The generation of biofortified and weed-resistant cereal plants through genetic engineering

Eduard Pérez Massot

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UNIVERSITAT DE LLEIDA

ESCOLA TÈCNICA SUPERIOR D'ENGINYERIA AGRÀRIA

DEPARTAMENT DE PRODUCCIÓ VEGETAL I CIÈNCIA FORESTAL

GRUP DE RECERCA DE BIOTECNOLOGIA VEGETAL APLICADA

THE GENERATION OF BIOFORTIFIED AND WEED-RESISTANT CEREAL PLANTS THROUGH GENETIC ENGINEERING

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DOCTORAL DISSERTATION

Lleida, November 2014

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Teresa Capell i Ludovic Bassié, catedràtica i professor, respectivament, del Departament de Producció Vegetal i Ciència Forestal, i directors de la tesi realitzada pel senyor Eduard Pérez Massot, "The generation of biofortified and weed-resistant cereal plants through genetic engineering",

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.

Lleida, 30 de setembre de 2014

Dra. Teresa Capell Dr. Ludovic Bassié

A la meva mare i al meu pare

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ABSTRACT

Mineral malnutrition is widespread in developing countries, where over-dependence on staple cereal crops lacking many key nutrients, including essential minerals, is prevalent because poor people cannot afford to diversify their diets. Poverty is the main underlying cause of limited access to food, and therefore it should be tackled in any long-term strategy to address malnutrition. Biofortified staple crops with enhanced mineral content through genetic engineering are a promising approach to counter malnutrition in the short- and middle-term.

We aimed to create a population of transgenic rice plants with increased capacity for calcium (Ca) and selenium (Se) accumulation in the grain. Particle bombardment was used to transform two different rice varieties with two genes involved in Ca accumulation and one gene related with Se assimilation. Transgenic rice lines were characterized molecularly, and the impact of transgene expression on mineral accumulation and endogenous Ca and Se metabolism was evaluated in preliminary experiments. Calcium accumulation was not significantly increased in transgenic lines overexpressing vacuolar Ca transporters, although levels of other minerals were enhanced in different plant organs. Transgenic lines over-expressing the first enzyme in the Se assimilation pathway exhibited enhanced selenate reduction capacity in leaves, which translated into moderate increases in grain Se content in two of the transgenic lines. In addition, transgene expression induced down-regulation of the endogenous Se assimilation pathway in response to high selenate supply.

Cereal production in Africa is severely hampered by *Striga* infection. This parasitic weed is responsible for substantial yield losses and affects the livelihood of millions in Sub-Saharan Africa. Strigolactones are terpenoid hormones that plants secrete into the rhizosphere, where they can act as germination stimulants for *Striga* seeds. Genetic engineering can be used to develop *Striga* resistant cereal varieties through reducing strigolactone production.

We generated transgenic maize plants expressing RNAi constructs targeting two genes involved in the strigolactone biosynthetic pathway, the *Zea mays dwarf27* (*Zmd27)* and the *Zea mays carotenoid cleavage dioxygenase 8* (*Zmccd8*). Expression of target genes was down-regulated, and distinct phenotypes were observed in transgenic lines expressing either gene. Strigolactone levels were drastically reduced in hte *Zmccd8* line;

however the *Zmd27* line produced the same amount of strigolactones as the wild-type plants. A *Striga* germination assay with seeds from transgenic lines will constitute the ultimate proof on whether decreased strigolactone secretion in the *Zmccd8* line is sufficient to limit or block the incidence of *Striga* parasitism.

RESUM

La ingesta insuficient de minerals és quelcom comú als països en vies de desenvolupament, on bona part de la població basa la seva dieta en cereals amb poc contingut de minerals al no poder adquirir altres aliments més rics en nutrients. La pobresa és el factor que més limita l'accés a una dieta variada entre la població dels països en vies de desenvolupament; per tant, l'erradicació de la malnutrició a llarg termini passa necessàriament per eliminar la pobresa. No obstant això, el desenvolupament de cultius biofortificats amb un major contingut de minerals mitjançant l'enginyeria genètica pot resultar útil per combatre la malnutrició a curt o mitjà termini.

En aquest projecte ens vam proposar desenvolupar plantes d'arròs transgèniques que acumulessin més calci (Ca) i seleni (Se) al gra. Mitjançant la biolística vam transformar dues varietats d'arròs amb dos gens relacionats amb l'acumulació de Ca, i un gen involucrat en l'assimilació de Se. Les línies transgèniques resultants van ser caracteritzades a nivell molecular, i es va avaluar com afectava l'expressió dels transgens a l'acumulació de minerals i al metabolisme del Ca i del Se en les plantes transgèniques. L'acumulació de Ca no va incrementar en cap de les línies que sobreexpressaven els dos transportadors de Ca, però, en canvi, van augmentar els nivells d'altres minerals. Les línies transgèniques que sobreexpressaven el primer enzim de la ruta d'assimilació del Se van mostrar una major capacitat per reduir el selenat, fet que es va traduir en un augment del Se en gra en dues línies transgèniques. A més, es va veure que l'expressió del transgen reduïa l'activitat de la ruta endògena d'assimilació del Se en presència d'altes concentracions de selenat.

El gènere *Striga* perjudica greument la producció de cereals a l'Àfrica. Aquesta mala herba provoca grans pèrdues i amenaça el mitjà de vida de milions de persones a l'Àfrica sub-Sahariana. Les estrigolactones són hormones terpenoides que les plantes secreten a la rizosfera, on poden actuar com a estimulants de la germinació de llavors d'*Striga*. L'ús de l'enginyeria genètica pot permetre el desenvolupament de noves varietats de cereals que produeixin menys quantitat d'estrigolactones, fent-les així resistents a l'*Striga*.

En aquest estudi vam desenvolupar línies de blat de moro transgèniques que expressaven una construcció genètica de RNAi, per tal de disminuir l'expressió de dos

dels gens involucrats en la biosíntesi d'estrigolactones, el *Zea mays dwarf27* (*Zmd27)* i el *Zea mays carotenoid cleavage dioxygenase 8* (*Zmccd8*). L'expressió d'aquests dos gens va disminuir en les corresponents línies transgèniques, que a més exhibien un fenotip característic. Els nivells d'estrigolactones van disminuir dràsticament en la línia *Zmccd8* i, en canvi, van restar inalterats en la línia *Zmd27*. La realització d'un assaig d'infecció confirmarà si la reducció dels nivells d'estrigolactones observada en la línia *Zmccd8* es tradueix en un augment de la resistència a la infecció per *Striga.*

RESUMEN

La ingesta insuficiente de minerales es un problema común en países en vías de desarrollo, dónde la mayor parte de la población basa su dieta en cereales con bajo contenido en minerales al no poder adquirir otros alimentos más ricos en nutrientes. La pobreza es el factor que más limita el acceso a una dieta variada entre la población de los países en vías de desarrollo; por lo tanto la erradicación de la malnutrición a largo plazo pasa necesariamente por eliminar la pobreza. No obstante, el desarrollo de cultivos biofortificados con un mayor contenido de minerales mediante la ingeniería genética puede resultar útil para combatir la malnutrición a corto o medio plazo.

En este proyecto nos propusimos desarrollar plantas de arroz transgénicas que acumulasen más calcio (Ca) y selenio (Se) en el grano. Mediante la biolística transformamos dos variedades de arroz con dos genes relacionados con la acumulación de Ca y un gen involucrado en la asimilación de Se. Las líneas transgénicas resultantes fueron caracterizadas a nivel molecular, y se evaluó cómo afectaba la expressión de los transgenes en la acumulación de minerales, así como en el metabolismo del Ca y del Se en las plantas transgénicas. La acumulación de Ca no incrementó en ninguna de las líneas que sobreexpresaban los dos transportadores de Ca, aunque sí aumentaron los niveles de otros minerales. Las líneas transgénicas que sobreexpresaban la primera enzima de la ruta de asimilación del Se mostraron una mayor capacidad para reducir el selenato, hecho que se tradució en un aumento del Se en grano en dos de las líneas transgénicas. Además, se vió que la expresión del transgen disminuía la actividad de la ruta de asimilación del Se en presencia de altas concentraciones de selenato.

El género *Striga* perjudica gravemente la producción de cereales en África. Esta mala hierba provoca grandes pérdidas y amenaza el medio de vida de millones de personas en la África sub-Sahariana. Las estrigolactonas son hormonas terpenoides que las plantas secretan a la rizosfera, dónde pueden actuar cómo estimulantes de la germinación de semillas de *Striga*. El uso de la ingeniería genética puede permitir el desarrollo de nuevas variedades de cereales que produzcan menos cantidad de estrigolactonas, haciéndolas así resistentes a la *Striga*.

En éste estudio desarrollamos líneas de maíz transgénicas que expresaban una construcción genética de RNAi para disminuir la expresión de dos de los genes involucrados en la biosíntesis de estrigolactonas, el *Zea mays dwarf27* (*Zmd27)* i el *Zea*

mays carotenoid cleavage dioxygenase 8 (*Zmccd8*). La expresión de estos dos genes disminuyó en las correspondientes líneas transgénicas, que mostraban un fenotipo característico. Los niveles de estrigolactonas disminuyeron drásticamente en la línea *Zmccd8* y, en cambio, quedaron inalterados in la línea *Zmd27*. La realización de un ensayo de infección confirmará si la reducción en los niveles de estrigolactonas observada en la línea *Zmccd8* se traduce en un aumento de la resistencia a la infección por *Striga*.

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2,4-D .	2,4-Dichlorophenoxy acetic acid
ACA	Autoinhibited calcium ATP-ase
AMP	Adenosine monophosphate
APS	Adenosine 5'-phosphosulfate
APSe	Adenosine 5'-phosphoselenate
ATP .	Adenosine triphosphate
BAP	6-Benzylaminopurine
CAX	Calcium exchanger
CCD	Carotenoid cleavage dioxygenase
cDNA.	Complementary DNA
Cys .	Cysteine
DAP	Days after pollination
DMSO	Dimehtyl sulfoxide
dNTP .	Deoxynucleoside 5'-triphosphate
DW	Dry weight
EDTA	Ethylenediaminotetraacetic acid
EFSA	European Food Safety Authority
FAO	The Food and Agricultural Organization
FAOSTAT	Statistical Database from FAO
GE .	Genetic engineering / engineered
GR24 .	Strigolactone analog. Chemical name: ((3aR*,8bS*,E)-3-(((R*)-4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one
GSH	Glutathione
HPLC-ICP-MS	High-performance liquid chromatography-inductively coupled plasma-mass spectrometry
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LB	Lysogeny Broth
MeSeCys	Methylselenocysteine
Met .	Methionine
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA

MS	Murashige and Skoog
NAA	1-Naphthalene acetic acid
ND	Non determined
OAS	O-acetylserine
PCR .	Polymerase chain reaction
PPT	Phosphinothricin
RNAi .	RNA interference
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAT .	Serine acetyltransferase
SDS .	Sodium dodecylsulfate
SeCys.	Selenocysteine
SeMet.	Selenomethionine
SLC	Strigolactones-like compound
SSC .	Saline sodium citrate
TAE	Tris-acetate-EDTA buffer
TBE .	Tris-borate-EDTA buffer
tRNA .	Transference RNA
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectometry
X-Gal .	5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

General Introduction

General introduction

A. Mineral biofortification

A.1. Food insecurity, malnutrition and poverty

Food security exists when all people, at all times, have physical and economic access to sufficient amounts of safe and nutritious food that meets their dietary needs and preferences (World Food Summit 1996). As such, the availability of food must be stable over time and everyone should have access to it (FAO 2006). The nutritional quality of this food is as important if not more so than the calorific value (Farré et al. 2010). Food insecurity therefore not only affects the 870 million hungry people in the world (FAO 2012b) but also the approximately 3 billion people who achieve the minimum dietary energy requirement (MDER) but nevertheless suffer from diseases caused by inadequate nutrition (Christou and Twyman 2004; Gómez Galera et al. 2010). Food insecurity is prevalent in developing countries, particularly sub-Saharan Africa and South Asia, which account for 98% of the world's hungry and the largest proportion of undernourished people (FAO 2012a).

Despite isolated episodes of unpredictable food insecurity caused by sudden production deficits (FAO 2011a), global food production is generally sufficient to feed everyone in the world with an average of 2,790 calories per person per day (FAO 2012b). Therefore today's food insecurity has not to do with limited availability but to restricted access to food (Smith et al. 2000). Factors preventing access to food in developing countries include natural disasters (caused by biotic and abiotic stresses), conflict, civil strife, lack of infrastructure, land ownership issues, unsafe water and poorly-developed health and education systems (POST 2006). Poverty is considered the main underlying factor restricting access to food and therefore, the main cause to chronic food insecurity in the developing world (Van Wijk 2002; Christou and Twyman 2004). More than a quarter of the developing world population subsist on less than $US 1 per day, increasing to at least half the population in sub-Saharan Africa and in the least developed countries on each continent (Yuan et al. 2011).

This situation is the result of the complex interaction of multiple factors. However, an array of policies implemented at international level during the last decades, that have been promoted by international institutions and accepted by national governments,

have played an important role in reinforcing the inequities between developing and industrialized countries (Ziegler 2004, 2006). Developing countries under International Monetary Fund and World Bank programs were persuaded to liberalize their agricultural sectors (Naiman and Watkins 1999). In many occasions, this resulted in the devastation of local markets by artificial low prices caused by the 'dumping' of highly subsidized agricultural products from industrialized countries, especially the EU which affected the livelihood of millions of subsistence farmers (Ziegler 2004). Being unable to compete with subsidized products from industrialized countries, farmers in developing countries focused on producing tropical cash crops like coffe, peanuts, cotton or flowers. These were then exported to generate income to import food staples, which were mainly dominated by industrialized countries (Zeigler 2004). The 49 least developed countries in 2003 had shifted from net food exporters to net food importers over the last 30 years, while the cost of their food imports was rising up to 75% of their exports income (FAO 2003). In this situation, the population of poor developing countries are in constant risk of food insecurity.

As stated above, poverty is the main underlying cause of limited access to food. Therefore, any long-term strategy to erradicate hunger and malnutrition must tackle poverty (Christou and Twyman 2004). For this reason, multiple policy reforms are required to revert the current dynamics in global agricultural trade, focusing on reviewing export-oriented agricultural sectors in developing countries, the establishment of mechanisms to effectively guarantee the right-to-food, reinforce public health, education and social protection systems, as well as empowering women and local communities through rural development (De Schutter 2014).

In this context, science and technology can contribute to tackle malnutrition in the short and middle term through the development of a plethora of technologies to provide the required nutrients to those malnourished; however, their role should not be overestimated and they should not be approached as a substitute for the needed policy reforms.

A.2. Mineral dietary requirements and deficiency diseases

In the context of human nutrition, minerals are inorganic chemical elements that are required for biological or biochemical processes taking place in the human body (Gómez-Galera et al. 2010). Up to 22 minerals are needed for human wellbeing;

however, 17 of them are required in so minute amounts and/or are so abundant in food and drinking water, that deficiencies are very rare (White and Broadley 2005; Gómez-Galera et al. 2010). Iodine, Fe and Zn are the elements most frequently lacking in human diets (Kennedy et al. 2003), although Ca and Se can be deficient in the diets of some populations (Dayod et al. 2010; Combs 2001). Mineral intake does not only depend on the amount of mineral present in the diet, but also in its bioavailability, that is the portion of an ingested element that can be absorbed in the human gut (Welch and Graham 2005). Mineral bioavailability can be reduced by the presence of antinutrients such as polyphenolics and organic acids (White and Broadley 2005). Cereal grains and other staple foods have generally low bioavailable mineral content (Christou and Twyman 2004), therefore in cases where staple crop-based diets cannot be diversified with fruits, vegetables or animal-source food, mineral deficiencies are inevitable (Kennedy et al. 2003; Table 1).

Table 1. *Food sources and RDIs of 5 most important minerals lacking in human diets. Source: Office of Dietary Supplements (2011) National Institutes of Health, Bethesda*

Mineral	Food source	RDI
Iron	Red meat, fish, poultry, soybeans, lentils, beans	8-27 mg/day
Zinc	Seafood, meat, poultry, beans, nuts, whole grains	8-13 mg/day
Selenium	Nuts and peanuts, seafood, some meats and cereals	40-70 μg/day
Calcium	Dairy products, nuts, chinese cabbage, kale, broccoli	800-1300 mg/day
Iodine	Fish and seafood, dairy products and some vegetables	90-290 mg/day

A.2.1. Calcium

Calcium is the most abundant mineral in the human body and a critical mineral nutrient for bone and teeth health, where 99% of the body's Ca is stored (ODS 2013). The remaining 1% of Ca in the human body is required for vascular contraction and vasodilation, muscle movement, nerve transmission, intracellular signaling and hormonal secretions (ODS 2013). Dairy products are the main potential source of

dietary Ca; however, because of dietary preferences, dairy-product intolerances, or poor acquisition capacity, their consumption may be minimal in many cases (Dayod et al. 2010). Plants are generally poor contributors to Ca intake. Most staple cereals have low Ca content (12-15 mg/g in rice on average), and despite the fact that nuts, fruits and vegetables contain higher total calcium levels, the fraction of bioavailable Ca in these foods is low due to the presence of antinutrients such as phytate or oxalate (Hirshi 2009). The recommended Ca daily intake (RDI) ranges between 800-1300 mg for adults and 1300 mg for children above 9 years old, although in the majority of cases Ca intake is below the RDI (Jeong and Guerinot 2008). Human skeleton acts as a calcium reserve, therefore calcium deficiency results in low bone density, which is a major cause of osteoporosis and rickets (bone deformities; ODS 2013). Whereas rickets caused by severe infantile Ca deficiency are rare and confined in regions of South Africa, Nigeria and Bangladesh (Pettifor 2008), osteoporosis is very frequent among older people throughout the globe (Dayod et al. 2010). Osteoporosis is linked to enhanced decalcification of bones, which generally occurs after the age of 35. It is widely accepted that keeping an appropriate Ca RDI throughout life and increasing Ca intake in later life is beneficial and decreases the occurrence of osteoporotic fractures (Michaelsson et al. 2005; Michaelsson 2009). Calcium deficiency has also been related to hypertension and colorectal and other cancers (Heaney and Bargerlux 1994; Centeno et al. 2009), as well as to fetal skeleton development disorders, pre-eclampsia and prenatal hypertension (Chan et al. 2006).

A.2.2. Selenium

Se is an essential mineral for all mammals (Schwarz and Foltz 1957). It is mainly found in two rare amino acids - selenocysteine (SeCys) and selenomethionine (SeMet) - that are key components of selenoproteins. These proteins include selenoenzymes with antioxidant activity (e.g. glutathione peroxidase) and those that play a role in protein stability, mRNA transcription and other biochemical processes (Broadley et al. 2006). Selenium RDI ranges from 40-70 μg in adults, and intakes below this threshold can lead to several physiological disorders such as Keshan disease (a cardiomyopathy) and Kashin-Beck disease (an osteoarthritis disorder; Fordyce 2005), cardiovascular diseases, pancreatitis, asthma, inflammatory response syndrome, as well as several alterations of the immune system such as reduced immunocompetence or thyroid functioning (if Iodine is also deficient; Rayman 2000;

2002). In addition, there is a huge body of evidence supporting a potential anti-carcinogen activity of Se, with *in vitro* studies showing cell growth inhibition and programmed cell death induction, and epidemiological evidence relating optimal Se status with reduced cancer incidence (Combs 2005). Human Se intake varies around the globe, mainly depending on the Se levels in the soil, which in turn determine the Se content of plants and their edible parts (Lyons et al 2003). Se deficiency is rare in industrialized countries, where Se-fertilizers have been applied in Se-depleted soils; however, it is estimated that 0.5-1 billion people in developing countries are at risk, especially in South-East Asia (Combs 2001).

A.3. Technical strategies to address mineral deficiencies

The most effective intervention to address malnutrition is the implementation of a varied diet including fresh fruit, vegetables, fish and meat. So far this is impractical in many developing countries because such food cannot be purchased by most of the population (UN 2011). Alternative strategies based on technological applications have been developed and implemented, such as supplementation and fortification, which consist on supplying micronutrients either directly to the population or to processed foods; or biofortification, where the intervention takes place before plants are harvested. In the following sections I will review these approaches.

A.3.1. Conventional strategies

Supplementation and food fortification have been successfully implemented in a number of countries to address acute or chronic micronutrient deficiencies (Nantel and Tontisirin 2002). These two strategies are recommended and followed by several international platforms and initiatives, such as the Micronutrient Initiative (MI 2010), the Global Aliance for Improved Nutrition (GAIN 2011) and the Sprinkles Global Health Initiative (SGHI 2009), which aim to eradicate micronutrient deficiency in developing countries.

A.3.1.1. Supplementation

Supplementation consists of periodic administration of nutrients as capsules, pills, tablets, nutrient solutions or injections for immediate consumption and alleviation of deficiencies (WHO/FAO 1998). It is considered to be the most effective short-term intervention and is recommended in developing countries to alleviate acute nutritional

deficiencies; however, it is unsustainable for large populations and should be replaced by fortification at the earliest opportunity (Nantel and Tontisirin 2002). In industrialized countries mineral malnutrition is not a widespread problem; therefore supplementation is focused on certain population subsets that have adequate energy but inadequate micronutrient intake due to specific medical conditions (Kant 2000). Calcium supplements are often supplied in the form of Ca-carbonate or Ca-citrate (ODS 2013). In the developing world, where acute and chronic micronutrient deficiencies are frequent, large-scale supplementation interventions for Fe and vitamin A targeting the entire population have been implemented. The latter is the most cost effective and successful, with near one million young children's lives saved over a decade (Shrimpton and Schultnik 2002). Zinc supplementation has also been implemented in regions with Zn-depleted soils to counter acute diarrhea (WHO/UNICEF 2009). Supplementation has especially been recommended in extreme situations such as refugees camps, where it can contribute to the reduction of the incidence of several diseases (Hotz and Brown 2004); as well as during pregnancy and childhood, where can strongly contribute to a better physical and mental development in children (Smuts et al. 2005). In addition, evidence has been generated supporting the effectiveness of addressing malnutrition through multiple micronutrient supplementation rather than supplementing each nutrient individually (Li et al. 2009).

In order to ensure a successful implementation of this intervention, periodic delivery and consumption of supplements by target populations must be ensured and monitored (Hotz and Brown 2004). This requires a robust infrastructure and governmental will to improve the health of its population (Shrimpton and Schultink 2002); however, logistical and economic constraints currently limit the provision of supplements to the target population and fail to reach people in remote rural areas (Allen 2002).

