backcrossing. For example, crossing several existing transgenic maize plants within a conventional plant breeding program achieves homozygosity in 6–8 years, as shown for the SmartStax maize variety containing eight transgenes, including six *Bt* genes (encoding the toxins Cry1A.105, Cry2Ab2, Cry11F, Cry3Bb1 and Cry34/35Ab1). SmartStax was generated by crossing the existing varieties MON 89034, TC 1507, MON 88017 and DAS-59122-7, to stack all the events in one line (Sanahuja et al., 2011).

DNA blot analysis confirmed the integration of all three transgenes, and the different banding patterns indicated that each plant originated from an independent transformation event.

6.6.2 Factors affecting the expression of multiple transgenes

We analyzed transgene expression at mRNA and protein levels. The synthesis of full-length transcripts corresponding to each of the transgenes was confirmed by mRNA blot analysis, whereas western blots confirmed the synthesis of heterologous proteins with the predicted molecular mass of Cry1Ac. We were unable to confirm the expression of Cry1C and Vip3 proteins directly because suitable antibodies were unavailable.

Whereas most of the transgenic plants expressed all integrated transgenes, some showed no transgene expression at all and there was significant variation in the level of transgene expression among the different transgenic lines. This phenomenon has been observed in transgenic plants expressing Cry1C and Cry1Ac individually (Cao et al., 1999; Nayak et al., 1997) and in plants expressing multiple insecticidal genes, although in this case all lines

carried all the transgenes (Maqbool and Christou, 1999). The expression of Cry1Ac protein was confirmed in transgenic plant 4 (which may also express Cry1C and Vip3 although these cannot be detected) but the level was lower than Cry1Ab expression in the positive control line MON810. Similar results were observed for the Cry1C protein in transgenic broccoli (Cao et al., 1998) and in other transgenic crops such as rice, potato and tobacco expressing other Bt toxins (Maqbool and Christou, 1999; Perlak et al., 1991; 1993). The range of the expression levels in independent transformants has been attributed to position effects and is not generally correlated with gene copy number or epigenetic modification (Perlak et al., 1993; Maqbool and Christou, 1999). Transgene expression levels are also influenced by the orientation of adjacent transgenes, promoter occlusion and the potential for mutations and rearrangements to occur during integration.

6.7 Conclusions

We have produced transgenic maize plants by co-transforming immature embryos with three different insecticidal genes: *cry1Ac, cry1C* and *vip3*. We regenerated four different transgenic plants expressing one, two and three insecticidal proteins at the mRNA level. Cry1Ac protein was expressed in transgenic plant 4, although at levels were lower than Cry1Ab in the commercial line MON810 (which we used as positive control). The accumulation of Cry1C and Vip3 could not be tested directly because there are no antibodies available for detection. This chapter describes preliminary work in the development of a novel maize variety with pyramiding resistance but more plants must be recovered for comprehensive screening and phenotypic evaluation. However, the results are consistent with previous reports describing transgenic maize plants generated by combinatorial genetic transformation showing that this approach is suitable for the rapid production of maize plants carrying diverse combinations of input transgenes.

6.8 Future prospects and recommendations

Further experiments are required to generate a large population of transgenic plants for further screening and evaluation. In addition, further analysis is needed to evaluate the levels of Cry1C and Vip3 proteins in the four transgenic plants we have already described. This transgenic maize population will be tested for its ability to control major lepidopteran pests and to identify the best transgenic lines for pest management. This will involve insect bioassays to determine the efficacy of the toxin or toxins produced by each transgenic plant, and the overall efficacy of pyramiding resistance against different maize pests.

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GENERAL CONCLUSIONS

General conclusions

1. Ascorbic acid tends to accumulate more in the leaves than the roots and endosperm tissues in maize. Its accumulation in young leaves correlates with the higher expression of L-galactose pathway genes, but the rate of loss by oxidation (dehydroascorbate degradation) exceeds the rate of ascorbic acid biosynthesis in mature leaves.

2. The ascorbic acid content was higher in young tissues (endosperm at 20 dap, young leaves and roots at 4 days after germination) than in older tissues, reflecting the rates of cell expansion and cell division in young tissues.

3. Ascorbic acid accumulation during root and endosperm development is complex, potentially involving ascorbic acid transport from leaves to sink tissues and intersections with alternative pathways.

4. The maize genome contains two GDP-mannose-3',5'-epimerase (GME) genes: *gme1* located on chromosome 1 and *gme2* located in chromosome 4.

5. The expression of *gme1* was higher in leaves than roots, whereas *gme2* was more strongly expressed in the roots. Neither gene was expressed at high levels in the endosperm. This suggests that maize *gme1* may have a specific role in leaves and *gme2* in roots, but they may also be co-regulated.

6. During endosperm development, ascorbic acid biosynthesis via the L-galactose pathway declines and causes the total ascorbic acid content of the seeds to decline. The loss of total ascorbic acid after 20 dap predominantly reflects the loss of reduced ascorbic acid, because dehydroascorbate levels do not change significantly during the development and maturation of seeds.

7. During endosperm development, ascorbic acid levels are under strict yet complex and genotype-dependent feedback regulation which appears to be controlled predominantly by ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR). These enzymes may in turn be regulated at the level of transcription by the abundance of dehydroascorbate, suggesting a novel strategy by which plants control the redox state in their seeds.

8. Different transgenic maize plants were recovered expressing genes involved in vitamin C biosynthesis. The plants were characterized at the DNA, RNA and metabolite levels (abundance of ascorbic acid) in preparation for more detailed experiments.

9. Most nutritionally-enhanced crops developed thus far provide an improvement over conventional crops in terms of the dietary reference intake of essential nutrients even after food processing, but their efficacy (biological impact under controlled conditions) and effectiveness (biological impact in real life) need to be evaluated in more detail.

10. Combinatorial transformation is a useful strategy to create libraries of maize plants containing and expressing different combination of insecticidal transgenes. The transgenic plants were characterized at the DNA, RNA and (to some extent) protein levels. This sets the stage for more detailed experiments to determine the efficacy of these plants against different maize pest. The plants will be crossed with other maize plants expressing different nutritional traits to provide protection against pests when deployed in the field.

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1. Farré G, Gómez-Galera S, Naqvi S, Bai C, **Sanahuja G**, Yuan D, Zórrilla-López U, Tutusaus L, Rojas E, Fibla M, Capell T, Christou P and Zhu C (2012) Nutritional improvement of crops using biotechnology. Enclyclopedia of Sustainability Science and Technology. (in press)