A.3.1.2. Fortification

Food fortification is one of the most cost-effective long-term intervention methods for micronutrient nutrition (Horton 2006). It consists of the addition of the target nutrient to the food item during the time of processing, which can increase the price of the final product; it relies heavily on a strong food processing and distribution

infrastructure. These factors, however, prevent the most impoverished people living in remote rural areas from enjoying the benefits of fortification (Gómez-Galera et al. 2010). In addition, government awareness and robust policies are also needed for implementation and compliance monitoring. The Flour Fortification Initiative recommends the implementation of mandatory flour fortification programs at national level, involving both the public and private sectors, and establishing partnerships among countries (FFI 2014). Research efforts are required in order to identify the most suitable fortification vehicles to deliver minerals, selecting those food items that are most widely and frequently consumed among the population. In addition, an appropriate fortificant must be selected: one that is stable and readily absorbed in the gut; that does not alter the quality of the food and that can be stored and distributed easily (Zimmermann et al. 2004). As for supplementation, food fortification with several micronutrients simultaneously would be more efficient and cost-effective, although it should be ensured that no adverse interactions take place between the different micronutrients (Alavi et al. 2008; Sivakumar et al. 2001).

There are several examples of successful food fortification. One of the most successful is the case of iodine fortification of salt, which helped to erase iodine deficiency disorders (IDD) in many parts of the world (UNICEF 2008). In only six years, iodized salt reached 70% of households in the developing world. Those countries without nutrition policies require external funding and implementation, which is not constant, and therefore IDD reappear periodically (WHO 2004). Another case of successful fortification approach is the Fe fortification of wheat flour, which was established as mandatory in several countries in Latin America resulting in a decrease in iron deficiency anemia (IDA) (Shrimpton et al. 2005). It is estimated that in 2007 Fe-fortified wheat flour helped 540 million people to avoid IDA (CDCP 2008). Iron fortification presents some technical challenges, since those Fe compounds that are easily absorbed in the gut have a substantial impact on food taste, whereas those that do not change food taste are more difficult to absorb in the gut (Frossard et al. 2000).

There are few examples of Ca and Se fortification in developing countries; however, much interest has been raised in industrialized countries. Several Ca-fortified products such as fruit and vegetable juices and concentrates have been produced (Weaver 1998). Several products such as margarine and soft drinks have been fortified with Se,

although they have not been widely distributed (Combs 2001). Selenium-fortified salt has been used in several parts of China with Se-depleted soils but it has not been supported as the iodized salt (Lyons et al. 2004b).

A.3.2. Biofortification

Although some conventional interventions have been successful in alleviating mineral malnutrition even in the long term (i.e. iodine-fortified salt), in many cases they have a limited impact; therefore, biofortification has been suggested as a long-term alternative to improve mineral nutrition. Biofortification consists of increasing mineral content and bioavailability in the edible parts of staple crops (Zhu et al. 2007), and it can be achieved by agronomic intervention, plant breeding, genetic engineering (GE), or a combination of them.

Agronomic intervention only allows increasing the mineral content in the crop but cannot alter its bioavailability. In addition, continuous investment is needed to ensure a continued supply of the mineral, which also raises environmental concerns (Zhu et al. 2007). In contrast, plant breeding and GE strategies can enhance the micronutrient content as well as bioavailability in the crop and require an initial investment restricted to research and development stages, being entirely sustainable thereafter (Cakmak 2008). Therefore, cost-effectiveness of biofortification through plant breeding and GE would not exclude the most impoverished farmers to use these technologies (Welch and Graham 2004).

Other agricultural practices can also contribute in the success of biofortification programs and should be considered. These include field selection according to mineral availability, diversification of cropping systems (e.g. legume-cereal rotation systems) and the use of traditional micronutrient-enriched indigenous crops (Welch and Graham 2005).

A.3.2.1. Agronomic intervention

Agronomic intervention involves the addition of the appropriate mineral as an inorganic compound to the fertilizer. This strategy should only be implemented in those cases where the mineral deficiency in the crop reflects the lack of the mineral in the cultivated soil and, preferably, the added mineral is highly mobile within the soil. Moreover, crop plants should have the capacity to store the mineral in edible parts in a

bioavailable form, otherwise the nutritional status of the crop will not be affected (Frossard et al. 2000).

Selenium is highly mobile in different soil types, it is efficiently absorbed by plants, and it accumulates in grains in a very bioavailable form, SeMet; therefore, it is an excellent candidate for this strategy (Lyons et al. 2004b). Selenium-enriched NPK fertilizers have been successfully used in Finland and in New Zealand to increase the Se content of several crops and thus reverse the Se deficiency of their populations (Rayman 2002; Broadley et al. 2006). This approach failed in the US due to leaching and accumulation in aquatic biota (Maier et al. 1998). Trials are underway in Se-depleted regions of China to evaluate Se accumulation in rice plants after application of Se fertilizers either to the soil or by foliar spraying (Chen et al. 2002; Fang et al. 2008; Wang et al. 2013).

Calcium content in soils is generally high enough to meet plant demands, and Ca deficiency is very rare. Exogenous application of Ca fertilizers is mainly used in horticulture to avoid temporary Ca deficiencies that can decrease quality and yield (Dayod et al. 2010), and it might increase Ca concentration in tubers and leaves, but this will hardly be the case in fruits and seeds (White and Broadley 2008).

A.3.2.2. Plant breeding

Conventional breeding in the context of biofortification aims to improve the nutrient content and bioavailability of staple crops using their natural variation, i.e. by crossing the best performing plants and selecting those with desired traits over many generations (Welch and Graham 2005). Breeding of staple crops has traditionally been focused on increasing yields and disease resistance (Genc et al. 2005). In the shift to breeding for nutritional improvement, certain criteria must be considered to target breeding efforts into the right direction: (I) the mineral enrichment level must be sufficient to solve the nutritional deficiency; (II) crop productivity must not decrease; (III) the mineral enrichment must be stable within a wide range of climate and enviroment diversity; and (IV) nutrient bioavailability in crops should be assessed to ensure that nutritional improvement is effectively implemented (Welch and Graham 2005). Breeding programs require long development times, although the introduction of molecular biology techniques such as quantitative trait loci (QTL) maps and marker-assisted selection (MAS) has contributed to accelerate the

identification of high mineral varieties (Gómez-Galera 2011).

Plant breeding requires the existence of substantial genetic variation in the concentration of the mineral elements in the edible parts of crop species. Substantial variation has been detected in Fe and Zn content in rice, wheat and maize grains, that has been used in breeding programs to generate high-mineral varieties. However, comercial varieties released so far still have low mineral levels that are insufficient to provide the RDIs (White and Broadley 2005). Variation in Ca content in staple crops reported so far does not allow the implementation of breeding programs, although significant variation between wheat cultivated varieties and wild relatives in Se grain concentration has been reported (White and Broadley 2008; Genc et al. 2005).

A.3.2.3. Genetic engineering

Genetic engineering uses biotechnology to introduce target genes directly into breeding varieties (Gómez-Galera et al. 2010). Genetic engineering is not limited to the genetic pool of sexually-compatible plants. Rather, it draws on an unrestricted genetic pool to select the most appropriate gene(s) for a specific trait, and even synthetic genes can be used. The option to design specific genetic constructs permits to target the expression of the desired trait in the edible parts of the plant (Naqvi et al. 2011). Genetic engineering allows a relatively rapid generation of improved elite varieties with the desired traits, in contrast to conventional breeding programs, especially if the targeted trait needs to be introgressed from a wild relative (Zhu et al. 2007). In addition, due to advances in multiple gene transfer methods, different nutritional traits can be engineered simultaneously in the same plant, thus avoiding the need for complex breeding programmes (Gómez-Galera et al. 2010). Therefore, GE provides the most straightforward way to produce nutritionally complete crops, at least conceptually and technically. Potential strategies to increase Ca and Se content and bioavailability in edible parts of staple crops will be discussed in Section 1.1.5.

A.4. Mineral availability in soils and impact on plants

Plants can only take up mineral elements in specific chemical forms. Therefore, to ensure the success of a biofortification strategy it is necessary to have a comprehensive knowledge of the forms of mineral elements aquired by plant roots, as well as their supply and availability in the rizhosphere solution (White and Broadley

2008). Mineral elements can be present in the soil in the form of free ions, as ions adsorbed onto mineral or organic surfaces, dissolved compounds, precipitates, lattice structures or contained in the rizhosphere biota (White and Broadley 2008). The most important soil properties determining mineral availability are soil pH, redox conditions, cation exchange capacity, microbial activity, soil structure, organic matter and water content (Frossard et al. 2000). Iron and Zn concentrations are high in many soils; however, soil properties often restrict their phytoavailability, which is determined by soil-specific precipitation, complexation and adsoption reactions, as well as by soil pH (White and Broadley 2008).

Plants require minerals for their development and reproduction. Some of them play important structural roles, such as Ca, while others act as cofactors or key components of enzymes and other proteins, such as Fe. For this reason, plants rely on complex mechanisms to regulate mineral uptake and homeostasis, in order to ensure an adequate amount of each mineral while avoiding accumulation to toxic levels (Grusak et al. 1999).

A.4.1. Calcium uptake, distribution and accumulation in plants

The supply of Ca to field crops is determined by the soil cation exchange capacity, the portion of soluble Ca ions (Ca^{2+}) in the base cation pool, the rate of mineralization of soil organic matter, and the pH of the soil solution (McLaughlin and Wimmer 1999). Calcium supply is generally sufficient to meet plant needs, and Ca^{2+} concentration in the rizhosphere normally ids in the mill molar range (White and Broadley 2003). Nevertheless, calcium deficiency can occur on strongly weathered tropical soils due to their low total Ca content (Richey et al. 1982); on highly acidic soils, where Ca^{2+} uptake can be inhibited by Al^{3+}; and on alkaline or saline soils, where excess of Na^{+} inhibits Ca^{2+} uptake (Marschner 1995). Saline soils are present worldwide, with special prevalence in the arid subtropics (Frossard et al. 2000). Acute Ca deficiency is a costly issue in horticulture, when Ca supply is temporarily not sufficient to meet the demands of growing tissues (Ho and White 2005). Plants growing in soils with excessive Ca can suffer from Ca toxicity, which may prevent seed germination and reduce growth rates (White and Broadley 2003).

Calcium content in plants differs markedly between species, and much of this variation is attributed to the phylogenetic division between eudicots and monocots

(White and Broadley 2003). Monocots tend to have less cation exchange capacity in their roots and therefore take up less Ca from the soil than eudicots. For this reason, Ca^{2+} concentration in eudicots is generally higher than in monocots (Broadley et al. 2003).

Calcium is an essential plant macronutrient. Calcium ions are required for plant membrane stability and cell wall integrity; it is a key component of multiple cell signalling events and it acts as a major osmoticum agent in some cells (White and Broadley 2003). Calcium is taken up by roots from the rizhosphere solution through Ca^{2+}-permeable ion channels present on root cell membranes (White 2000). It is then delivered to the shoot via the xylem, through both the symplast and apoplast pathways (White 2001). Xylem transports Ca^{2+} to the leaves, which constitute the main Ca^{2+} sink in plant structure because redistribution to other tissues via phloem is very slow. This means that growing tissues cannot rely on Ca^{2+} reserves in older tissues, but instead they depend exclusively on the immediate Ca^{2+} supply in the xylem, which ultimately depends on the transpiration rate and the soil Ca^{2+} supply (White and Broadley 2003).

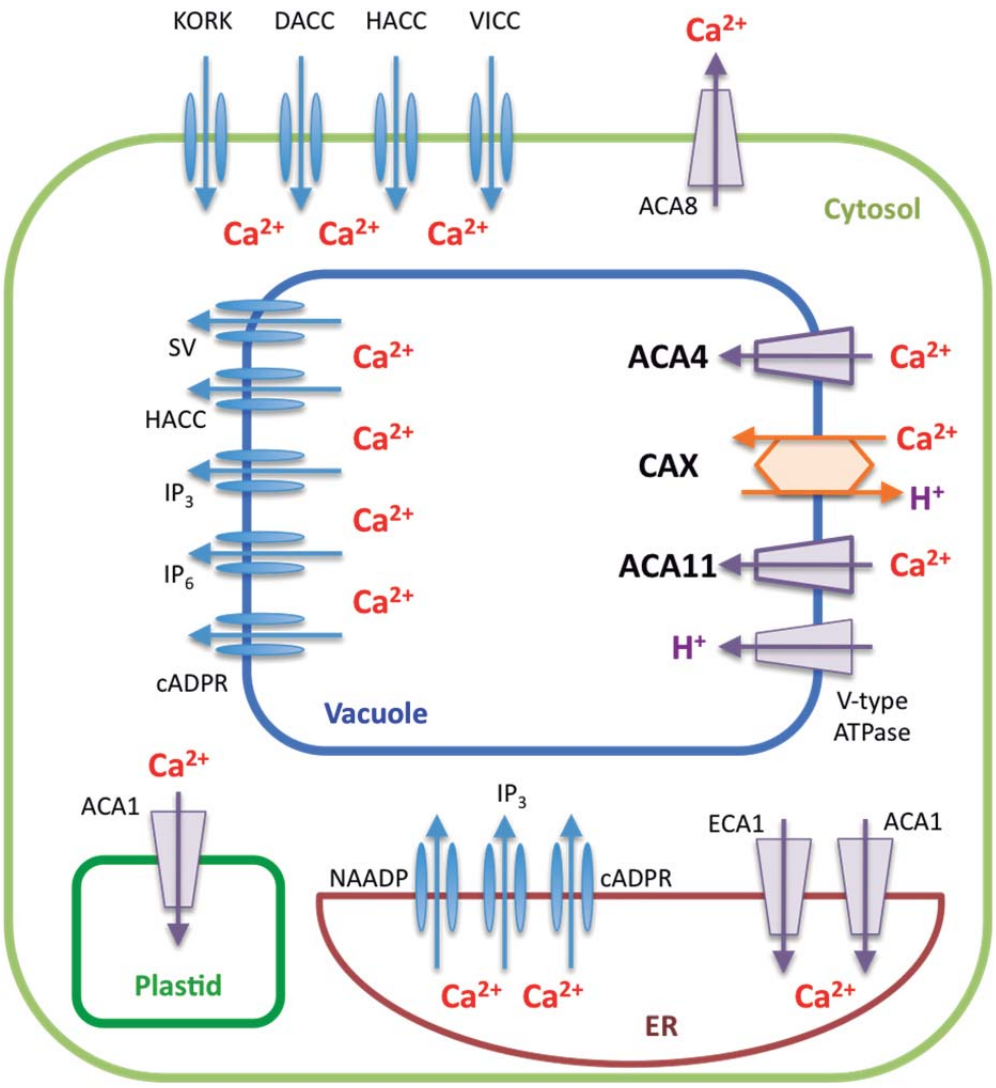

Figure 1. *Calcium transporters in plant cells. Adapted from White and Broadley (2003). Hyperpolarization-activated Ca^{2+} channels (HACC), depolarization activated Ca^{2+} channels (DACC), voltage-independent cation channels (VICC) and Ca^{2+}-permeable outward rectifying K^+ channels (KORC) located in the plasma membrane; HACCs, inositol triphosphate (IP_3), inositol hexakiphosphate (IP_6), cyclic ADP ribose (cADPR)-activated, and depolarization-activated SV receptors embedded in the tonoplast membrane; and nicotinic acid adenine dinucleotide phosphate (NAADP) receptors, IP3, and cADPR receptors located in the ER membrane allow Ca^{2+} influx into the cytosol. Energy-dependent calcium efflux is mediated by Ca^{2+}-ATPases (ACAs and ECAs) and H^+/Ca^{2+}-antiporters (CAX).*

Calcium-permeable channels in cell membranes allow Ca^{2+} entrance into plant cells (Figure 1), where it is rapidly stored in intracellular organelles, such as vacuoles and the endoplasmic reticulum (ER). This requires active transport involving Ca^{2+}-ATPases and H^+/Ca^{2+}-antiporters (Figure 1), which keep cytosolic Ca^{2+} concentration

($[Ca^{2+}]_{cyt}$) below the micromolar range in unstimulated cells (Hirshi 2001). Several environmental challenges and developmental cues can trigger the rapid influx of Ca^{2+} to the cytosol through cation channels in tonoplast and ER membranes, generating perturbations in $[Ca^{2+}]_{cyt}$ that can be restored by the subsequent efflux into storage organelles through active transport (Sanders et al. 2002). This variation in $[Ca^{2+}]_{cyt}$ may initiate signaling pathways that lead to diverse cellular responses involving multiple processes (Tuteja and Mahajan 2007). The intensity, period and localization of $[Ca^{2+}]_{cyt}$ perturbations shape the Ca signal and determine the cell response. The information encoded in the Ca signal is decoded by an array of Ca^{2+}-binding proteins, such as calmodulin (CaM), calcineurin B-like proteins (CBLs) and Ca^{2+}-dependent protein kinases (CDPKs), which are known as 'Ca^{2+} sensors' (McAinsh and Pittman 2009; White and Broadley 2003).

This signaling mechanism needs a complex and fine-regulated system of transporters to increase and decrease $[Ca^{2+}]_{cyt}$ precisely (Tuteja and Mahajan 2007; Figure 1). Calcium influx into the cytosol relies on several non-selective Ca^{2+}-permeable channels, many of which have been characterized electrophysiologically, but their molecular identity has not been elucidated yet (McAinsh and Pittman 2009). Efflux of cytosolic Ca back to the tonoplast and the ER is mediated by two important and well-characterized families of transporters: Ca^{2+}-ATPases and H^+/Ca^{2+}-antiporters (Pittman 2011). Plant Ca pumps use ATP to selectively transport Ca across membranes, and are encoded by members of the P-type ATPase gene family, which includes the ER-type Ca^{2+}-ATPases (*ECA*) and the autoinhibited Ca^{2+}-ATPases (*ACA*), located in tonoplast membranes (McAinsh and Pittman 2009). This last group of Ca pumps contain an N-terminal autoinhibitory domain which is regulated by CaM binding (Geisler et al. 2000). Calcium exangers (CAX) are high-capacity non-selective cation transporters energized by a cross-membrane pH gradient, and encoded by the *CAX* gene family. As ACAs, they are regulated by an N-terminal autoinhibitory domain that binds to CaM (Manohar et al. 2011).

A.4.2. Selenium uptake, distribution and accumulation in plants

Selenium concentration in soils is heterogeneous and site-specific, mainly determined by soil geochemistry, and generally it ranges between 0.01 and 2.0 mg/kg (Wang and Gao 2001; Fordyce et al. 2005). Selenium speciation depends on soil properties: while

selenate is the major Se species in alkaline and oxidized soils (pE + pH > 15; pE is analogous to pH and is defined as the –log of electron activity), selenite predominates in drainage systems with a neutral to acidic pH (pe + pH = 7.5-15), and selenide is only stable under low redox conditions (pe + pH < 7.5; Broadley et al. 2006). Selenate is relatively highly mobile in soils, although reduction to selenite will decrease mobility and enhance absorption by Fe and Al oxides/hydroxides (White and Broadley 2008). Selenate is the most available Se form in most cultivated soils. However, this is not the case for paddy rice fields with suboxic soils where most fraction of Se will be present in the form of selenite (Premarathna et al. 2010).

Selenium content in food crops varies greatly between countries and regions, since it depends directly on Se soil content (Zhu et al. 2009). Although some reports suggest beneficial effects of Se in plants, there is still no conclusive data to confirm that Se is required for essential functions in plants (Lyons et al. 2009). Plants take up Se mainly in the form of selenate and selenite, although organo-Se compounds such as SeCys and SeMet can also be absorbed by plant roots (Zayed et al. 1998). Plants absorb selenate and selenite through two independent uptake mechanisms: selenate is actively transported into the root cells by high-affinity sulphate transporters (HASTs; Figure 2) due to the similarity between selenate and sulfate; whereas selenite is thought to be absorbed either by passive diffusion or by phosphate transporters (Terry et al. 2000; Li et al. 2008; Zhu et al. 2009). Selenium metabolism follows the sulfur assimilation pathway up to incorporation into organic compounds (Sors et al. 2005). However, selenate and selenite distribution within the plant differs greatly. Selenite is rapidly converted to organo-Se compounds in the roots, whereas selenate is loaded into the xylem and transported to the shoot, where it will be assimilated into organo-Se compounds and distributed similarly to sulphur (S) compounds (Sors et al. 2005; Terry et al. 2000; Figure 2). This has relevant consequences in the implementation of an agronomic biofortification program, especially in choice of Se species that should be supplied in the fertilizer. It has been reported that roots are the main organs for Se volatilization (Zayed and Terry 1994). When selenite is supplied as Se source a higher proportion of Se remains in the roots, and this can lead to higher rates of Se volatilization. Instead, selenate supply allows a more efficient distribution of Se throughout the plant, including the edible parts (Zayed et al. 1998).

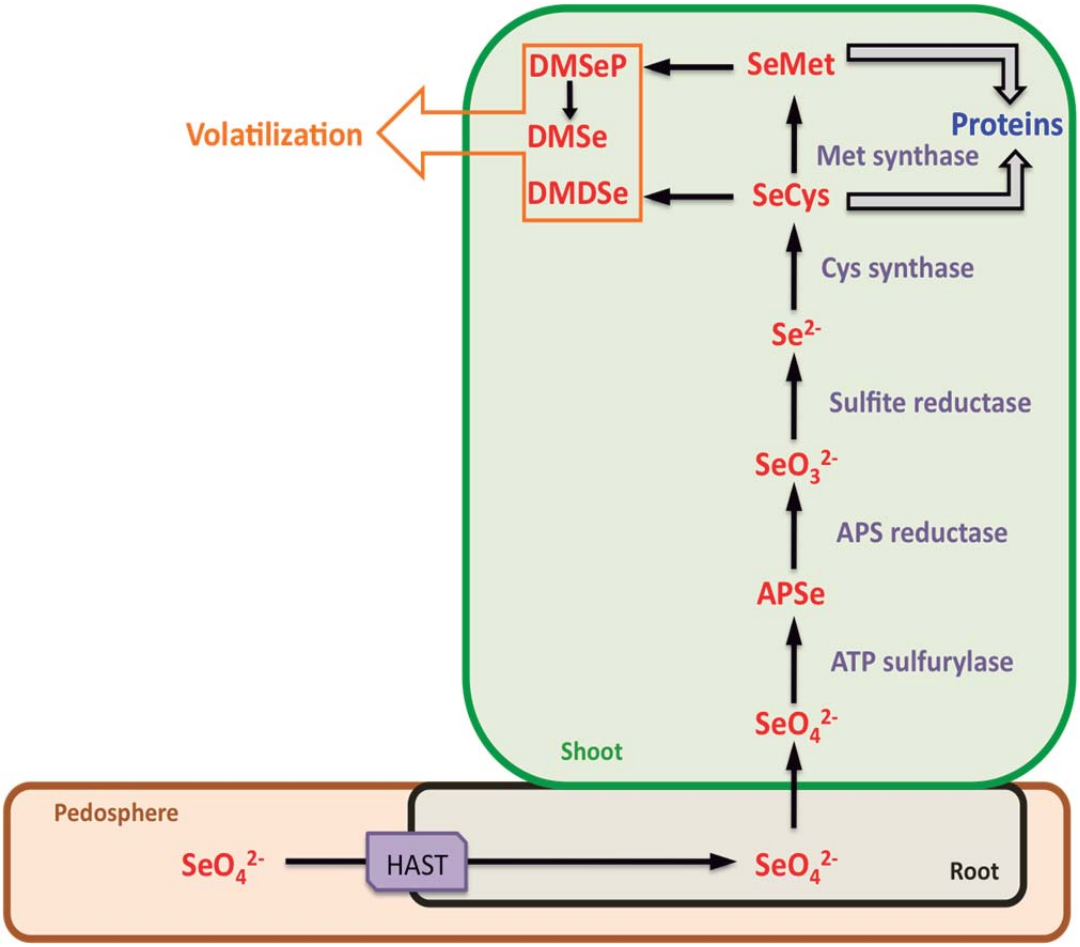

Figure 2. *Selenate assimilation pathway in plants. Adapted from Sors et al. (2005). High-affinity sulphate transporters (HASTs) take up selenate from the soil solution. It is then rapidly transported into the shoot, where it is reduced and incorporated into organic compounds for protein synthesis or volatilization. DMSe: dimethylselenide; DMSeP: dimethylselenoniopropionate; DMDSe: dimethyldiselenide.*

Seleno-amino acids can be non-specifically incorporated into proteins, and this is considered to be the major form of Se toxicity in plants (Brown and Shrift 1981). The ability of Se accumulators to tolerate high concentrations of Se is based on their capacity to divert Se away from protein synthesis. Consistent with this hypothesis, SeMet appears as the major Se-compound in rice grains, whereas in Se-enriched food crops the non-protein Se-amino acid methylselenocysteine (MeSeCys) is the major Se-compound (Brown and Shrift 1981). In these plants, the synthesis of SeMet is limited by the methylation of the precursor SeCys to form MeSeCys (Sors et al. 2005).

A.5. Transgenic strategies to increase mineral uptake and bioavailability in plants

A.5.1. Calcium

Calcium biofortification by genetic engineering has focused on increasing Ca loading into vacuoles through the overexpression of H^+/Ca^{2+}-antiporters (Dayod et al. 2010). The *Arabidopsis* CAX1 was the first such antiporter to be identified (Hirschi et al. 1996) and fully characterized (Pittman and Hirschi 2001; Mei et al. 2007). A truncated version of *AtCAX1* (*AtsCAX1*) lacking the N-terminal autoinhibitory domain was overexpressed in a number of different vegetables leading to increases in Ca content in different organs. Transgenic carrots expressing *AtsCAX1* accumulated 1.4-1.6 fold more Ca in roots than wild-type (Park et al. 2004). Up to 3 fold increases in Ca levels over wild-type were reported in *AtsCAX1*-expressing potato tubers (Park et al. 2005b). Transgenic tomatoes expressing *AtsCAX1* accumulated 2-2.5 fold higher Ca levels than wild-type and had a prolonged shelf life (Park et al. 2005a). More recently *AtsCAX1* has been overexpressed in lettuce, increasing Ca content in leaves by 1.3 fold over wild-type without affecting flavor, bitterness or crispness (Park et al. 2009). However, in parallel with Ca increases, detrimental effects were observed in some cases (Hirschi 1999, Park et al. 2005a). In order to alleviate such impairments, the use of weaker promoters (such as AtCDC2a promoter), other CAX antiporters (such as CAX4 and the chimeric CAX2A/B) and full-lenght CAX variants containing the auto-inhibitory N-terminal domain were approached (Park et al. 2004; Park et al. 2005a, Kim et al. 2006).

It has been suggested that expressing Ca^{2+}-binding proteins in the tonoplast would result in high Ca content in the edible parts of crops (White and Broadley 2008). In agreement with this hypothesis, a preliminary study showed that overexpression of the Ca^{2+}-binding domain of calreticulin, the main Ca^{2+}-binding protein of the ER, in *Arabidopsis* resulted in increased amounts of bioavailable Ca compared to wild-type plants (Wyatt et al. 2002).

A.5.2. Selenium

Higher Se content of Se-accumulator plants compared to non-accumulators relies heavily on their capacity to store and mitigate the toxicty of high Se concentrations

(Sors et al. 2005; Terry et al. 2000). Therefore, GE strategies to increase Se levels in crops focus mainly on assimilation and accumulation rather than increasing uptake capacity. Heterologous expression of Arabidopsis ATP sulfurylase, a rate-limiting enzyme in the selenate assimilation pathway that is responsible of reducing selenate to selenite (Zayed et al. 1998) confered greater Se tolerance and increases by 2-3 fold Se content in roots and shoots in Indian mustard plants (Pilon-Smits et al. 1999). Enhancing the production of non-toxic, sink metabolites, such as MeSeCys, through the overexpression of a SeCys methyltransferase (SMT) resulted in increased Se tolerance and foliar Se accumulation in Arabidopsis (LeDuc et al. 2004; Ellis et al. 2004). Moreover, the combination of these two strategies in Indian mustard resulted in much higher Se concentration and greater total Se, due to the high rate conversion of selenate to MeSeCys (LeDuc et al. 2006). Another strategy to divert Se from incorporation into proteins involved expression of a mouse selenoCys lyase (SeCysL), an enzyme that catalyzes the conversion of SeCys to alanine and elemental Se. Transgenic plants accumulated 2-fold greater leaf Se concentrations and exhibited increased Se tolerance (Garifullina et al. 2003; Pilon et al. 2003; Van Hoewyk et al. 2005). Importantly, some of these transgenic lines were evaluated under field conditions. Indian mustard plants overexpressing ATP sulfurylase performed well in terms of biomass production and exhibited a 4-fold increase in Se accumulation over wild-type plants (Bañuelos et al. 2005). Another field study evaluated SMT and SeCysL transgenic plants and demonstrated 2-fold higher Se accumulation than wild-type plants (Bañuelos et al. 2007).

B. *Striga*

B.1. Biology of *Striga*

The genus *Striga* belongs to the *Scrophulariaceae* family and comprises more than 40 species, 11 of which are considered parasitic on food crops (Aly 2007). The most damaging species are *Striga hermonthica*, followed by *Striga asiatica* and *Striga gesnerioides* (Cardoso et al. 2011). The first propagates through cross-pollination while both *S. asiatica* and *S. gesnerioides* are self-fertile, resulting in reduced genetic variability compared to the obligately out-crossing *S. hermontica* (Ejeta 2007). *Striga* witchweeds are obligate hemi-parasites. Even though they retain some photosynthetic capacity their dependency on hosts to develop and complete their life cycle is absolute

(Cardoso et al. 2011). *Striga* produce large amounts of seeds that can remain viable in the soil up to 14 years under dormancy (Bebawi et al. 1984). Under specific temperature and moisture conditions dormancy is broken through a reversible process called conditioning, which makes *Striga* seeds competent to respond to root-secreted plant host signals, mainly strigolactones (Rich and Ejeta 2007). Once germination has been stimulated, radicle cells differentiate into parasitic organs referred to as haustoria, which are able to attach to and penetrate into the host root to establish a xylem-xylem connection from where to take up water and nutrients from the host, including photoassimilates. This allows *Striga* to develop shoots and flowers and to produce a large amount of seeds that will increase the seed bank of the soil (Rich and Ejeta 2007; Figure 3). Host-derived water and nutrients permit a rapid development of *Striga* in detriment of the host plant, which remains stunted. However, the decrease in plant host growth cannot be only explained by the withdrawal of resources by *Striga*, since it is already observed even before the witch weed has emerged from the soil (Cardoso et al. 2011). Therefore, it has been suggested that *Striga* exhibit a pathological or phytotoxic effect on host plants that could be related with reduced transpiration and photosynthesis, and increased levels of ABA (Frost et al. 1997; Hibberd et al. 1996).

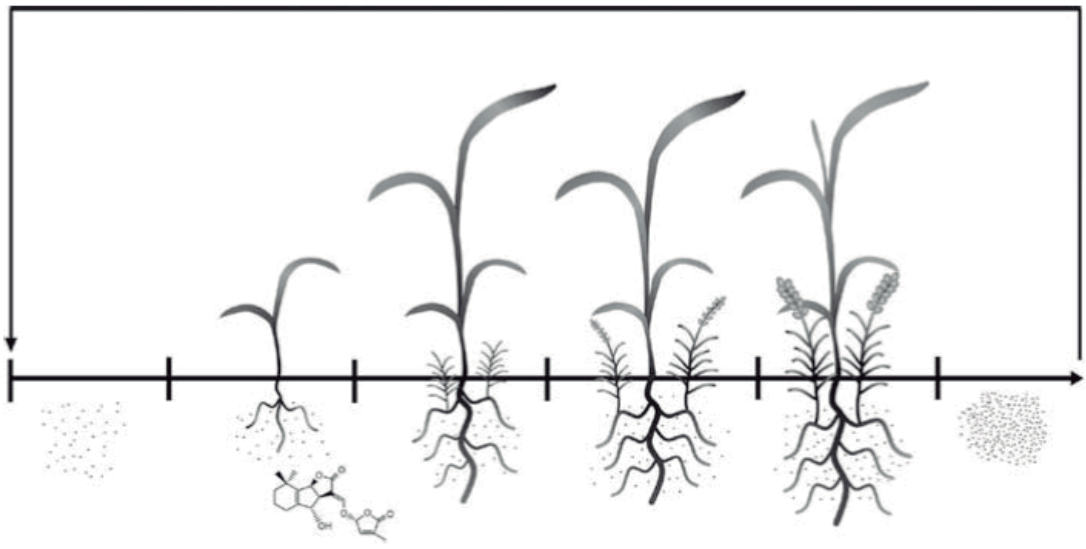

Figure 3. Striga *life cycle (Cardoso et al. 2011). Conditioned* Striga *seeds germinate in response to host plant root-secreted signals, infect host plants, develop and produce a large amount of seeds that enrich the* Striga *seed bank existing in the soil.*

B.2. Global prevalence of *Striga*

Striga spp. are found mainly in tropical and subtropical regions, with the highest prevalence in Sub-Saharan Africa (Cardoso et al. 2011). *S. hermonthica* is widely distributed throughout this continent with special prevalence in western, central and eastern Africa, in addition to the south-western part of the Arabian Peninsula across the Red Sea. *S. asiatica* is widely distributed in southern and eastern Africa, with presence in other continents such as Asia, in southern India; America, in the United States; and Oceania, in Australia. *S. gesnerioides* is also present in various continents, being abundant in Africa, the Arabian Peninsula and the Indian subcontinent, and has also been introduced into the United States (Ejeta 2007).

B.2.1. Impact of *Striga* on crop cultivation in Africa

Africa is considered to be the center of origin for *Striga* (Ejeta 2007), which has reached its higher diversity in regions where it has co-evolved with cereals, especially with sorghum, millets and upland rice (Gressel et al. 2004). At present *Striga* is spread in most regions of Sub-Saharan Africa, except in those with high rainfall or high altitude with low temperatures (Sauerborn 1991; Figure 1). *Striga* infestation is especially severe in nutrient-depleted soils with low rainfall, which are relatively abundant in the semi-arid tropics, becoming the greatest biotic constraint for cereal production in Africa (Aly 2007). The cereal production area with severe to moderate *Striga* infestation is estimated to be 50 million hectares (Ejeta 2007), with production losses in these regions ranging 10-90%, depending on the crop cultivar, degree of infestation, rainfall pattern and soil degradation (Gressel et al. 2004). Yield losses are estimated to cost more than $7 billion annually and affect the livelihood of 300 million people (Aly 2007). Most of these people belong to poor farmer communities that rely on subsistence agriculture; with very few resources to counter the different biotic, climatic and edaphic problems that reduce crop yield in African climates (Ejeta 2007); therefore, *Striga* infestation often overlaps with food insecurity (Figure 4). Many factors have contributed to the spread of *Striga*. A major one is the distribution of food grain and seed aid within food insecure areas, since 20 to 40% of commercial seed lots in the market have been found to be contaminated with *Striga* seeds (Berner et al. 1994). Seed exchange between farmers also contributes in dispersing the witchweed in a local level (Ejeta 2007). Another major reason of *Striga* dispersal is

the lack of adoption of available control methods to limit its proliferation. Even though some potential control methods have been developed in the last decades, most of them are unaffordable to poor subsistence farmers (Gressler et al. 2004), reflecting the fact that *Striga* is a poor farmer's problem: the direct result of broader demographic and economic pressures that must be solved (Ejeta 2007).

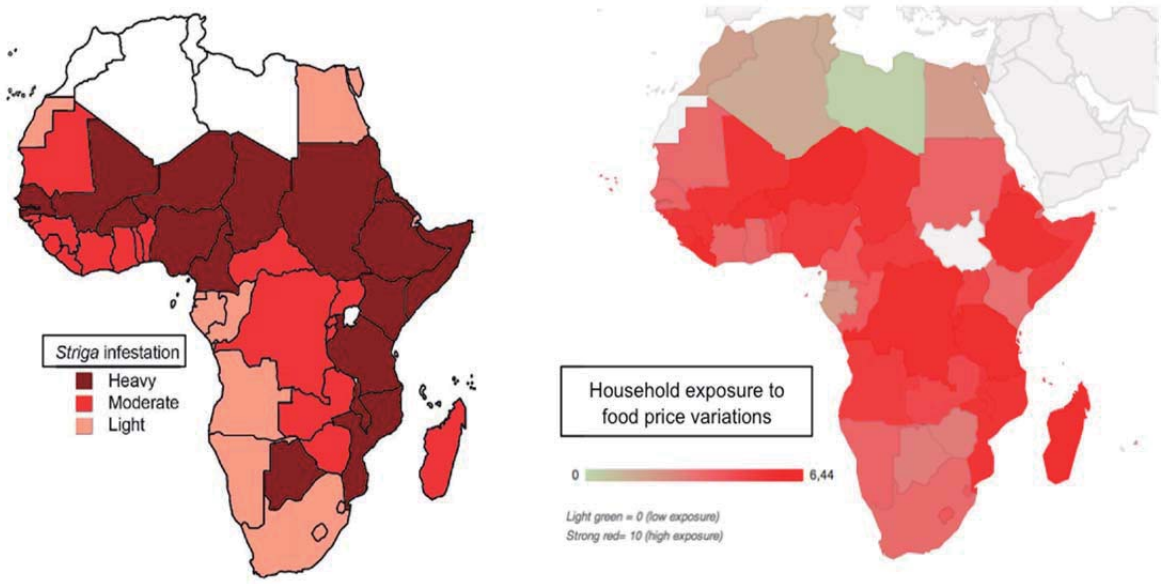

Figure 4. Striga *infestation in Africa is most prevalent in those countries where households are most exposed to fluctuation in food prices, which is an indicator of food insecurity. Sources: Ejeta (2007) and Food Security Portal (2007; www.foodsecurityportal.com) using data from the World Bank, FAO, USDA and IPFRI.*

B.3. Strategies for *Striga* control

Several parasitic weed management practices have been developed in order to control and limit *Striga* infestation and propagation (Aly 2007; Gressel et al. 2004). These can be broadly classified into the general categories of chemical approaches, cultural and physical techniques, plant breeding and biological control. In addition to these techniques, new biotechnological approaches have also been explored. All these will be reviewed in the following sections.

B.3.1. Conventional approaches

Conventional techniques for *Striga* control are based on three main principles: reducing the amount of Striga seeds within the soil; preventing production of new seeds; and avoiding spread from infested to non-infested fields (Obilana and Ramaiah

1992). Tackling each one of these principles alone has brought limited success. However, the simultaneous targeting of all the three through different combinations of conventional techniques has resulted in acceptable levels of *Striga* control in Sub-Saharan Africa if practiced long enough (Gressler et al. 2004).

B.3.1.1. Cultural / physical techniques

Different cultural practices have been used for decades to control *Striga* infestation in Sub-Saharan Africa. These include crop rotation with "trap crops", which stimulate *Striga* germination without being themselves parasitized (Odhiambo and Ransom 1994; Sauerborn et al. 2000; Hess and Dodo 2004), and "catch crops" that are parasite-susceptible (Oswald et al. 1999). Trap and catch crops help to reduce *Striga* infection and increase yields in subsequent cereal crops; however, they are only useful when parasite levels in soil are low (Gressler et al. 2004). Intercropping cereals with legumes and other crops has also been practiced in Sub-Saharan Africa for a long time and has been shown to reduce *Striga* infestation (Kroschel 2001; Oswald et al. 2002). Transplanting plants to the field after seedling establishment can double yields in *Striga*-infested fields, since larger host plants can resist better the parasite (Elzein and Kroschel 2003). However, due to its high labor requirement, this technique is limited to small fields (0.1 ha). Hand pulling of mature parasite plants has been useful to control infection, especially in fields with relatively low infestation levels (Verkleij and Kuiper 2000), although in the case of *Striga* this method is less effective because much of the host damage occurs before parasite emergence from the soil. Therefore the benefit resides mainly in reducing the *Striga* seedbank in the soil (Ramaiah 1985). This is also the objective of solarization, a technique that uses the sunlight in the summer to increase the temperature of the soil under polyethylene mulch (Rubiales et al. 2009). The lethal effect is caused by the fluctuations of temperature throughout the day and the accumulation of certain volatiles within the soil atmosphere (Jacobson et al. 1980). Although it has been effective under optimal conditions (Abu-Irmaileh 1991; Mauromicale 2005) it is expensive and difficult to implement in many rural areas (Joel et al. 2007).

Despite being used in some specific cases, these practices have not been widely adopted, since poor farmers perceive them as uneconomical, labour intensive, impractical, or not congruent with their other farm operations (Oswald 2005).

B.3.1.2. Chemical approaches

In the last decades, a number of chemicals have been developed for parasitic weed control (García-Torres 1998). Dicamba and 2,4-D are the most widely used herbicides against *Striga*. Both are systemic herbicide, however, dicamba is sprayed to crop foliage 35 days after crop emergence, while 2,4-D is applied directly to the parasitic weed throughout the growing season (Gressler et al. 2004). Nevertheless, the application of herbicides poses many hurdles: chemical damage to the host, continuous parasite seed germination along the season, marginal crop selectivity, environmental pollution and low persistence (Gressler et al. 2004). In addition, due to their relatively high cost and the technology required for their application, most of the chemicals are not accessible to small-scale subsistence farmers (Verkleij and Kuiper 2000). In the last decade, slow release herbicide formulations for seed dressing have been developed, aiming to achieve season-long control of the parasitic weed. The seed dressing allows the herbicide to spread along the crop root zone as the roots grow, prevents herbicide leaching away from the rizhosphere, and requires less chemicals (Kanampiu et al. 2002). This approach was successfully implemented for the control of *S. hermonthica* and *S. asiatica* in maize fields in four African countries, by using mutation-derived imidazolinone resistant maize seeds dressed with imazapyr, resulting in 3-4 fold yield increase under severe infestation levels (Kanampiu et al. 2003; Kanampiu et al. 2009). Nevertheless, *Striga* resistance to herbicides is expected to arise rapidly due to its prolific seed production (Aly et al 2007).

Fumigation with methyl bromide has been reported to effectively reduce the parasite weed seedbank in soil (Jacobsohn 1994; Goldwasser et al. 1995) although its use has been phased out by international agreement due to its environmental toxicity (McDonald 2002). Potential substitutes for methyl bromide have been explored, but they are much less effective and more expensive (McDonald 2002; Goldwasser and Kleifeld 2004).

Nitrogen and phosphorus fertilization can also play a role in integrated parasitic weed control (Rubiales et al. 2009). *Striga spp*. is more problematic in nutrient-depleted soils, therefore, increasing the nutrient content of the soil can increase yields in *Striga* infested fields: on one hand, the nutrient status of the crops is improved, which allows the host plant to increase the competitive ability for sap flux versus parasitic demand

(Gworgwor and Weber 1991). On the other hand, the plant host root system development is reduced, as well as the secretion of germination stimulants (Jamil et al. 2012). The effect of soil fertility on *Striga* infection of maize plants was evaluated in the greenhouse and under field conditions. Substantial yield increases were reported in the greenhouse; however, results were less consistent under open-field conditions, possibly due to heterogeneity in rainfall regime, soil properties, *Striga* density and climate (Jamil et al. 2012).

Induction of suicidal germination of *Striga* seeds offers an attractive alternative to reduce the parasite seed bank in the soil (Aly 2007). This technique relies on the application of germination stimulants in the field in the absence of any host crop, thus forcing the premature death of parasite seedlings due to their inability to survive without nutritional supply from their host (Rubiales et al. 2009). Different strigol analogues, such as GR24 or Nijmegen-1, have been used for this purpose with *Striga* and *Orobanche* parasitic weeds, although with limited success under field conditions so far (Wigchert et al. 1999; Plakhine et al. 2009). Ethylene has been reported to stimulate germination of *Striga* seeds; however, it had little effect in inducing suicidal germination of *S. hermonthica* (Ransom and Njoroge 1991). Novel approaches are being developed, aiming to combine different pathogens in a single formulation, or even combining bioherbicides with chemical herbicides to achieve higher efficacy in parasitic weed control (Müller-Stöver et al. 2005).

B.3.1.3. Plant breeding

The development of resistant crop varieties is considered as the most practical and economically feasible long-term strategy for limiting damage by parasitic weeds (Cubero and Hernández 1991). Eleven high-yielding *Striga*-resistant sorghum cultivars with multiple resistance mechanisms pyramided together were developed and released for use in *Striga* endemic areas in several African countries, either alone or synergistically with other agronomic interventions in an integrated management program (Ejeta et al. 2007; Tesso et al. 2007; Mbwaga et al. 2007).

Several *Striga* resistant maize lines were developed by recurrent selection under artificial *S. hermonthica* infestation (reviewed in Menkir et al. 2007). Two of these varieties showing highest yield increases under *Striga* infestation were then released to farmers in order to conduct multiple on-farm trials to assess their performance,

either alone or in rotation with legumes. An average of 88% increase in grain yield in *Striga* resistant varieties was reported, and these integrated approaches spread through farmer-to-farmer diffusion (Ellis-Jones et al. 2004; Franke et al. 2006).

B.3.1.4. Biological control

This approach is based on the use of living organisms (insects, fungi, etc.) to suppress parasitic weeds. Although several insects have been collected on *Striga* in India and Africa, most of them are not specific for the parasitic weeds, and they are unable to provide the level of control desired by farmers (Traoré et al. 1999; Kroschel et al. 1999). More recently, the potential of some fungal isolates to act as biocontrol agents for *Striga* was reported (Joel et al. 2007). *Fusarium oxysporum* was able to significantly reduce the emergence of *S. asiatica* and *S. hermonthica*, with visible disease symptoms on the latter (Elzein and Kroschel 2004). Field trials testing different formulations of *F. oxysporum* bioherbicides were conducted in 2006 in four Sub-Saharan countries, revealing that granule-based formulation was more effective than seed coating (Beed et al. 2007).

B.3.2. Biotechnological approaches

Within the previous sections, several conventional approaches for *Striga* control have been reviewed. So far none of them has been successful in suppressing the parasite with enough efficacy to stop its spread and revert the yield drop affecting millions of subsistence farmers in Sub-Saharan Africa. As mentioned earlier, breeding of resistant crop cultivars is the most economically feasible long-term strategy for controlling parasitic weeds; however, breeding for parasitic plant resistance is complex, laborious and time-consuming (Aly 2007) and currently resistant cultivars for most crops are not available. Most likely, sustainable *Striga* control will be the outcome of integrated management programs combining multiple and diverse approaches tackling *Striga* infection at different levels (Tesso et al. 2007; Ransom et al. 2007; Mbwaga et al. 2007) therefore it is important to increase the repertoire of available strategies to combat *Striga*. The development of molecular biology and genetic engineering offers the possibility to develop several novel biotechnological approaches that can pave the way towards the development of new resistant cultivars.

B.3.2.1. Molecular breeding

Most resistance to *Striga* appears to be polygenic, which makes it difficult to breed because several genes must be selected at the same time and its improvement takes a long time (Menkir et al. 2007). The use of mapping populations, quantitative trait loci (QTL) analysis and advanced backcross QTL (AB-QTL) analysis combined with marker-assisted selection (MAS) offers many opportunities for the development of new resistant cultivars (Scholes and Press 2008). Some QTL and AB-QTL studies have been performed to identify the genetic basis of resistance in cultivated and wild relatives of rice (Gurney et al. 2006) and sorghum (Grenier et al. 2007; Haussman 2004). In addition, MAS appears as a very useful tool for introgression and pyramiding of *Striga* resistance into improved, local African landraces through the use of robust molecular markers (Grenier et al. 2007). As more QTLs underlying resistance traits are characterized, comparative genomic approaches will allow the identification of *Striga* resistance genes in crop host genomes (Scholes and Press 2008) which will be ready to use in further genetic engineering strategies.

B.3.2.2. Genetic engineering

One of the first strategies for parasitic weed control through genetic engineering was the generation of herbicide tolerant crops overexpressing a 5-enolpyruvoyl-shikimate-3-phosphate synthase (EPSPS) resistant to glyphosate inhibition. This approach was used with transgenic oilseed rape treated with glyphosate to successfully control the parasitic weed *Orobanche aegyptiaca* (Nandula et al. 1999). *Orobanche* was also effectively controlled when asulam tolerant transgenic potatoes expressing a modified dihydropterate synthase (DHPS) - the target of the herbicide asulam - were treated with the herbicide (Surov et al. 1998). Transgenic glyphosate tolerant maize has been introduced into South Africa for general weed control, although it has been grown in *Striga*-free areas so far (Gressel 2009). Nevertheless, the success with *Orobanche* control in glyphosate tolerant crops suggests that glyphosate tolerant maize would perform as well for *Striga* control.

Another strategy that has been applied for *Orobanche* control - and could potentially work with *Striga* - is the expression of an antimicrobial polypeptide of the cecropin family (Kanai and Natory 1989) in crop host plants. Transgenic tobacco plants expressing a sarcotoxin IA gene under the control of the *HMG2* promoter, which is specifically activated during infection, demonstrated increased resistance to

Orobanche infection (Hamamouch et al. 2005).

RNAi-based gene silencing has been used in an attempt to suppress different essential endogenous *Striga* genes involved in aromatic amino acids biosynthesis, fatty acid biosynthesis, vacuole formation and nucleotide biosynthesis (Framond et al. 2007). Maize plants were engineered with RNAi constructs targeting 5 different *Striga* genes; however, none of the regenerated maize events showed obvious resistance to *Striga* infection (Framond et al. 2007).

There have been some attempts to increase the virulence of biocontrol agents through genetic engineering (Gressel et al. 2007). Transgenic *F. oxisporum* overexpressing single genes encoding host lytic enzymes and different toxins showed increased virulence against the parasite, although not enough to achieve pest control (Gressel et al. 2007). It has been suggested that synergies among the various virulence genes would help in achieving the desired levels of virulence.

B.4. Role of strigolactones in *Striga* infection

Striga seeds germinate in response to plant host secreted signals (Section 1.2.1). Strigol was the first *Striga* germination stimulant that was identified, after being isolated from cotton exudates (Cook et al. 1966). Since then, many other compounds with similar structure and germination stimulatory properties have been identified in different plant species. These are known as strigolactones (Xie et al. 2010). Host plants produce and secrete strigolactones in minute amounts into the rizhosphere, where they can trigger the germination of parasitic weeds at extremely low concentrations (Cardoso et al. 2011). However, in addition of stimulating plant parasitism, strigolactones play another role in the rizhosphere that benefits the host plant: they stimulate hyphal branching of arbuscular micorrhizal fungi (Akiyama et al. 2005, Beserer et al. 2006). AM fungi establish symbiotic relations with nearly 80% of land plants (Parniske 2008). In the symbiosis the plant provides photo assimilates to the fungus in exchange of water and minerals, which can be obtained very efficiently from the soil by the fungus. In case of nutrient starvation, the plant increases the production and secretion of strigolactones into the rizhosphere, and AM fungi perceive this signal and respond extending their hyphae beyond the rizhosphere (Cardoso et al. 2011). Not surprisingly, strigolactone production is induced under phosphate starvation (Yoneyama et al. 2007). Experimental evidence suggests that

AM fungal colonization induces resistance to plant parasitism, either by reducing strigolactone secretion by the plant host, or by strigolactone uptake by the AM fungi (Lendzemo et al. 2005; López-Ráez et al. 2011). At present 15 different strigolactones have been characterized structurally and more have been tentatively identified (Xie et al. 2010). One plant species produces many different strigolactones in various quantities. Different varieties within the same species may produce different combinations of strigolactones (Awad et al. 2006). Such diversity might be the result of a selective pressure for new AM fungi colonization stimulants without parasitic plant germination stimulant activity (Ruyter-Spira et al. 2013). Each structural variant of strigolactones may have different germination inducing activity on different parasitic plants, as well as different influence in AM fungal branching stimulant activity (Akiyama et al. 2010). In addition to their signaling role in the rizhosphere, strigolactones are also responsible for regulating plant architecture both above and below ground, and they are directly involved in the control of shoot branching (Umehara et al. 2008; Gómez-Roldan et al. 2008).

B.5. Down-regulation of endogenous strigolactone pathway

It has been reported that plant strigolactone biosynthesis mutants are less susceptible to infection by parasitic plants (López-Ráez et al. 2008; Koltai et al. 2010). This suggested that the reduction of strigolactone production in host plants could be considered as an alternative strategy to control parasitic weeds (Cardoso et al. 2011; Kohlen et al. 2012). Silencing genes encoding enzymes involved in the strigolactone biosynthetic pathway could be expected to reduce or eliminate parasitism. Strigolactone biosynthesis takes place in the plastid where carotenoid precursors are synthesized (Matusova et al. 2005). The iron-containing protein D27 converts all-*trans*-β-carotene to 9-*cis*-β-carotene (Alder et al. 2012) which is subsequently sequentially cleaved by the carotenoid cleavage dioxygenases (CCDs) CCD7 and CCD8 to form the strigolactone-like compound carlactone (Alder et al. 2012). The conversion of carlactone to the likely precursor of all other strigolactones, 5-deoxystrigol, requires dioxygenation followed by dehydrogenation and two ring closure steps, which might be carried out by MAX1, the only strigolactone biosynthetic enzyme without any reported specific function (Ruyter-Spira et al. 2013; Figure 5).

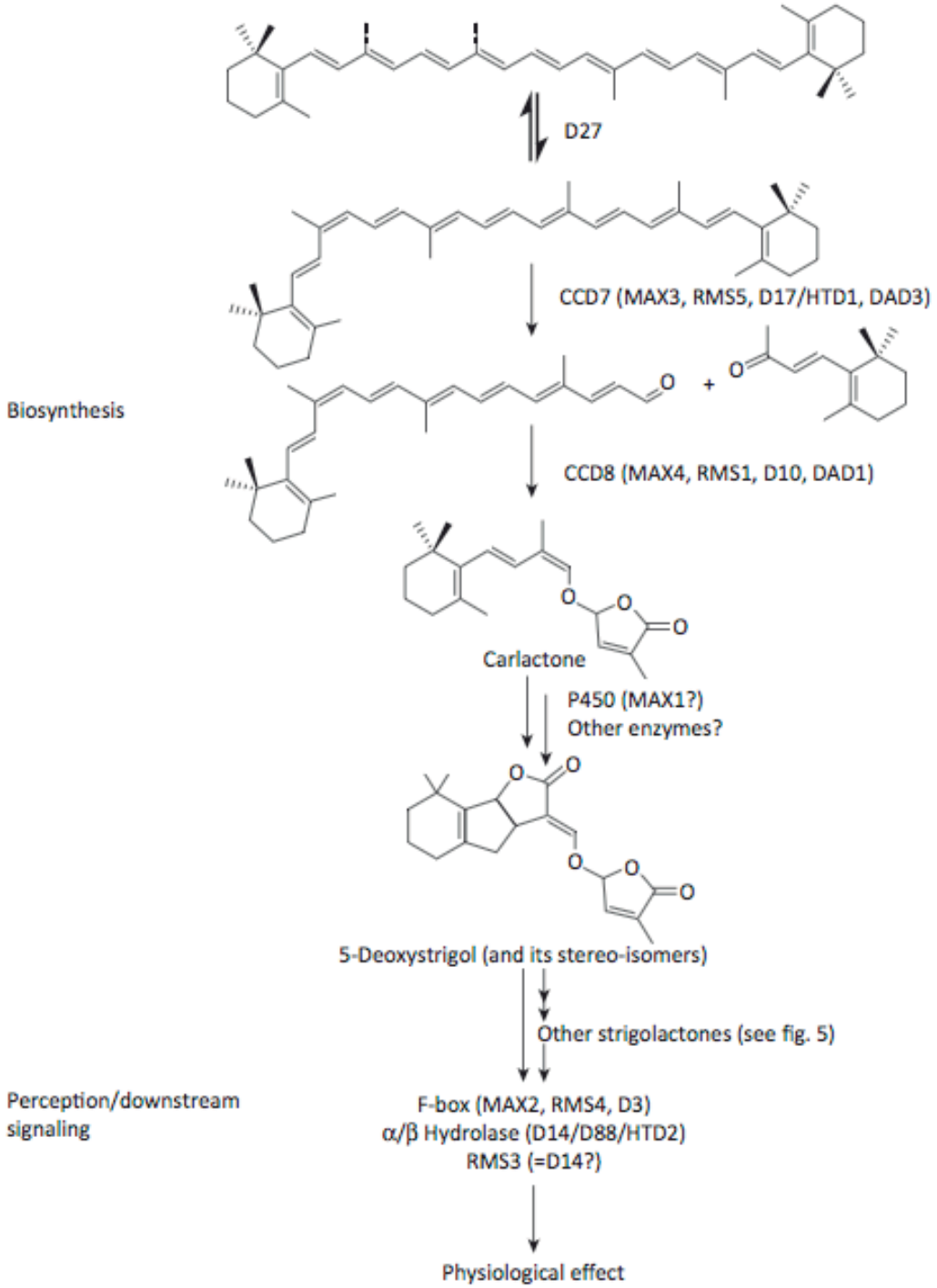

Figure 5. *Strigolactone biosynthetic and signaling pathways (Ruyter-Spira et al. 2013). Mutants in* Arabidopsis thaliana *(MAX),* Pisum sativum *(RMS),* Oryza sativa *(D and HTD) and* Petunia hybrida *(DAD) have been identified and characterized for several steps in the pathways.*

RNAi-mediated silencing of CCD7 and CCD8 has been reported in tomato (Vogel et al. 2010; Kohlen et al. 2012). Expression of target genes in transgenic tomatoes was dramatically reduced, which correlated with diminished strigolactone production, and

germination of parasitic weeds was reduced by up to 90% in both cases. Interestingly, arbuscular mycorrhizal symbiosis was only mildly affected in CCD8 knock-down lines, demonstrating that reduction of strigolactone levels may be a suitable strategy for parasitic weed control.

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Chapter 1

The generation of transgenic rice plants overexpressing genes involved in calcium storage and selenium assimilation

The generation of transgenic rice plants overexpressing genes involved in calcium storage and selenium assimilation

1.1. Abstract

The biofortification of staple crops with key nutrients is a potential short and middle term solution to malnutrition in developing countries. Genetic engineering is a versatile and efficient method that can be used to generate plants with an enhanced capacity for mineral accumulation. In the present study, particle bombardment was used to generate a population of transgenic rice plants constitutively expressing two genes controlling cellular Ca storage and one gene controlling Se assimilation. Regenerated plants were characterized at the molecular level and fertile lines expressing different transgene combinations were selected for further in-depth characterization. The analysis of Ca and Se metabolism in the transgenic lines will allow the identification of lead events that promote mineral accumulation as well as the preliminary characterization of endogenous regulatory mechanisms.

1.2. Introduction

Rice is one of the most important food crops in the world and is an essential part of the economy and culture of South-East Asia where more than 90% of production occurs. It is the main staple for more than 3 billion people, providing 50–70% of dietary carbohydrates and proteins over large parts of Asia. Large amounts of rice are also consumed in many South American and Caribbean countries, and it is rapidly growing as a staple food in a number of African countries (Table 1). Rice is therefore a major food as well as a source of wealth and employment for many poor people in developing countries (FAO 2002).

Table 1. *FAO 2012 statistics on paddy rice production, harvested area and yield (Source: FAOSTAT 2014)*

	Production (millions tons) [% of global production]	Harvested Area (millions ha) [% of global area]	Yield (tons/ha)
World	719.7	163.2	4.41
Asia	651.5 [90.5%]	145.2 [89.0%]	4.48
Africa	26.8 [3.7%]	10.5 [6.4%]	2.54
Americas	36.0 [5.0%]	6.5 [3.9%]	5.46
Europe	4.3 [0.6%]	0.7 [0.4%]	6.30
Oceania	0.9 [0.1%]	0.1 [0.06%]	8.66

Like other cereals, rice grain is deficient in many important nutrients including essential minerals. The world's poorest people are dependent on staples such as rice as their only source of food, and with no purchasing capacity to diversify their diet they often suffer from malnutrition (Christou and Twyman 2004). Biofortification has been proposed as a short-term and mid-term strategy to alleviate malnutrition. The development of crops with higher levels of key nutrients usually requires the simultaneous modulation of several metabolic pathways (Zhu et al. 2007). Plant breeding programs that seek to improve mineral nutrition are limited to the exploitation of natural variation within sexually-compatible species. Genetic engineering is a more versatile approach because it can source genes from diverse species, it allows multiple nutritional deficiencies to be tackled simultaneously, and nutrient accumulation can be directed to the edible parts of the plant (Naqvi et al. 2009a).

Co-transformation by direct DNA transfer is an efficient and reliable method to simultaneously introduce several genes into plants at a single locus, thus preventing segregation in subsequent generations (Alpeter et al. 2005). In this context, our group began a research program several years ago aiming to biofortify rice and maize with four vitamins (pro-vitamin A, vitamin E, folate and ascorbate) and four minerals (Fe, Zn, Ca and Se) by genetic engineering (Naqvi et al. 2009b; Naqvi et al. 2010). In the first part of this project, I focused on the generation of rice plants with a higher capacity for Ca and Se accumulation by simultaneously overexpressing three *Arabidopsis thaliana* genes encoding deregulated versions of a $Ca^{2}/^{+}H^{+}$-antiporter (sCAX1) and a Ca^{2+}-ATPase (sACA4) controlling calcium accumulation in the vacuole, and the plastid-located ATP sulfurylase (APS) to overcome the principal bottleneck in the endogenous selenium assimilation pathway.

1.3. Aims

The major aim of the work described in this chapter was to generate a population of transgenic rice plants constitutively expressing three genes involved in Ca and Se accumulation. The specific objectives were:

1) To transform rice embryos with the three target genes by particle bombardment and regenerate putative transgenic rice plants.

2) To analyze transgene expression in each of the regenerated plants to identify useful lines for further analysis of Ca and Se metabolism.

1.4. Materials and methods

1.4.1. Transformation vectors

The constructs for *Arabidopsis thaliana scax1*, *saca4* and *aps1* were generated by Sonia Gómez-Galera in 2011. The corresponding cDNAs were transferred individually to vector pAL76 (Christensen and Quail 1996), which contains the constitutive maize ubiquitin-1 (*Ubi-1*) promoter, its first intron and the nopaline synthase (*nos*) transcriptional terminator. A fourth plasmid containing the hygromycin phosphotransferase (*hpt*) selectable marker gene (Sudhakar et al. 1998) was used for the generation of transgenic rice plants.

1.4.2. Rice combinatorial transformation and plant regeneration

Mature rice seeds (*Oryza sativa* L. *cv* EYI 105 and Nipponbare) were cultured as previously described (Sudhakar et al. 1998; Valdez et al. 1998). After 7 days, the embryos were excised and bombarded with DNA-coated gold particles (Christou et al. 1991). Gold particles (10 mg) were coated with 40 µg of a DNA mixture containing the three individual pAL76 plasmids and the *hpt* marker in a 3:3:3:1 molar ratio. Rice embryos were incubated on high-osmoticum MS medium (0.2 M mannitol) for 4 h before bombardment. Bombarded embryos were cultured on MS medium supplemented with 30 mg/l hygromycin (Roche, Mannheim, Germany) in the dark for 1 month, and subcultured every 2 weeks. Embryogenic callus was transferred onto shoot induction medium and then root induction medium supplemented with 30 mg/l hygromycin in the light (900 µmohn/m^2/s photosynthetically-active radiation). After regeneration, plantlets were transferred to pots (containing soil supplied by Traysubstract, Klasmann-Deilmann GmbH, Geeste, Germany) and were grown in a growth chamber at 26 ± 2°C, with a 12-h photoperiod (900 µmohn/m^2/s photosynthetically-active radiation) and 80% relative humidity. Plants were initially irrigated with a soluble Fe solution (Sequestrene 138 Fe G-100; Syngenta Agro SA, Madrid, Spain), and then watered with tap water until seed harvest. Table 2 shows the media used in each step of the process, which is illustrated in Figure 1.

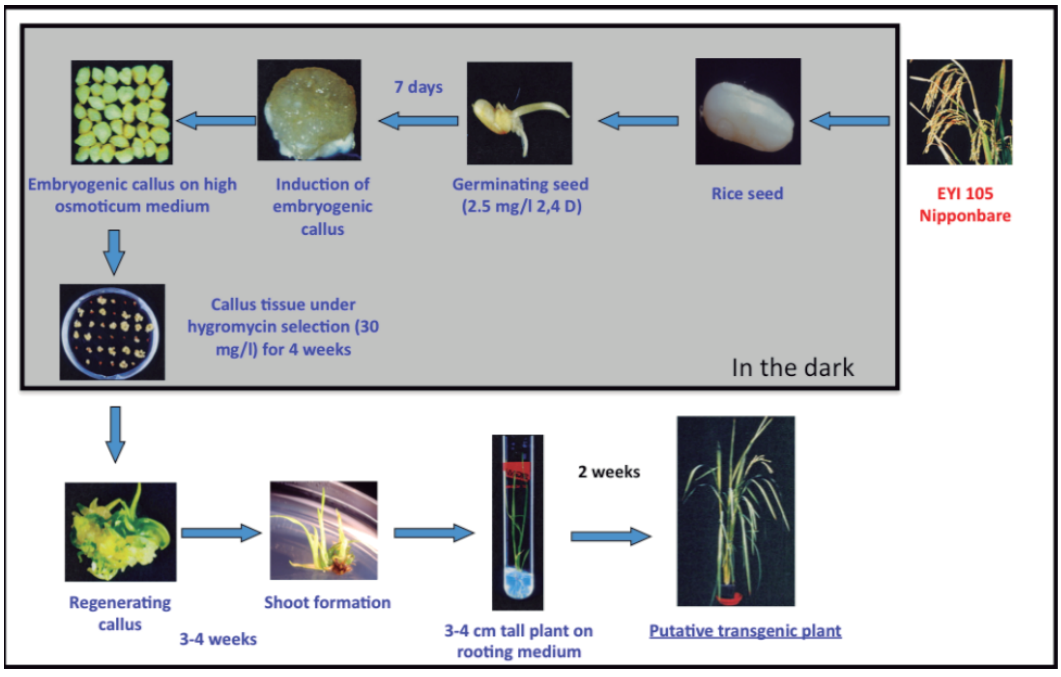

Figure 1. *Rice transformation process*

Table 2. *Medium composition (1 l) for rice transformation and regeneration.*

	Callus induction	Osmoticum medium	Selection medium	Shoot induction medium	Root induction medium
Approx. time in culture	5 to 7 days	24h	Four weeks (two weeks subcultures)	21 to 25 days	2 to 3 weeks
MS powder *	4.4 g	4.4 g	4.4 g	4.4 g	2.2 g
Casein hydrolysate	300 mg	300 mg	300 mg	100 mg	--
Proline	500 mg	500 mg	500 mg	--	--
Sucrose	30 g	30 g	30 g	--	10 g
Maltose	--	--	--	30 g	--
Mannitol	--	72.8 g	--	--	--
2,4-D (5mg/ml)	500 µl	500 µl	500 µl	--	--
Adjust pH to 5.8 using KOH					
Phytagel (Sigma)	5 g	3 g	5 g	4 g	3 g + 2 g agar
Autoclave at 121ºC for 20 min					
BAP (Sigma, 1 mg/ml)	--	--	--	3 mg (3 ml)	--
NAA (Sigma, 1 mg/ml)	--	--	--	0.5 mg (0.5 ml)	--
Hygromycin (50 mg/ml)	--	--	600 µl	600 µl	600 µl
Vitamin B5 (200x)**	--	--	--	--	2.5 ml

*MS Medium with Gamborg's B5 vitamins (Duchefa Biochemie, Haarlem, The Netherlands)

**B5 Vit (200x stock): dissolve 1 g myo-inositol, 10 mg nicotinic acid, 10 mg pyridoxine HCl and 100 mg thiamine HCl in 50 ml sterile water

1.4.3. RNA extraction

Total RNA was extracted from 120 mg of frozen, ground leaf material using a modification of a previously reported method (Vicient and Delseny 1999). The ground leaf tissue was mixed with 900 µl of extraction buffer (1 M Tris-HCl pH 9.0, 1 M LiCl, 0.5 M EDTA pH 8.0, 20% SDS) and incubated at room temperature for 5 min before extracting with one volume of 25:24:1 phenol:chloroform:isoamylalcohol (Sigma-Aldrich, St. Louis, US). Samples were centrifuged at 10,000 x g for 10 min at 4ºC and the supernatant was recovered. After two further rounds of extraction, the supernatant was mixed with one volume of 4 M LiCl and incubated at –20ºC for 1 h. RNA was precipitated by centrifuging at 10,000 x g for 20 min at 4ºC and the resulting pellet was washed with 500 µl 75% ethanol for 1 h on ice. After centrifugation at 10,000 x g for 10 min at 4ºC, the RNA pellet was air-dried and resuspended in 70 µl sterile distilled water. RNA integrity was assessed by 1.2% TBE agarose gel electrophoresis and the total RNA concentration was estimated using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, US).

1.4.4. mRNA blot analysis

Total RNA (25 μg) was denatured and fractionated by 1.2% agarose-formaldehyde gel electrophoresis in 1x MOPS buffer (Sambrook et al. 1989). RNA was then transferred to a positively-charged nylon membrane (Roche, Mannheim, Germany) and fixed by UV cross-linking. Probes (Table 3) were DIG-labeled as described by Capell et al. (2004) and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Membranes were pre-hybridized at 50ºC for 2 h and DIG-labeled probes were denatured at 95ºC for 10 min before dilution in 10 ml EasyHyb solution, which was used for hybridization at 50ºC overnight. Membranes were washed twice for 10 min in 2x SSC + 0.1% SDS at room temperature, twice for 30 min in 0.5x SSC + 0.1% SDS at 68ºC, once for 20 min in 0.2x SSC + 0.1% SDS at 68ºC, and once for 10 min in 0.1x SSC + 0.1% SDS at 68ºC. Subsequent steps were carried out according to the manufacturer's instructions (DIG Luminescent Detection Kit; Roche, Mannheim, Germany). Membranes were first incubated in blocking solution for 1 h at room temperature and then in the presence of the DIG-specific antibody for 30 min at room temperature. After three 20-min washes, membranes were incubated with CSPD chemiluminescent substrate (Roche, Mannheim, Germany) and exposed to BioMax light film (Sigma-Aldrich, St. Louis, US) at 37ºC.

Table 3. *Primers used to synthesize DIG-labelled probes for mRNA blot analysis.*

Gene	Probe primers	
	Position	**Sequence**
Atscax1	Forward	5'-GCTGAGCGAGTCAGCTTTTT-3'
	Reverse	5'-ACCCGGGCAACACATTGGTACTTGTCTTCGT-3'
Atsaca4	Forward	5'-AATGGTAGCAGTTGTTGGAATC- 3'
	Reverse	5'-ACCCGGGTCAGGCAGAGTTGGAA- 3'
Ataps1	Forward	5'-AGGATCCATGGCTTCAATG GCTGCCGTCTTAA-3'
	Reverse	5'-AAAGCTTTTACACCGGAACCACTTCTGGTAGT-3'

1.5. Results

1.5.1. Recovery of transgenic plants

In the first round of transformation experiments, rice embryos were co-bombarded with *AtAPS1*, *AtsCAX1*, *AtsACA4* and *hpt*, and 60 hygromycin-resistant putative transgenic lines were generated. A second round of transformation experiments was carried out using separate transgene combinations for each mineral. Nineteen hygromycin-resistant putative transgenic lines were generated after bombardment with the Ca-related genes *AtsCAX1* and *AtsACA4*, whereas 17 hygromycin-resistant lines were recovered following transformation with *AtAPS1*.

1.5.2. Expression analysis of putative transgenic plants

The leaves of the putative transgenic plantlets were screened directly for transgene expression by mRNA blotting. Transcript accumulation was confirmed by the presence of a single band in the *AtsCAX1* and *AtsACA4* mRNA blots (with the exception of line 11) or multiple bands in *AtAPS1* mRNA blot (Figure 3). The band size indicated the presence of the full-length *AtAPS1* transcript. The mRNA blots revealed substantial differences in expression levels among the transgenic lines.

Half of the lines generated in the first round of bombardment did not express any of the transgenes. Fourteen lines expressed *AtAPS1* alone, whereas only a single line was recovered expressing *AtsCAX1* and *AtsACA4*. The remaining 14 lines expressed different combinations of the transgenes (Figure 2A). In the second round of bombardment, nearly half of the putative transgenic lines bombarded with *AtAPS1* expressed the transgene, whereas only four of the 19 putative transgenic lines bombarded with the Ca-related genes expressed one or both of the transgenes (Figure 2B). Only those lines expressing one or more transgenes were grown until maturity to confirm their fertility status. The fertility rate in the first round of bombardments was 37%, showing no obvious correlation with specific transgene expression. In the second round of bombardments, 38% of the lines expressing *AtAPS1* were fertile whereas all four lines expressing the Ca-related genes were sterile (Figure 2).

All fertile lines expressing the different transgene combinations are shown in Figure 3, and the mRNA blot data are summarized in Table 4. Line 84 showed little or no expression of *AtAPS1*, but bands were detected in the mRNA blot used in the

preliminary screening of the regenerated lines so this line was included in further experiments (Figure 3D). Two of the four fertile lines that expressed all three transgenes in the T_0 generation suffered loss of transgene expression in the T_1 generation (data not shown) so these lines were discarded in subsequent experiments. Line 11 only expressed a single *AtAPS1* transcript that was present in most of the lines but did not correspond to the full-lenght transcript (Figure 3C). Further experiments demonstrated that this transcript was dysfunctional (Chapter 2). Transgene expression was monitored in independent T_1 plants representing some of the fertile lines to determine the segregation pattern (Figure 3-E).

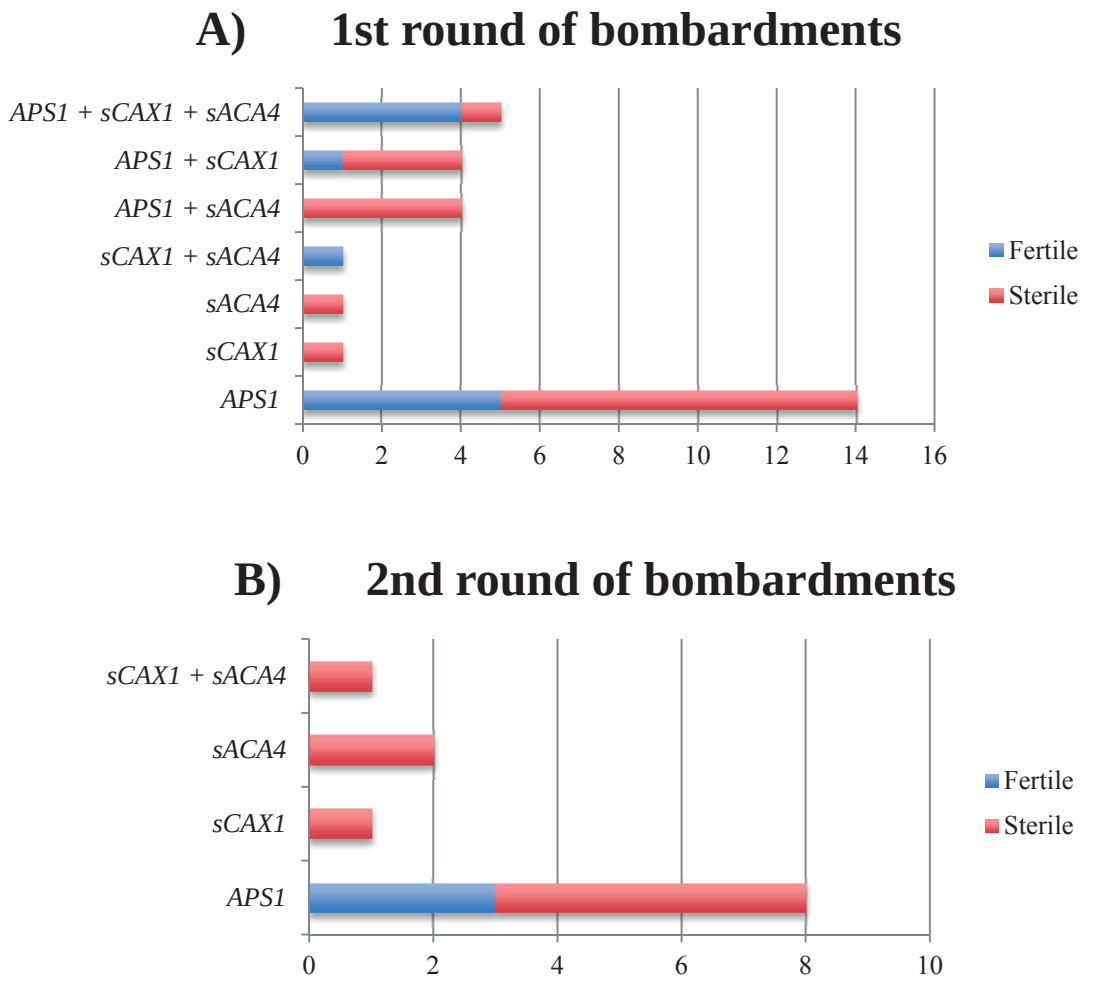

Figure 2. *Frequency of fertile (blue) and sterile (red) transgenic lines expressing different transgene combinations in two rounds of transformation experiments. The* hpt *gene is assumed to be expressed in all transgenic lines. A) Transgenic lines bombarded with* AtAPS1, AtsCAX1 *and* AtsACA4 *plus* hpt *gene. B) Transgenic lines bombarded with either* AtsCAX1 *and* AtsACA4 *plus* hpt, *or* AtAPS1 *plus* hpt.

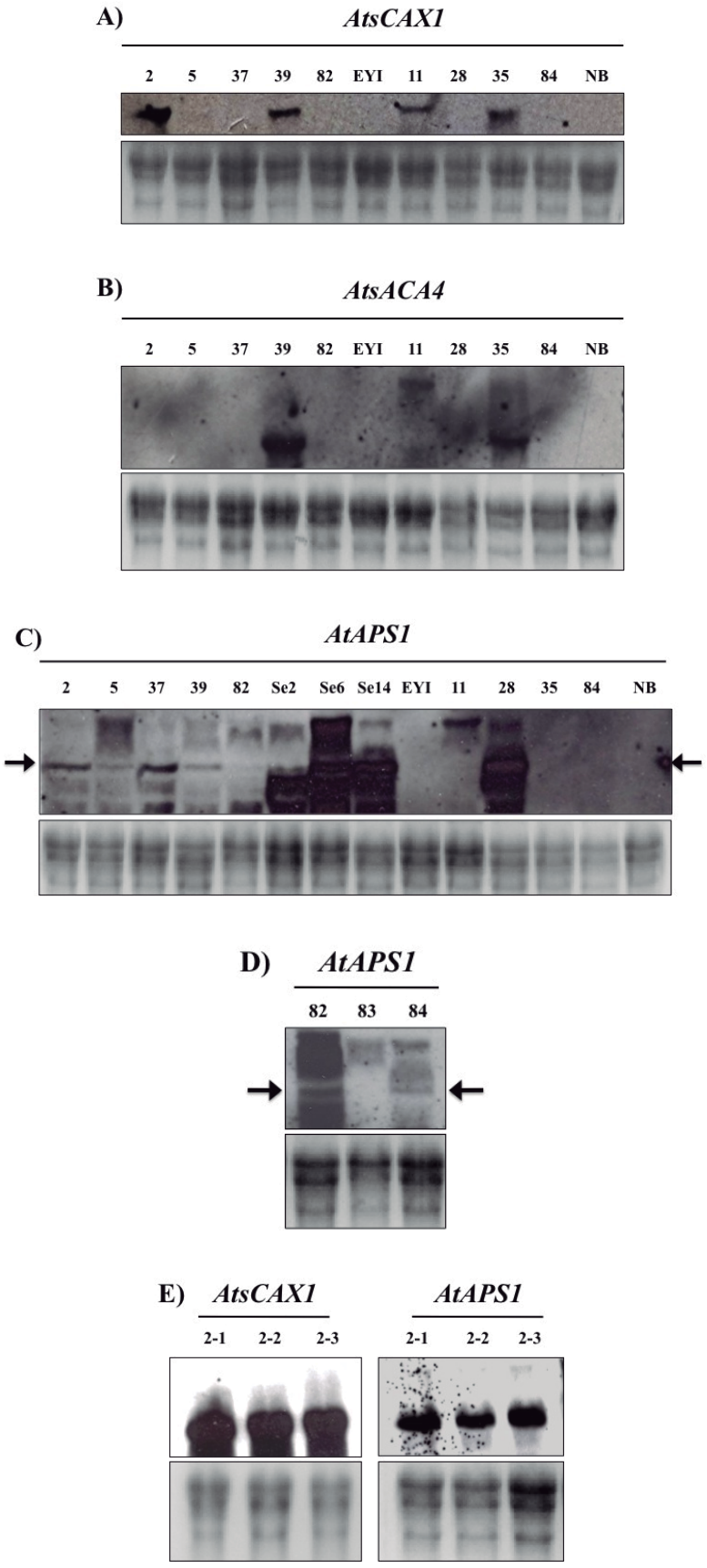
A)
AtsCAX1
2 5 37 39 82 EYI 11 28 35 84 NB
B)
AtsACA4
2 5 37 39 82 EYI 11 28 35 84 NB
C)
AtAPS1
2 5 37 39 82 Se2 Se6 Se14 EYI 11 28 35 84 NB
D)
AtAPS1
82 83 84
E)
AtsCAX1
2-1 2-2 2-3
AtAPS1
2-1 2-2 2-3

Figure 3. *mRNA blot analysis showing the expression of* AtsCAX1 *(A),* AtsACA4 *(B) and* AtAPS1 *(C) in the leaf tissue of T_1 plants of transgenic fertile lines. Lines bombarded with* AtAPS1 *alone (Se2, Se6 and Se14) were not screened for* AtsCAX1 *and* AtsACA4 *expression. Lines 2, 5, 37, 39, 82, Se2, Se6 and Se14 have an EYI-105 (EYI) background, and lines 11, 28, 35 and 82 have a Nipponbare (NB) background. D) mRNA blot analysis showing* AtAPS1 *expression in leaf tissue of T_0 plants of lines 82, 83 and 84. E) mRNA blot analysis showing expression of AtsCAX1 and AtAPS1 in independent T_1 plants of line 2. Arrows indicate the full-length* AtAPS1 *transcript*.

Table 4. *Summary of transgene expression in fertile lines*.

Line	*AtAPS1*	*AtsCAX1*	*AtsACA4*
EYI-105			
2	**X**	**X**	
5	**X**		
37	**X**		
39	**X**	**X**	**X**
82	**X**		
Se2	**X**		
Se6	**X**		
Se14	**X**		
Nipponbare			
11	**X**	**X**	**X**
28	**X**		
35		**X**	**X**
84	**X**		

1.6. Discussion

The generation of nutritionally-enriched staple crop varieties has been envisaged as a potential solution to tackle malnutrition in the short term (Zhu et al. 2007). In order for this approach to be effective, crops must accumulate not one but several micronutrients in their edible parts (Christou and Twyman 2004). As discussed above, the use of GE technology is a versatile and practical approach to generate crops with multiple nutritional traits (see General Introduction). In this study, I aimed to generate transgenic rice plants with the ability to accumulate higher levels of Ca and Se in the

grain. Unlike organic nutrients which are synthesized de novo, minerals need to be taken up by plants from the environment. The availability of minerals in the rhizosphere is determined by soil properties and the characteristics of the minerals themselves, which in turn influences the efficiency of plant uptake mechanisms (White and Broadley 2008). In addition, the distribution and storage of minerals within plants depend on the properties of each mineral and their specific physiological role. Therefore GE strategies to enhance mineral accumulation in crops need to consider specific aspects of mineral uptake, distribution and storage and will need to be determined on a case-by-case basis (Gómez-Galera et al. 2010).

Calcium is taken up by roots in the form of soluble Ca^{2+}, which is transported to the leaves in the transpiration stream. After xylem unloading, Ca^{2+} enters the cells and is imported into the vacuoles via energy-dependent processes (White and Broadley 2003). Therefore, strategies to increase Ca accumulation in plants could focus on (I) increasing Ca uptake from the rhizosphere; (II) increasing Ca uptake into cells; and/or (III) enhancing the Ca storage capacity of cells (Dayod et al. 2010). Plants growing in Ca^{2+}-rich soils can suffer Ca toxicity (White and Broadley 2003), suggesting that uptake from the rhizosphere does not need to be enhanced. Calcium uptake into cells is mediated by Ca^{2+}-permeable channels in the plasma membrane (White 2000). Although Ca^{2+} channels have been found in all plant cell types and many have been characterized electrophysiologically, the genes encoding these proteins are largely unknown. Candidate genes include plant homologs of cyclic nucleotide-gated channels and glutamate receptor-like proteins (Demidchik and Maathuis 2007). However, the overexpression and knockout of such genes has not generally resulted in significant changes in the levels of Ca in the shoot, but has instead led to Ca-sensitive phenotypes, suggesting that these genes are most likely involved in Ca^{2+} signaling rather than in Ca storage (Conn and Gilliham 2010). Therefore, it seems unlikely that enhancing Ca transport into cells would increase the overall Ca content.

Cellular Ca is mainly stored in tonoplasts, the largest storage compartment in plants. Several reports have shown that the expression of tonoplast membrane-spanning transporters was increased at the mRNA and protein levels in Ca-accumulating cells (Carter et al. 2004; Richardson et al. 2007; Yang et al. 2008). Calcium loading into vacuoles is an energy-demanding process driven by Ca^{2+}-ATPases (ACA) and H^+/Ca^{2+}-antiporters (CAX) (Pittman 2011). An *Arabidopsis thaliana* knockout mutant

lacking ACA4 and ACA11 was characterized by lower levels of Ca^{2+} in the shoot, and the same phenotype was observed for another mutant deficient in CAX1 and CAX3 expression (Conn and Gilliham 2010). Therefore, these transporters appear to be suitable candidates for Ca biofortification.

As described in the General Introduction, GE-based attempts to enhance the Ca storage capacity of crops have thus far included the expression of *AtsCAX1*, *AtCAX4* and *AtCAX2A/B* under the control of either the CaMV 35S promoter or the *AtCDC2a* promoter. The most dramatic increases in Ca content were reported when *AtsCAX1* was constitutively overexpressed, so we used the same strategy for our rice biofortification experiments. Ca^{2+}-ATPases have not been used previously in biofortification experiments, but they play an essential role in Ca^{2+} efflux from the cytosol into tonoplasts (Conn and Gilliham 2010), so we decided to include a deregulated version of the well-characterized *Arabidopsis* ACA4 protein in our experiments (Geisler et al. 2000).

Selenium, unlike Ca, has not been shown to play any key role in higher plants, and most likely its uptake and accumulation is a consequence of the similarity between Se and the essential macronutrient sulfur (Sors et al. 2005). Plant roots take up Se mainly in the form of selenate and selenite, depending on the predominant Se species in the rhizosphere. Selenate is easily translocated to the shoots, where it is then reduced to selenite and assimilated into organic Se compounds through the sulfur assimilation pathway. In contrast, selenite is rapidly converted into organic Se compounds within the roots, and only a small portion is transported to the shoots (Terry et al. 2000). Strategies to enhance Se accumulation in plants will depend on the Se species that is predominantly taken up by each crop. In most cases, selenate is the prevalent Se species in the soil and, given that it is efficiently taken up by sulfate transporters, biofortification strategies have focused on this assimilation pathway (Sors et al. 2005). The reduction of selenate to selenite is the major rate-limiting step in the sulfate/selenate assimilation pathway in four different crops including Indian mustard and rice (Zayed et al. 1998). In order to overcome this rate-limiting step, the ATP sulfurylase gene *AtAPS1* was overexpressed in Indian mustard plants, resulting in increased Se accumulation in leaves and enhanced Se tolerance (Pilon-Smits et al. 1999). Alternative strategies focused on the diversion of Se from protein incorporation, and this also increased Se accumulation and tolerance in Indian

mustard plants (Valdez-Barillas et al. 2011).

Rice takes up Se mostly in the form of selenite, which is the dominant Se species in anoxic paddy soils (Zhang et al. 2006). Therefore, under normal conditions, the normal rate-limiting step of selenate reduction is already overcome in rice plants, although the uptake of selenite results in less efficient Se translocation from root to shoot and grain (Zayed et al. 1998). In regions where Se deficiency is widespread, the soil Se content tends to be limiting and Se-enriched fertilizers are required (Wang and Gao 2001). The addition of Se fertilizers to flooded soils in the form of selenate or selenite is highly inefficient because selenate is rapidly converted to selenite, and more than 80% of the selenite is fixed in non-labile pools (Premarathna et al. 2010). Alternative fertilization strategies such as foliar spraying have been tested (Hu et al. 2002; Xu et al. 2004; Wang et al. 2013) and one report has shown that that Se content of rice grains is 36% higher when the plants are sprayed with selenate rather than selenite, although no explanation was proposed (Chen et al. 2002). Furthermore, a promising fertilization method involving the addition of selenate-enriched urea granules to the floodwater has been developed recently (Premarathna et al. 2012). Urea granules prevented the reduction of selenate under anaerobic conditions, allowing the selenate to be taken up by the rice plants and translocated to the shoot and grain. In regions with Se-depleted soils where rice is treated with selenate-enriched fertilizers, the rice plants will therefore take up Se mainly in the form of selenate. Se assimilation into organic compounds will then be limited by the reduction of selenate to selenite (Zayed et al. 1998). In this scenario, the overexpression of *AtAPS1* in rice plants could enhance Se accumulation in the grain, increasing the efficiency of Se use and thus reducing the amount of Se fertilizer required in biofortification programs.

In order to generate transgenic rice lines with a higher capacity for Ca and Se accumulation, EYI-105 and Nipponbare rice embryos were bombarded with *AtsCAX1*, *AtsACA4* and *AtAPS1* simultaneously. Particle bombardment is the most prominent method for direct DNA transfer and it promotes the stable integration of multiple genes carried by independent vectors (Chen et al. 1998; Zhu et al. 2008). A population of 96 putative transgenic lines was generated. DNA analysis was omitted and plants were screened directly for transgene expression at the mRNA level. Expression analysis provides a practical advantage because it not only allows the

elimination of untransformed lines, but also those which have been transformed but in which the transgenes are non-functional or silenced.

Co-transformation of rice embryos with *AtAPS1*, *AtsCAX1* and *AtsACA4* generated 60 putative transgenic lines, 50% of which expressed different combinations of the transgenes. In a second round of transformation experiments, four of the 19 lines bombarded with *AtsCAX1* and *AtsACA4* expressed one or both transgenes, whereas transgene expression was detected in half of the lines bombarded with *AtAPS1* alone. Adding the results from the two rounds of bombardments, it is clear that 45% of the regenerated lines bombarded with *AtAPS1* alone expressed the transgene, whereas only 18% of those bombarded with the Ca-related genes showed transgene expression (Figure 2). The recovery of transgenic plants expressing the Ca-related genes was therefore less efficient than the recovery of those expressing *AtAPS1*. It is unclear if this phenomenon was observed in previous studies using CAX antiporters because the percentage of regenerated lines expressing the transgenes was not reported. However, the detrimental effects of CAX overexpression have been reported. *AtsCAX1* was first overexpressed in tobacco plants, which displayed increased sensitivity to stress (Hirschi 1999). The expression of a highly-active deregulated version of *AtCAX3* (which functionally complements CAX1, Cheng et al. 2005) in tobacco led to more dramatic phenotypes, including stunted growth and failure of flower development (Manohar et al. 2011). In addition, transgenic tomato plants expressing *AtsCAX1* showed growth defects (Park et al. 2005). CAX-induced impairments may be explained by considering the role of these antiporters in the regulation of $[Ca^{2+}]_{cyt}$, which is a key factor in many signaling pathways driving multiple cellular responses (Tuteja and Mahajan 2007). Excessive CAX activity could disturb endogenous fluctuations of $[Ca^{2+}]_{cyt}$, causing abnormal phenotypes (Hirschi 1999). Similarly, the overexpression of deregulated versions of *CAX1* and *ACA4* could exert a negative impact on rice cells by disrupting $[Ca^{2+}]_{cyt}$ and causing negative selection pressure for the regeneration of transgenic lines expressing these transgenes.

Interestingly, the generation of transgenic rice plants with enhanced capacity for Ca accumulation was attempted in a previous study, although the long form of *CAX1* containing the N-terminal auto-inhibitory domain (Pittman et al. 2002) was used instead of the deregulated and more active version, *sCAX1* (Kim et al. 2005). A population of 29 rice transgenic lines expressing long *CAX1* was generated with no

deleterious effects on plant growth and development, although Ca accumulation was not measured and therefore it remains unclear whether the transgenic plants had a higher CAX1 activity. It would be interesting to find out whether the authors tried to generate transgenic plants expressing *AtsCAX1*.

Excessive Ca sequestration into vacuoles can affect fertility in some crops. All transgenic tobacco lines expressing the highly active deregulated version of *AtCAX3* were sterile (Manohar et al. 2011), and only 15% of the 48 transgenic tobacco events expressing *AtsCAX1* were fertile (Hirschi et al. 1999). A similar fertility rate was observed in *AtsCAX1*-expressing tomato lines, with only one of six primary transformants producing viable seeds (Park et al. 2005). Nevertheless, the fertility rate was higher in my experiments, reaching 30% among transgenic rice lines expressing Ca-related genes (Figure 2). Similarly, 37% of the lines expressing *AtAPS1* alone were fertile, suggesting that in this case transgene expression did not cause sterility. *AtAPS1* expression has not previously been associated with reduced fertility (Pilon-Smits 1999). Therefore, the sterility of the transgenic rice lines generated in this study could reflect other factors, such as stress caused by tissue culture or handling (Gordon-Kamm et al. 1990).

It is remarkable that more than half of the regenerated lines, including those bombarded with *AtAPS1* alone, do not express any of the non-selected transgenes. This may reflect gene silencing, the recovery of transgenic lines containing only the selectable marker gene, and the regeneration of wild-type plants. The transformation system I used in my experiments was optimized in previous studies to inhibit the proliferation of wild-type cells by applying selection soon after bombardment (Christou and Ford 1995), and to maximize the chance of regenerating plants containing non-selected transgenes by using a 1:3 molar ratio of the selectable marker gene to the genes of interest (Maqbool and Christou 1999). Gene silencing is not unusual in transformation experiments, and has been repeatedly described in previous reports, so this seems the much more likely explanation of the absence of transgene expression (Assad et al. 1993; Wang et al. 2012).

Gene silencing was detected in the T_1 plants of lines 12 and 27, which expressed *AtsCAX1*, *AtsACA4* and *AtAPS1* in the T_0 transformants. All T_1 seeds were unable to germinate in the presence of hygromycin, and seedlings grown in the absence of the

selectable marker did not express any of the transgenes. Gene silencing after sexual reproduction in transgenic plants has been reported previously (Alpeter et al. 2004). All other lines expressing different combinations of the transgenes in T_0 plants showed stable expression in T_1 offspring. All hygromycin-resistant T_1 plants expressed the same transgenes as their corresponding T_0 transformants, showing an expression segregation pattern consistent with the Mendelian inheritance of a single dominant locus suggesting that all transgenes in each line were probably integrated at the same locus (Figure 3E). This phenomenon is consistently encountered in transgenic rice plants generated by particle bombardment (Cooley et al. 1995), and it can be explained by a multi-step gene integration model that involves the concatenation of different transgenes prior to integration, and the formation of 'integration hot spots' that join together multiple transgenes within the same locus (Kohli et al. 1998; Kohli et al. 2003; Altpeter et al. 2005).

Variations in transgene expression levels were observed among different lines, especially in the case of *AtAPS1* (Figure 3C). Transgene expression can be influenced by many factors, including position effects (Allen et al. 1993; Matzke and Matzke 1998), integrative fragmentation and rearrangements (Alpeter et al. 2005). Certain sequences are more frequently rearranged than others (Register et al. 1994). This could explain the more pronounced heterogeneity in the level of *AtAPS1* expression and the presence of multiple bands in the mRNA blot. Almost all the lines expressing *AtAPS1* showed similar band patterns, suggesting the existence of conserved and preferential rearrangements that generate both larger and incomplete mRNA molecules. A more in depth characterization of the 12 fertile transgenic lines generated in this experiment is required in order to determine whether transgene expression causes the anticipated changes in mineral accumulation in the rice grain.

1.7. Conclusions

I generated 12 fertile transgenic rice lines exhibiting stable expression of genes involved in the accumulation of Ca and Se. The recovery of plants expressing *AtAPS1* was more efficient than the recovery of plants expressing the two Ca-related genes, which seem to exert a negative effect on the rice cells consistent with previous reports using these and other Ca-related genes. The analysis of Ca and Se metabolism in the transgenic rice lines will determine whether transgenes play the expected role in

mineral accumulation, and will provide more insight into the endogenous mechanisms of mineral homeostasis.

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Chapter 2

Preliminary evaluation of transgenic rice plants overexpressing two calcium transporters and ATP-sulfurylase for mineral accumulation

Preliminary evaluation of transgenic rice plants overexpressing two calcium transporters and ATP-sulfurylase for mineral accumulation

2.1. Abstract

The development of genetically-engineered biofortified crops requires the assessment of nutrient accumulation and bioavailability. In the previous chapter, I described the generation of transgenic rice lines overexpressing *AtsCAX1* and *AtsACA4*, which encode vacuolar Ca transporters to promote Ca accumulation, and *AtAPS1*, encoding the first enzyme of the sulfate/selenate assimilation pathway to increase Se accumulation. In this chapter, the mineral content of the transgenic plants was analyzed to determine whether they accumulated higher levels of Ca and Se. The expression of endogenous genes involved in Ca and Se metabolism was also measured to assess the influence of transgene expression on mineral homeostasis in the transgenic plants. None of the lines showed significant and consistent enhanced Ca accumulation and although the lines expressing *AtAPS1* showed an enhanced selenate reduction capacity, this translated into increased Se levels in only two lines.

2.2. Introduction

Genetic engineering allows the development of crops with an enhanced capacity to accumulate minerals (Gómez-Galera et al. 2010). Thus far, Ca accumulation has been enhanced in the edible parts of four widely-consumed vegetables through the overexpression of a de-regulated version of an *Arabidopsis* Ca transporter (Park et al. 2004, 2005a, 2005b, 2009). *AtsCAX1*-expressing carrots provide a better source of bioavailable Ca for human consumption than wild-type carrots (Morris et al. 2008), demonstrating the feasibility of genetic engineering to effectively improve human diets. Conversely, most attempts to increase the Se accumulation capacity of crops through genetic engineering have been approached for phytoremediation rather than biofortification purposes (Pilon-Smits et al. 1999; LeDuc et al. 2006). Nevertheless, because the transgenic strategies used in these cases are usually valid for both purposes, any knowledge about Se metabolism gained from phyoremediation studies can also be used for the Se biofortification of staple crops (Zhu et al. 2009).

In the previous chapter, I described the development of rice plants expressing transgenes that increased their capacity to accumulate Ca and Se in the grain. I generated transgenic rice lines overexpressing *AtsCAX1* and *AtsACA4* to increase Ca accumulation, and *AtAPS1* to increase Se assimilation and accumulation. The next step was to evaluate the mineral storage capacity of different plant organs.

In the work described in this chapter, I assessed the influence of transgene expression on mineral accumulation and the expression of endogenous metabolic genes. Transgenic lines expressing *AtsCAX1* and *AtsACA4* were exposed to different Ca concentrations before analyzing the Ca content and the accumulation of other minerals. The expression of several endogenous Ca transporter genes was measured in order to assess the influence of *AtsCAX1* and *AtsACA4* expression on the regulation of endogenous Ca homeostasis. In contrast, *AtAPS1*-expressing lines were grown under normal conditions and Se was supplied in the form of selenate by foliar spraying, which is a prevalent method of Se biofortification (Chen et al. 2002). The total content of sulfur and Se was determined and I also investigated the Se speciation, which may better reflect the assimilation of Se than overall Se levels (Sors et al. 2005a). Se tolerance in the transgenic lines was also evaluated to gain some insight into the effect of *AtAPS1* expression on endogenous Se metabolism. Finally, the

expression of key genes in the Se assimilation pathway was analyzed in the presence and absence of Se.

2.3. Aims

The major aim of the work described in this chapter was to evaluate the mineral accumulation capacity of transgenic rice plants constitutively expressing genes involved in Ca and Se accumulation. The specific objectives were:

1) Analyze Ca levels and other minerals in different organs of transgenic rice lines expressing *AtsCAX1* and *AtsACA4*.

2) Analyze Se speciation, accumulation and tolerance in transgenic rice plants expressing *AtAPS1*.

3) Monitor the expression of endogenous rice genes involved in Ca and Se metabolism

2.4. Materials and methods

2.4.1. Plant material

Transgenic rice (*Oryza sativa* L. *cv* EYI-105 and Nipponbare) plant lines expressing different combinations of *AtsCAX1*, *AtsACA4* and *AtAPS1* were generated as described in Chapter 1. T_1 and T_2 seeds were used in the work described in this chapter.

2.4.2. Germination and growth conditions

Seeds from transgenic plants were sterilized in 70% ethanol for 3 min and commercial bleach for 20 min with agitation, and were germinated in sterile pots containing half-strength MS medium supplemented with 30 mg/l hygromycin (Roche, Mannheim, Germany) for 10 days (26 ± 2°C, with a 16-h photoperiod and 80% relative humidity). Wild-type seeds were germinated following the same procedure on medium lacking hygromycin. After 10 days, plantlets were transferred to pots (cultivation soil Traysubstract; Klasmann-Deilmann GmbH, Geeste, Germany) in a growth chamber at 26 ± 2°C, with a 12-h photoperiod (900 μmohn/m^2/s photosynthetically-active radiation) and 80% relative humidity. Plants were initially irrigated with a soluble Fe solution (Sequestrene 138 Fe G-100; Syngenta Agro SA, Madrid, Spain) and then

watered with tap water until seed harvest.

To measure Ca accumulation in different plant organs, 10 six-week-old plants from each transgenic line and the corresponding wild-type controls were grown in a single plastic container. Plants were watered using either tap water, or water supplemented with 2 mM or 10 mM $CaCl_2$ until seed harvest.

To measure Se accumulation and speciation, 10 six-week-old plants from each transgenic line and the corresponding wild-type controls were grown as above. Plants were regularly watered with tap water and sprayed with 200 ml of 40 mg/l Na_2SeO_4 every 2 weeks until seed harvest (six sprays in total).

To assess Se tolerance, transgenic seeds were germinated in pots containing half-strength MS medium supplemented with Se (400 μM or 50μM Na_2SeO_4), and 30 mg/l hygromycin whereas wild-type seeds were germinated using the same procedure but on medium lacking hygromycin. After 14 days, seedlings were collected and their shoots were weighed before freezing them in liquid nitrogen.

2.4.3. Expression analysis by quantitative real-time RT-PCR

Samples from plants of the same line were pooled and frozen before grinding. Total RNA was extracted from 120 mg of material as described in Section 1.4.3 and DNA was removed using the RNase-Free DNase kit (Qiagen, Hilden, Germany). RNA cleanup was carried out using the RNeasy® Plant Mini Kit (Qiagen) and 2 μg of total RNA was used for first-strand cDNA synthesis with the Omniscript® Reverse Transcription Kit (Qiagen) and a PTC200 thermal cycler (BioRad, Hercules, CA, US).

Quantitative real-time RT-PCR was carried out using a BioRad CFX96™ sequence detector system (BioRad). Triplicate PCR amplifications were carried out in 96-well optical reaction plates in 25-μl reactions comprising 12.5 μl 2X SYBR Green PCR master mix (BioRad), 1 μl of the 5 μM specific forward and reverse primers (Table 1), and 0.5 ng of the cDNA template. The amplification program started with a denaturation step at 95ºC for 3 min, followed by 40 cycles of 95ºC for 10 s, 59.4ºC for 30 s and 72ºC for 20 s. Fluorescence was measured at the end of each cycle. Amplification specificity was tested by product melt curve analysis over a

temperature gradient (50-90ºC) with fluorescence read after every 0.5ºC increase. Serial dilutions of cDNA (50–0.08 ng) were used to calculate amplification efficiency for each gene by plotting the C_t values of each dilution against the log of the starting template concentrations. The fluorescence threshold value and amplification efficiencies were calculated using the CFX96^{TM} software. Amplification efficiencies ranged from 80–100% and were always similar between the gene of interest and the reference gene (*actin*), which allowed the use of the $2^{-\Delta\Delta Ct}$ method to calculate relative expression levels (Livak and Schmittgen 2001). Values represent the mean of three replicates ± standard deviation. Two negative controls were included: (I) no enzyme in the reverse transcription reaction mixture in order to confirm the absence of genomic DNA contamination in the samples, and (II) no template in the real-time RT-PCR reaction mixture (water instead of cDNA) in order to confirm that none of the reaction components were contaminated. Neither control yielded any amplification fragments.

Table 1. *Primers used to determine transgene and endogenous Ca/Se-related gene expression levels by real-time RT-PCR.*

Mineral	Gene		Primers	PCR product
Ca	Transgenes	***AtsCAX1***	For: 5'-ATTGCCATCTGTTTCTTCGTC-3' Rev: 5'-GAAAACTCCTCCTCCTGTTGC-3'	108 bp
		AtsACA4	For: 5'-GTAGCCGTTATCCTCAAATGC-3' Rev: 5'-CAGGCAGAGTTGGAAGAAGAA-3'	98 bp
	Endogenous	***Oscax1a***	For: 5'-GCCAGCAAGACAGATCATCAT-3' Rev: 5'-CTGGGTATAGGATTGGGCACA-3'	150 bp
		Oscax1b	For: 5'-AGGATGATACCACGACCCAAC-3' Rev: 5'-CAAACCATTCCAAAACGACCT-3'	119 bp
		Oscax1c	For: 5'-AGCGACGGGAACAAGATTAC-3' Rev: 5'-GAAGATAACGCATTGACCCA-3'	144 bp
		Oscax2	For: 5'-GCATCGCCCTTTGATTGAGA-3' Rev: 5'-CCAGTTATCCAGTGTCCCTTG-3'	172 bp
		Oscax3	For: 5'-GAGCTGGCAGAATGTTGACA-3' Rev: 5'-TTACAACCACCCCTCCTCCT-3'	135 bp
		Osaca4	For: 5'-TCAGCCTGATTGTTGGTGTCA-3' Rev: 5'-GCAAGTGGCCTGTATCCATT-3'	88 bp
		Osaca11	For: 5'-ACCTGGCAGTTGTGGTTGGT-3' Rev: 5'-TTTACTTCCTGGGCAGCAA-3':	132 bp

Se	Transgene	***AtAPS1***	For: 5'-TGACAAGACGCAAGGCAAG-3' Rev: 5'-GTCTGGCGGGTTTTCGTTG-3'	115 bp
	Endogenous	***Osaps***	For: 5'-TCAGCGGACAGCAGCAAACT-3' Rev: 5'-GCTTCCAATCAAACCCAAAT-3'	176 bp
		Osapr	For: 5'-CGTGAAGGTGGGCAAGTTC-3' Rev: 5'-TTGACGAAGGCGAGGAGCGA-3'	168 bp
		Oscys	For: 5'-CTTTCGTCGGTGCTCTTCC-3' Rev: 5'-CACTGTGTTCCCCATTGCT-3'	181 bp
		Osmet	For: 5'-TGTGCGTTTCGTCTGAGTTCT-3' Rev: 5'-AACTTTGCCGCAGATTGTTT-3'	140 bp
		OsSAT	For: 5'-TTTCGAGCTGTGCTTGAACT-3' Rev: 5'-ACGACAATGTGCAGTACGGT-3'	144 bp
-	Endogenous	***Osactin***	For: 5'-GGAAGCTGCGGGTATCCATGAG-3' Rev: 5'-CCTGTCAGCAATGCCAGGGAAC-3'	179 bp

2.4.4. Determination of total mineral levels by ICP-MS

Leaf, shoot and root samples were collected at seed harvest. Root samples were washed in miliQ water to remove external mineral contamination, and seeds were dehusked by hand, and polished using a Pearlest Grain Polisher (Kett Electric Laboratory, Tokyo, Japan). Samples were ground to fine powder using mortars and pestles treated with 5% HNO_3, and were dried at 70ºC for 4 days in an oven. Then, 70 mg of root, 300 mg of shoot, 150 mg of leaf and 300 mg of seed samples were digested in 3.5 ml 70% HNO_3, 2.5 ml 30% H_2O_2 and 2 ml miliQ water using a MLS 1200 Mega high-performance microwave oven (Milestone, Sorisole, Italy). The digestion program started with 250 W for 1 min, followed by 0 W for 3 min, 250 W for 4 min, 400 W for 4 min and 600 W for 3 min. Digested samples were supplemented with miliQ water to a final volume of 20 ml, mixed and filtered through Puradisc 0.20-μm PES filter (Whatmann, Florham, NJ, US) and monitored using a 7700x ICP-MS spectrometer (Agilent Technologies, CA, US). The sample solution was quantified by external calibration by comparing the net signals against those of multi-element standards, and blank intensities were subtracted from the standard and the sample intensities. Analytical multi-element standard solutions were prepared by diluting single and multi-element stock solutions (High-Purity Standards, Charleston, US) containing 2 mg/ml Ca and Na, 500 μg/ml Mg, 100 μg/ml Fe and Zn, 50 μg/ml Se and 20 ug/ml Mn. Internal standard solution (Internal Standard Mix, Agilent

Technologies) was added online at a T-junction, located between the pump and the nebulizer. Values represent the mean of three replicates ± standard deviation.

2.4.5. Determination of selenium speciation by HPLC-ICP-MS

Samples were sent to the Speciation and Traces Determination group, Department of Analytical Chemistry, Universidad Complutense de Madrid (Spain). Selenium speciation in different samples was analyzed by HPLC-ICP-MS after the extraction of Se species following enzymatic hydrolysis. Proteolysis was carried out using two methods: incubation in a controlled temperature incubator and ultrasonic probe. In the controlled temperature incubation, 50 mg of sample were incubated with 20 mg of enzyme (Protease type XIV, Sigma-Aldrich) and 3 ml 30 mM Tris, pH 7.5 (Fluka, Sigma-Aldrich) in a Heraeus D-6450 gravity convection oven (Fischer Thermo Scientific, Wilmington, DE, US) for 24 h at 37ºC. In the ultrasonic probe method, 50 mg of samples were mixed with 20 mg Protease XIV and sonicated for 2 min at 40% ultrasound amplitude in a Sonoplus ultrasonic homogenizer (Bandenlin, Berlin, Germany) equipped with a 3-mm titanium microtip and a high-frequency generator (2200 W, 20 kHz). After proteolysis, samples were centrifuged at 11,000 rpm for 15 min using 10-kDa cut-off filters (Millipore, Billerica, MA, US). The aqueous extracts were filtered through a 0.22-μm filter before analysis by high-performance liquid chromatography (HPLC) using a PU-2089 HPLC pump (JASCO, Tokyo, Japan) fitted with a six-port injection valve (model 7725i, Rheodyne, Rohner Park, CA, US) with a 100-μl injection loop. A Hamilton PRP-X100 anion exchange column (Hamilton, Reno, Nevada, US) was used for sample separation. ICP-MS was carried out under conditions similar to those described above. Standard stock solutions of 1 g/L selenomethionine (SeMet) and selenocysteine (SeCys) (Sigma-Aldrich) were prepared in miliQ water and 3% HCl was added for better dissolution. Inorganic selenium solutions were prepared by dissolving sodium selenite (Na_2SeO_3) and selenate (Na_2SeO_4) (Merck, Darmstadt, Germany) in miliQ water.

2.4.6. Statistical analysis

Differences between transgenic and wild-type plants were tested by ANOVA and subsequent post-hoc comparison of means using the least significant difference (LSD) test ($P < 0.05$).

2.5. Results

2.5.1. Effect of *AtsCAX1* and *AtsACA4* expression

2.5.1.1. Mineral accumulation in plant organs

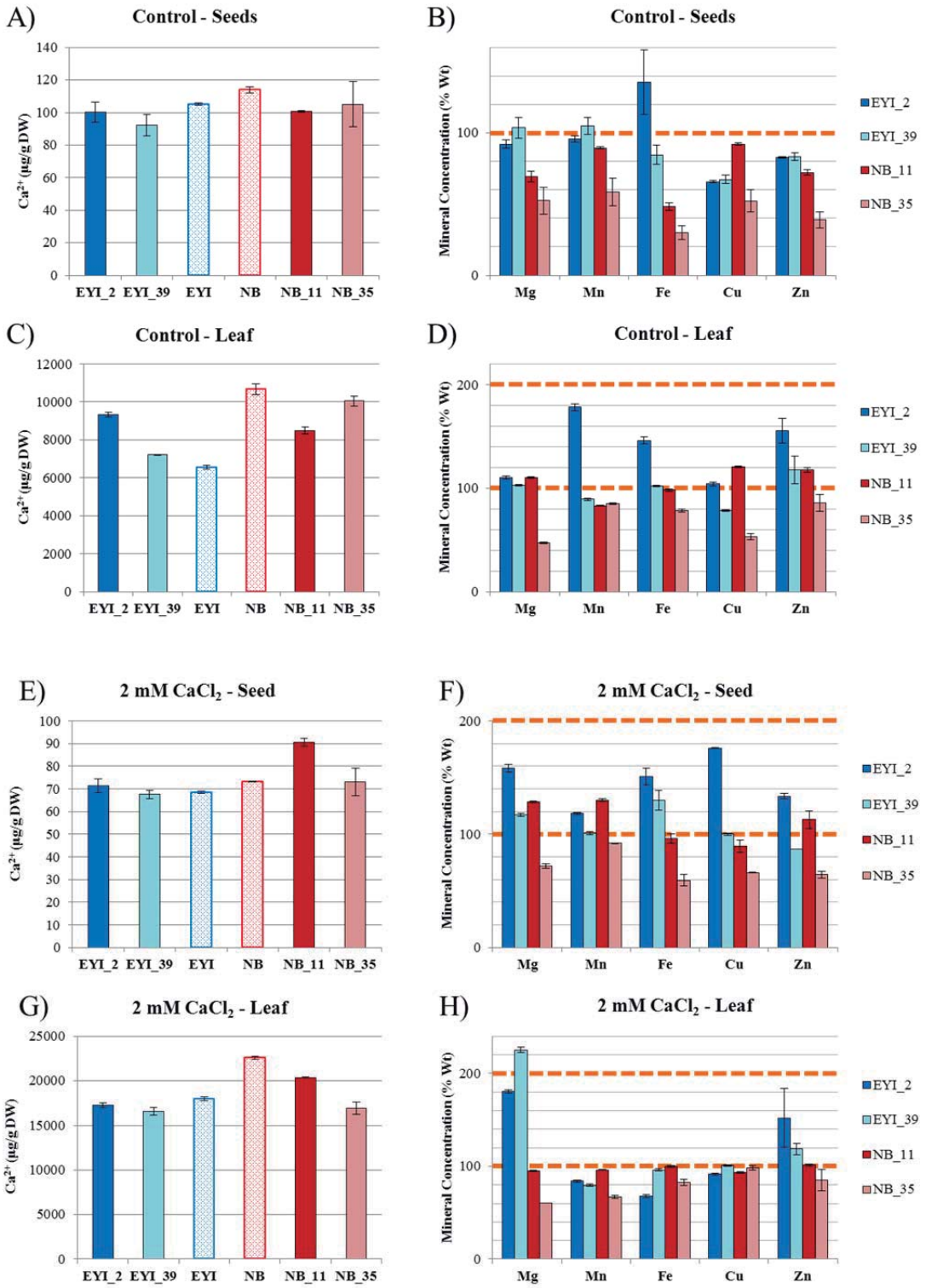
To ascertain whether the expression of *AtsCAX1* and *AtsACA4* could boost mineral accumulation, the levels of Ca^{2+} and other ions (Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}) were measured in transgenic lines 2, 11, 35 and 39. A preliminary evaluation was conducted with transgenic T_1 plants growing under normal conditions. No significant increases in the levels of Ca^{2+} or any other mineral were found in the seeds and leaves of lines 11, 35 and 39, but line 2 showed a 40% increase in Ca^{2+} accumulation in leaves, as well as enhanced levels of Mn^{2+}, Fe^{2+} and Zn^{2+} (Figures 1A-D).

The transgenic lines were then grown in the presence of 2 mM $CaCl_2$ and mineral levels were analyzed in seeds, leaves, shoots and roots. Although a 20% increase in Ca^{2+} was detected in the seeds of line 11, Ca^{2+} levels in other plant organs did not exceed that of the wild-type in any of the transgenic lines (Figures 1E-L). In contrast, other minerals accumulated to higher levels in certain organs of all four transgenic lines, particularly in line 2, which consistently showed 1.6–2-fold increases in Mg^{2+} levels in all organs (Figures 1E-L). This suggested the transgenic lines may have a higher ion storage capacity, although this was not reflected in the levels of Ca^{2+}.

I hypothesized that higher Ca^{2+} accumulation in the transgenic lines may occur only in the presence of excess environmental Ca, so the plants were grown in the presence of 10 mM $CaCl_2$. Both the transgenic and wild-type plants were severely affected by Ca toxicity, and most of the vegetative tissues were dead by seed harvesting time, so mineral levels were only measured in seeds. Lines 11 and 35 showed 30% and 50% increases in Ca^{2+} levels, respectively, whereas there was no difference in lines 2 and 39 (Figure 1M). Nevertheless, as in previous experiments, the accumulation of other minerals was superior in line 2, which contained 40% more Mg^{2+}, Cu^{2+} and Zn^{2+} that wild-type plants (Figure 1N).

There were slight differences in the Ca^{2+} levels detected in wild-type EYI-105 and Nipponbare plants. In the presence of normal calcium levels and 2 mM $CaCl_2$, Nipponbare plants tended to accumulate more Ca^{2+}, especially in the leaves (Figures 1A-L), whereas excess Ca caused more Ca^{2+} to accumulate in EYI-105 seeds (Figures

1M-N).


A)
Control - Seeds
Ca2+ (µg/g DW)
EYI_2 EYI_39 EYI NB NB_11 NB_35
B)
Control - Seeds
Mineral Concentration (% Wt)
Mg Mn Fe Cu Zn
EYI_2
EYI_39
NB_11
NB_35
C)
Control - Leaf
D)
Control - Leaf
E)
2 mM CaCl2 - Seed
F)
2 mM CaCl2 - Seed
G)
2 mM CaCl2 - Leaf
H)
2 mM CaCl2 - Leaf

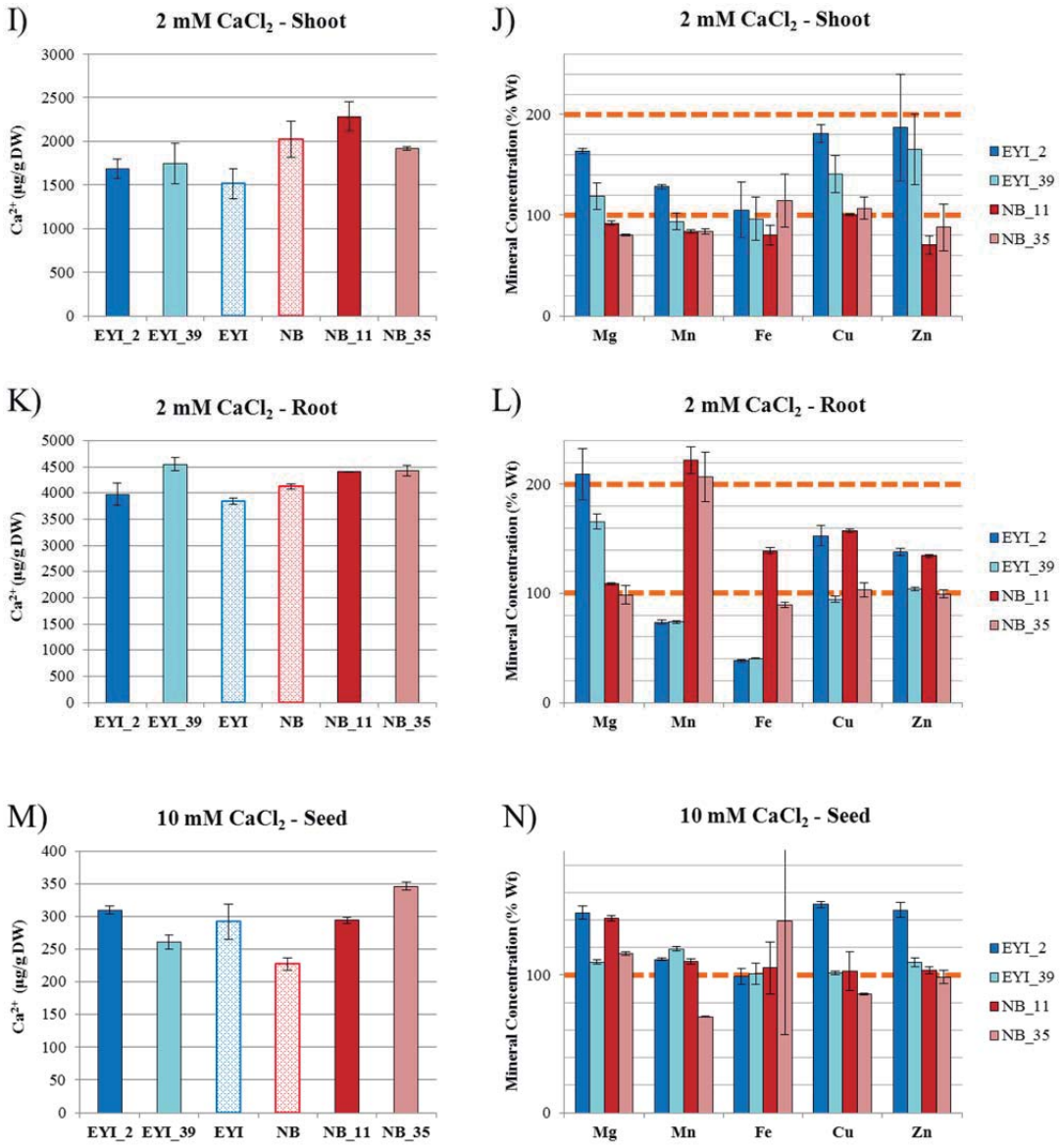


Figure 1. *Ion concentrations in different tissues of wild-type rice plants and transgenic lines 2, 11, 35 and 39, growing under normal conditions (A-D), and in the presence of 2 mM (E-L) and 10 mM (N and M) $CaCl_2$. Panels on the left show the relative mineral accumulation in each transgenic line with respect to the corresponding wild-type variety. Values are the mean of three replicates ± SD.*

2.5.1.2. Real-time RT-PCR analysis of Ca transporter genes

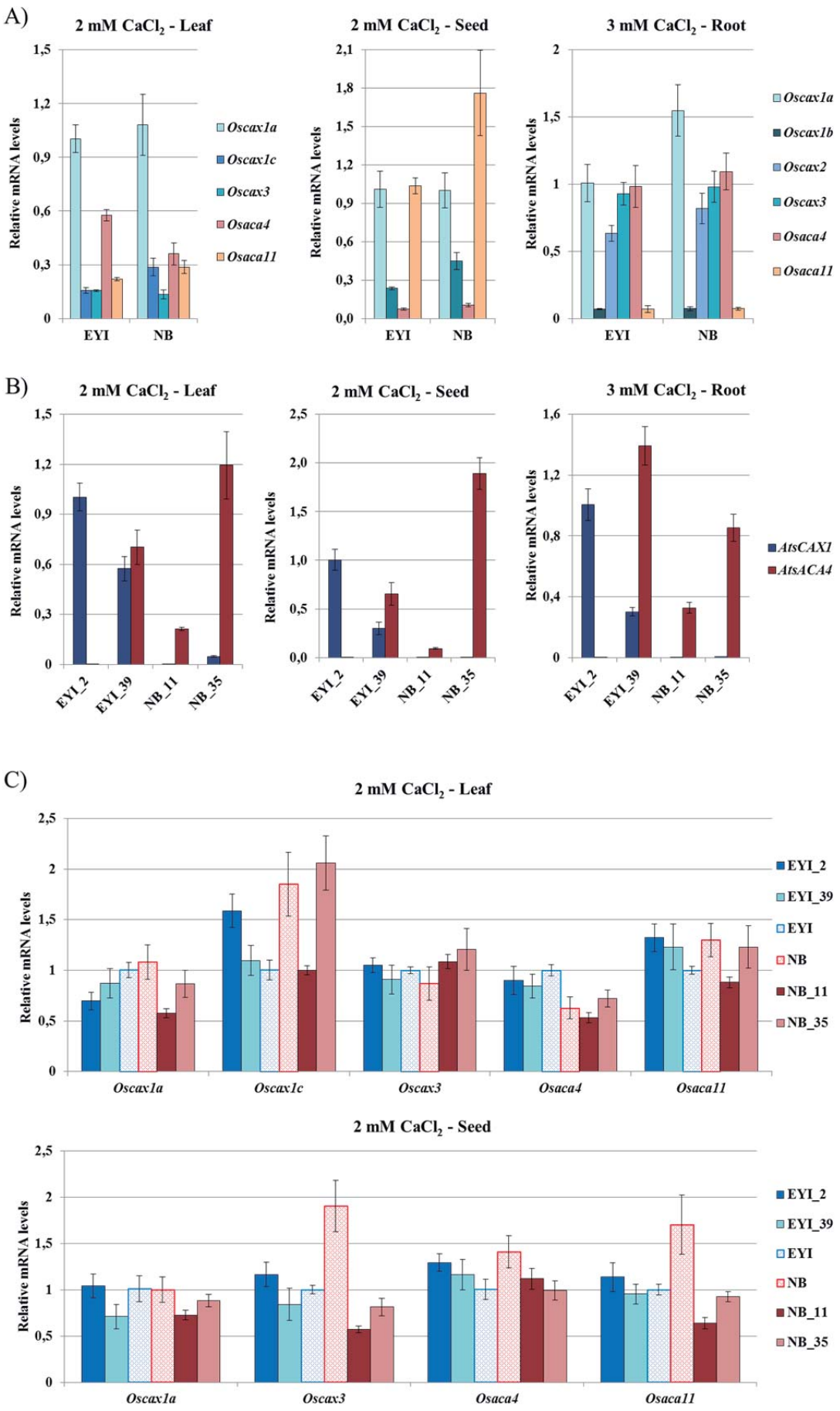
Calcium accumulation in transgenic plants did not increase as expected (Park et al. 2004, 2005a, 2005b, 2009). This may reflect compensatory responses to the altered CAX and ACA activities in the transgenic plants, which have been described in several CAX-deficient mutants (Manohar et al. 2011a). In order to determine whether the expression of endogenous Ca^{2+} transporters was downregulated at the mRNA

level in the transgenic lines, the transcripts of *Oscax1a*, *Oscax1c* and *Oscax3* vacuolar antiporters, and *Osaca4* and *Osaca11* vacuolar Ca^{2+}-ATPases were quantified by real-time RT-PCR in the leaves and immature seeds of plants growing in the presence of 2 mM $CaCl_2$. In addition, transgene expression was also measured to complete the analysis of vacuolar Ca transporter expression in each line.

The expression profile of vacuolar Ca transporters in Nipponbare and EYI-105 varieties, as well as transgene expression in the four transgenic lines, is presented in Figures 2A-B. Similar expression patterns were observed in both wild-type varieties in the leaves and seeds. As expected, expression profiles differed between tissues: *Oscax1c* was not expressed in seeds (Kamiya et al. 2005) whereas *Osaca11* was strongly expressed. Transgene expression in the four transgenic lines matched with the mRNA blots described in Chapter 1, except that the *AtsCAX1* transcript appeared to be absent in line 11, and was expressed at extremely low levels in line 35 (Figure 2B). The use of alternative primers targeting an upstream fragment of the *AtsCAX1* transcript yielded the same results (data not shown).

Expression of the different vacuolar Ca transporters in each transgenic line is compared to the wild-type varieties in Figure 2C. The expression levels of all five endogenous genes in the leaves of lines 35 and 39 were similar to wild-type levels. In contrast, line 2 showed a 50% increase in *Oscax1c* expression, whereas the expression of *Oscax1a* and *Oscax1c* was downregulated by 50% in line 11. The expression of all the Ca transporters was unaltered in the seeds of lines 2 and 39, whereas in lines 11 and 35 *Oscax3* and *Osaca11* expression was reduced by 50–70%.

The expression of endogenous genes and transgenes encoding several Ca transporters was also analyzed in roots of seedlings growing in the presence of 3 mM $CaCl_2$. Line 2 showed a 30–70% increase in *Oscax1a*, *Oscax2*, *Oscax3*, *Osaca4* and *Osaca11* expression, whereas line 11 showed a 50% increase in *Osaca11* expression. In contrast, all endogenous Ca transporters were downregulated by 30–50% in line 39, whereas the main CAX antiporters were downregulated in line 35 (Figure 2C).


A)
2 mM CaCl2 - Leaf
2 mM CaCl2 - Seed
3 mM CaCl2 - Root
Relative mRNA levels
EYI
NB
Oscax1a
Oscax1b
Oscax1c
Oscax2
Oscax3
Osaca4
Osaca11
B)
2 mM CaCl2 - Leaf
2 mM CaCl2 - Seed
3 mM CaCl2 - Root
Relative mRNA levels
EYI_2
EYI_39
NB_11
NB_35
AtsCAX1
AtsACA4
C)
2 mM CaCl2 - Leaf
2 mM CaCl2 - Seed
Relative mRNA levels
Oscax1a
Oscax1c
Oscax3
Osaca4
Osaca11
EYI_2
EYI_39
EYI
NB
NB_11
NB_35

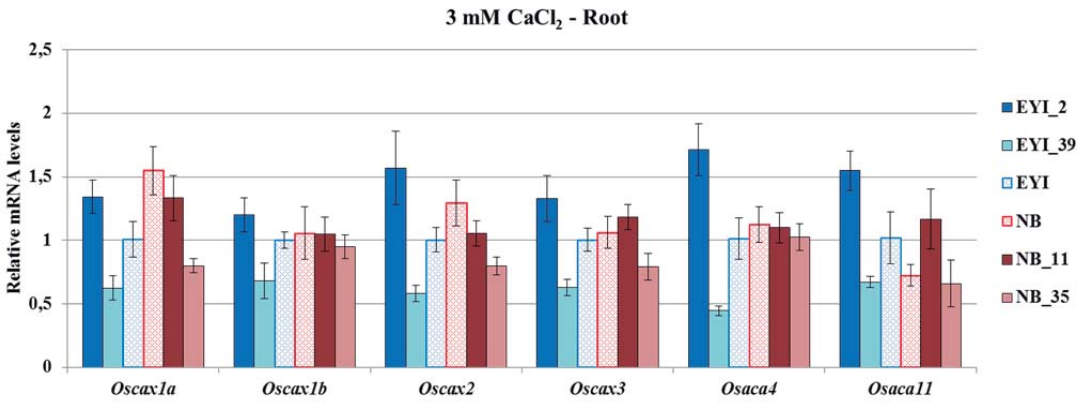


Figure 2. *Real-time RT-PCR analysis of vacuolar Ca transporters in different tissues of rice plants. A) Expression profile of endogenous Ca transporters in EYI-105 (EYI) and Nipponbare (NB) wild-type varieties. B) AtsCAX1 and AtsACA4 expression levels in transgenic lines 2, 11, 35 and 39. C) Expression levels of endogenous Ca transporters in each transgenic line compared to the wild-type. In A) and B) values were normalized against* Oscax1 *levels in EYI for each tissue, whereas in C) values for each gene were normalized against their expression in EYI. Actin was used as housekeeping gene. Values are the mean of three replicates ± SD.*

2.5.2. Effect of *AtAPS1* expression

In Chapter 1, I described the generation of 11 fertile transgenic rice lines expressing *AtAPS1*, but some of these were not included in the experiments described in this chapter. When I conducted the experiments to evaluate Se tolerance and speciation, only six of the transgenic lines produced enough viable seed for analysis, whereas the remaining five transgenic lines were included in later experiments which are currently ongoing. In contrast, the expression of Se-related genes was analyzed when enough seeds had been collected for all transgenic lines except line 82, which could only be included in the second Se speciation experiment. Line 11 was excluded from the expression analysis experiment because the transgene did not affect Se speciation (see Section 2.5.2.2).

2.5.2.1. Selenium tolerance

To evaluate whether the overexpression of *AtAPS1* in rice plants affected their Se tolerance, transgenic lines and wild-type controls were grown in half-strength MS supplemented with 400 μM Na_2SeO_4 for 2 weeks and the shoot weight was measured. The growth of both wild-type and transgenic seedlings was stunted compared to the

control treatment (data not shown), but there were no significant differences between wild-type and transgenic lines except for line 39, which showed reduced growth compared to EYI-105 (Figure 3A). The strong toxicity symptoms observed in all the lines suggested excess Se levels may obscure potential differences in Se tolerance, therefore I repeated the experiment using 50 μM Na_2SeO_4. The differences between the Se and control treatments were less pronounced than in the previous experiment (data not shown), and significantly reduced shoot weight was observed when lines 5, 28, 37 and 39 were compared to their corresponding wild-type variety (Figure 3B). Analysis of Se and sulfur levels in the seedlings exposed to 50 μM Na_2SeO_4 revealed that transgenic lines 5, 11, 28, 37 and 39 accumulated significantly lower levels of sulfur and Se than wild-type seedlings (Figures 3C-D).

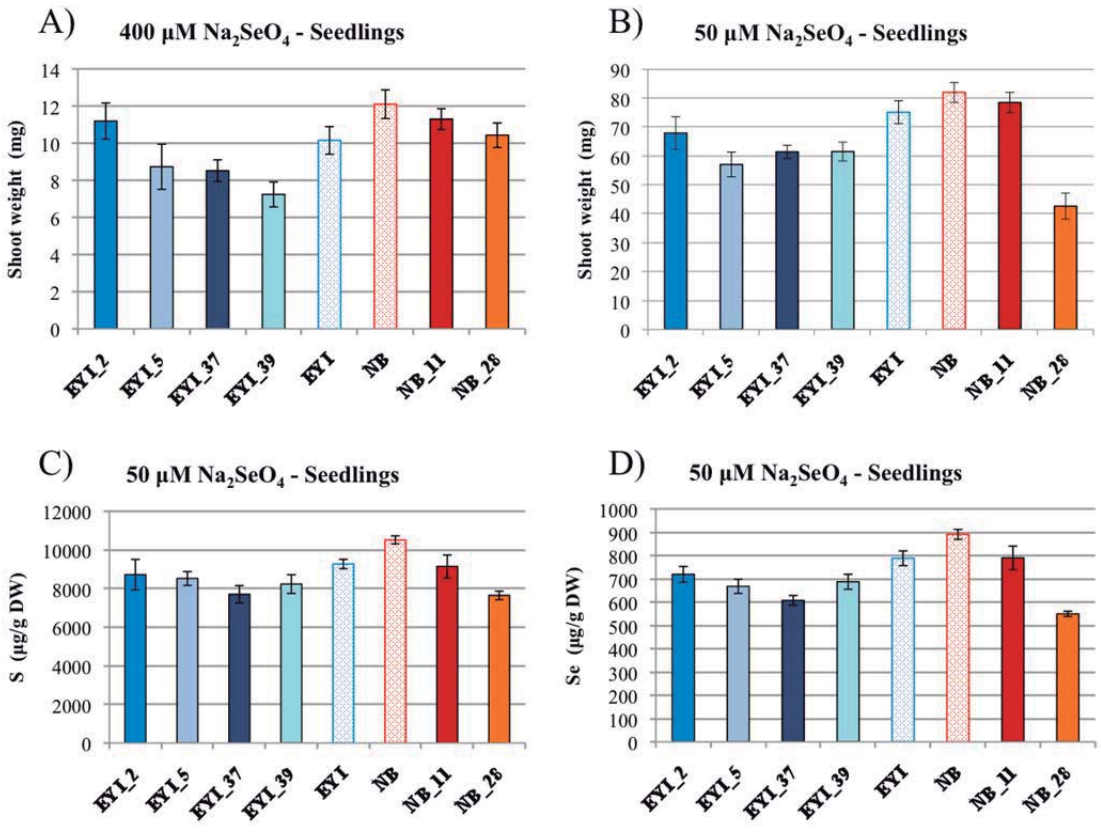


Figure 3. *Shoot weight of wild-type (EYI and NB) and* AtAPS1*-expressing rice lines growing on (A) 400 μM and (B) 50 μM selenate. Sulfur (C) and Se (D) levels were analyzed in seedlings growing on 50 μM of selenate. Values are the mean of ten seedlings ± SE.*

2.5.2.2. Selenium accumulation and speciation in transgenic rice plants

Transgenic rice seedlings expressing *AtAPS1* accumulated less Se than wild-type

plants, so it was necessary to test whether the Se content of the seeds was also lower in the transgenic lines. Selenate reduction in plants results in the incorporation of Se into organic compounds such as the amino acids SeCys and SeMet (Sors et al. 2005b). *AtAPS1* expression in rice could therefore potentially modify the ratio between inorganic and organic Se. The accumulation and speciation of Se in 8-week-old EYI-105 and Nipponbare wild-type plants and transgenic lines 2, 5, 11, 28, 37 and 39 was analyzed by growing the plants under normal conditions and spraying them with 230 μM Na_2SeO_4 every 2 weeks until seed harvest. The total Se and sulfur levels, and the proportions of selenate, selenite, SeCys and SeMet were then measured in the seeds and leaves.

Sulfur levels in the leaves of all six transgenic rice lines did not differ significantly from wild-type levels. In seeds, there were no significant differences in sulfur content between EYI-105 and lines 2, 5, 37 and 39, but lines 11 and 28 showed a 40% increase in the concentration of sulfur compared to Nipponbare wild-type seeds (Figure 4A). The total Se content in the leaves of lines 5, 37, 39, 11 and 28 was similar to wild-type levels, but there was a significantly lower concentration of Se in the leaves of line 2. Se accumulation in seeds was unaffected in lines 2, 11, 37 and 39, whereas lines 5 and 28 contained 30–40% more Se than wild-type seeds (Figure 4A). However, these increases did not affect Se speciation, which was the same in the seeds of all transgenic and wild-type lines (Figure 4B). As expected, most of the Se stored in seeds was assimilated in organic compounds such as SeCys and SeMet, which accounted for more than 95% of the total (Sun et al. 2010). In contrast, almost 70% of the Se in the leaves was present as selenate in both wild-type varieties, and the rest was assimilated into SeMet and to a lesser extent SeCys, whereas selenite was not detected (Figure 4B). Interestingly, transgenic lines 2, 5, 28, 37 and 39 contained greater proportions of organic Se, which accounted for 40–50% of total Se in the leaves, mainly in the form of SeMet. Inorganic Se was present as selenate, whereas selenite could not be detected. In contrast, line 11 showed the same speciation pattern as wild-type plants (Figure 4B) reflecting the absence of the full-length *AtAPS1* transcript in northern blots (Chapter 1).

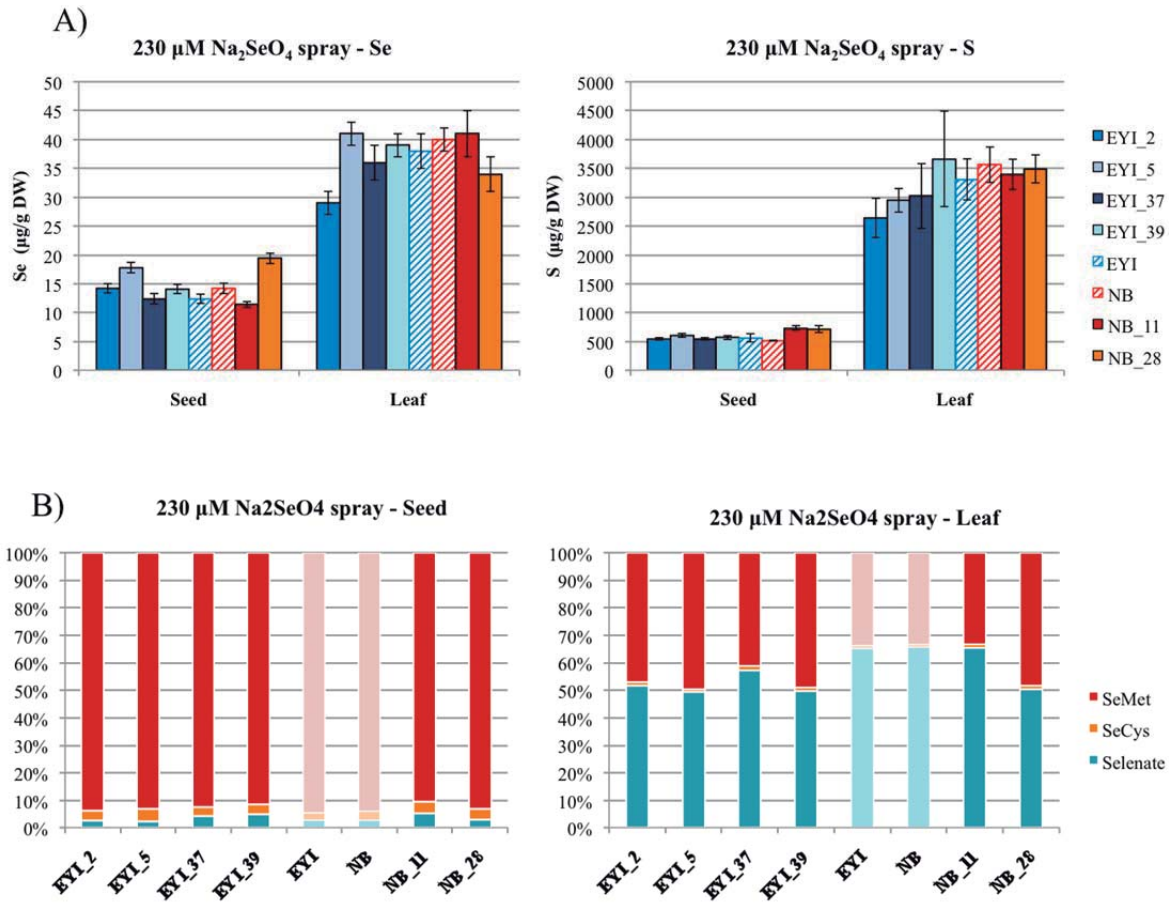


Figure 4. *Selenium and sulfur concentrations (A) and Se speciation (B) in seeds and leaves of wild-type and transgenic lines 2, 5, 11, 28, 37 and 39 sprayed with 230 µM selenate. Values are the mean of three replicates ± SD. SeMet: selenomethionine; SeCys: selenocysteine.*

2.5.2.3. Real-time RT-PCR analysis

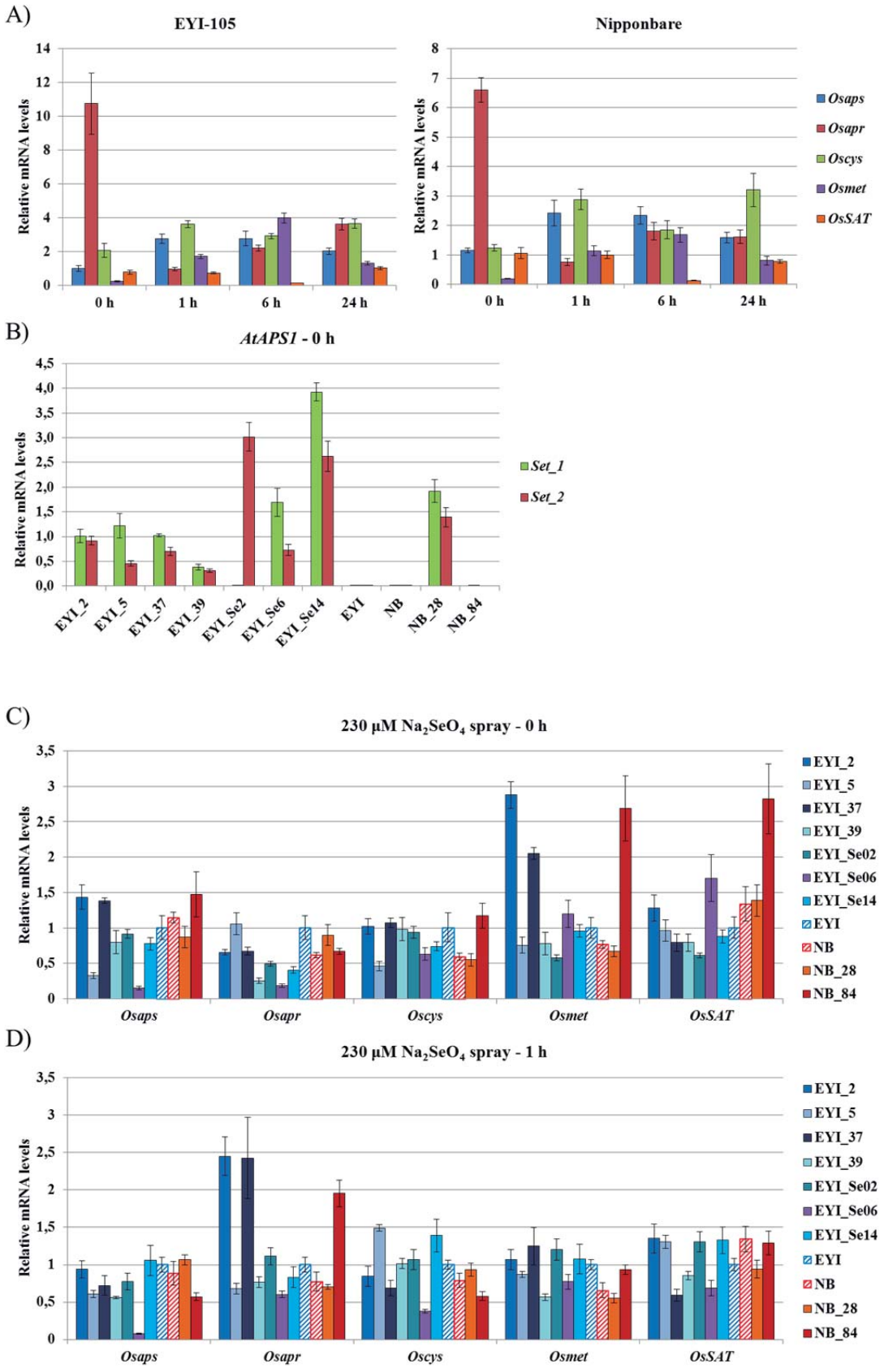
Speciation analysis revealed a correlation between *AtAPS1* expression and the enhanced assimilation of selenate into SeMet in leaves. This process involves several enzymatic steps that may be promoted in transgenic lines. In order to determine whether transgene expression influenced the key endogenous genes in the Se assimilation pathway, 8-week-old plants were sprayed with 230 µM Na_2SeO_4 and leaf samples were collected at 0, 1, 6 and 24 h after spraying. The expression of *AtAPS1*, *Osaps*, *Osapr*, *Oscys*, *Osmet* and *OsSAT* was monitored by real-time RT-PCR. The two wild-type varieties and transgenic lines 2, 5, 28, 37, 39, 84, Se2, Se6 and Se14 were included in this experiment.

Transgene expression was measured in samples collected before spraying (Figure 5B). As observed in the northern blot presented in Chapter 1, *AtAPS1* expression levels differed between the lines, with line Se14 showing the highest expression levels. However, the transgene was not detected in lines Se2 and 84, therefore another set of primers targeting an upstream fragment of the transcript was used. High levels of the amplification product were detected in line Se2 but no product was detected in line 84. Interestingly, the second set of primers reduced the amplification of the *AtAPS1* transcript in all transgenic lines (Figure 5B). Transgene expression before and after Se treatment was similar in each line (data not shown).

The expression of the five endogenous genes following exposure to the Se foliar spray was similar in both wild-type varieties (Figure 5A). Before Se treatment, *Osapr* expression levels were 5–10-fold higher than those of *Osaps*, *Oscys* and *OsSAT*, whereas *Osmet* was expressed at basal levels. One hour after Se treatment, *Osapr* expression dropped sharply whereas *Osaps* and *Oscys* transcript levels increased by 2–3-fold, and *Osmet* expression was induced by 6–7-fold. In contrast, *OsSAT* expression remained stable. Five hours later, moderate increases in *Osapr* and *Osmet* expression and a slight reduction in *Osaps* and *Oscys* expression resulted in similar transcript levels for all four genes, whereas *OsSAT* expression was reduced to basal levels. Eighteen hours later, *OsSAT* expression was restored and *Osaps* and *Osmet* levels were reduced, whereas *Osapr* and *Oscys* expression either increased or remained stable.

Osaps, *Osapr*, *Oscys*, *Osmet* and *OsSAT* expression levels in each transgenic line were compared to wild-type levels at each time point (Figures 5C-F). The results are summarized in Table 2. In all transgenic lines and all time points, the selenate assimilation pathway was controlled by downregulating one or more genes (except for line 84 before treatment) although one or more steps of the pathway were also upregulated, especially before and 1 h after Se treatment. Different trends were detected in the transgenic lines derived from EYI and NB cultivars. In most of the EYI transgenic lines, the selenate assimilation pathway was progressively downregulated compared to wild-type plants in response to Se treatment, and all lines showed the downregulation of either four or five genes 24 h after Se application. In contrast, downregulation did not increase after Se treatment in the NB transgenic

lines, and only one or two genes were downregulated compared to wild-type 24 h after Se application.



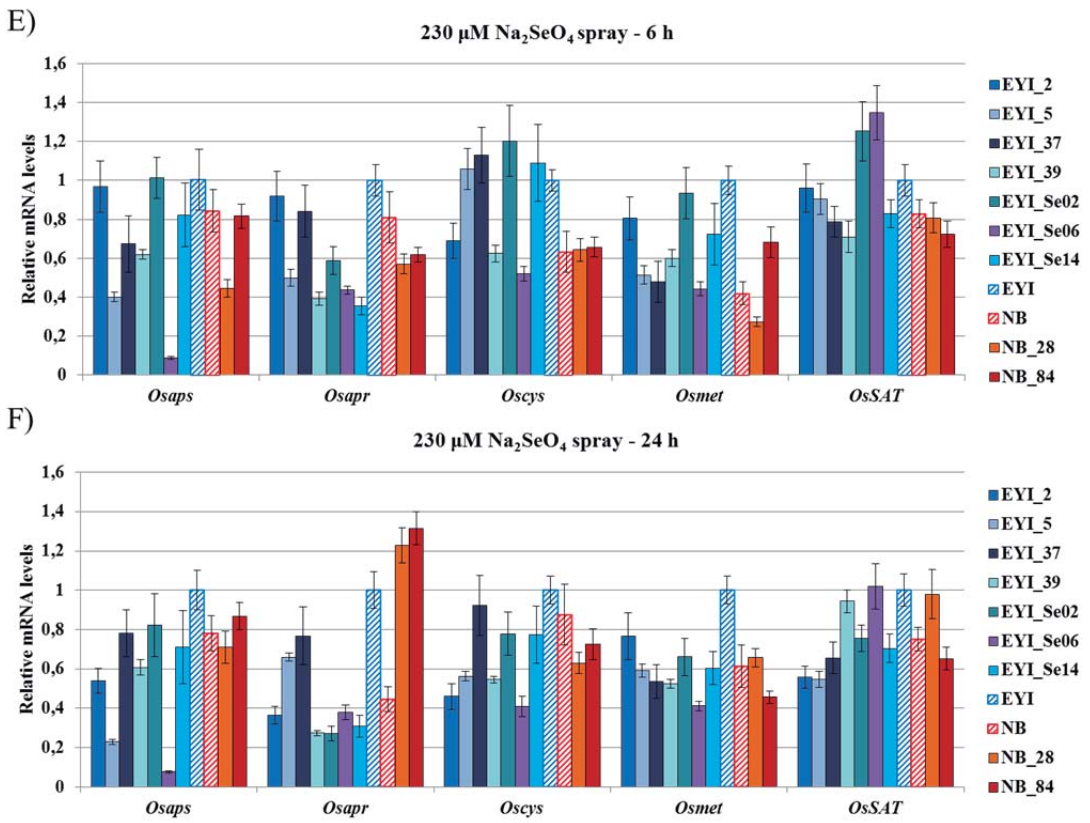


Figure 5. *Real-time RT-PCR analysis of genes involved in the Se assimilation pathway in the leaves of 8-week-old plants. A) Expression levels of five endogenous genes before and 1 h, 6 h and 24 h after Se spraying in the two wild-type varieties. B)* AtAPS1 *expression in transgenic lines 2, 5, 28, 37, 39, 84, Se2, Se6 and Se14 before spraying using two different sets of primers. C-F) Expression levels of the five endogenous genes in the transgenic lines compared to wild-type at each time point. In A) values were normalized against* Osaps *in EYI at 0 h, whereas in B) values were normalized against* AtAPS1 *in line 2. In C-F) values were normalized against EYI for each gene. Actin was used as housekeeping gene. Values are the mean of three replicates ± SD.* Osaps*: ATP sulfurylase;* Osapr*: adenosine 5'phosphosulfate reductase;* Oscys*: OAS(thiol)-lyase (cysteine synthase);* Osmet*: methionine synthase;* OsSAT*: serine acetyltransferase; Set_1: Downstream fragment of* AtAPS1*; Set_2: Upstream fragment of* AtAPS1.

Table 2. *Summary of expression analysis data for genes involved in the Se assimilation pathway (Figures 5C-F). Colors represent expression levels compared to wild-type. Blue: upregulation; Red: downregulation; Pale green: unchanged.*

	0 h	1 h	6 h	24 h
2	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT
5	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT
37	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT
39	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT
Se2	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT
Se6	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT
Se14	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT
28	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT
84	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT	APSAPRCysMet SAT

2.6. Discussion

In Chapter 1, I described the generation of 12 transgenic rice lines overexpressing *AtsCAX1* and *AtsACA4*, which encode vacuolar Ca transporters, and *AtAPS1*, which encodes the first enzyme in the sulfate/selenate assimilation pathway. The main objective of this experiment was to generate rice plants with enhanced capacity to accumulate Ca and Se in the grain as a biofortified staple crop. The next step was therefore to evaluate whether transgene expression was translated into increased mineral accumulation in transgenic plants. In addition, the influence of the transgenes on the corresponding mineral accumulation pathways was investigated to provide a mechanistic basis for the impact of transgene expression on the capacity of the plants to accumulate minerals.

2.6.1. Effect of *AtsCAX1* and *AtsACA4* expression

AtsCAX1 has successfully been used in previous studies to generate Ca-enriched crops (Table 3). Initially, tobacco was used to evaluate the effects of *AtsCAX1* overexpression *in planta*, and increases in Ca^{2+} content were reported in roots and leaves (Hirschi 1999). The transgenic tobacco plants also displayed strong phenotypic

changes such as necrotic lesions, chlorotic leaves and stunted root formation, contrasting with the more subtle phenotype observed in *AtsCAX1*-expressing *Arabidopsis* plants (Cheng et al. 2003). Therefore, tobacco was established as a model plant for the assessment of CAX function by phenotypic analysis (Mei et al. 2007; Manohar et al. 2011b). Carrot was the first edible crop to be transformed with *AtsCAX1*, and transgenic plants accumulated 50% more Ca^{2+} in roots than wild-type plants (Park et al. 2004). Further studies confirmed that total Ca^{2+} absorption increased in humans consuming *AtsCAX1*-expressing carrots compared to controls, demonstrating that bioavailable Ca^{2+} was effectively increased in transgenic plants (Morris et al. 2008). *AtsCAX1* was expressed in parallel under the control of CaMV35S promoter and a weaker promoter (AtCDC2a) in potato and lettuce (Park et al. 2005b, 2009). No difference in Ca^{2+} accumulation was detected between the two approaches in transgenic lettuce plants, whereas the use of CaMV35S in transgenic potato plants resulted in higher Ca^{2+} accumulation in tubers than in CDC2a plants. No concomitant increases in other minerals were detected in either the transgenic lettuce or potato plants (Park et al. 2005b, 2009). The CDC2a promoter was also used to express *AtsCAX1* in tomato, whereas *AtCAX4* was expressed using the CaMV35S promoter (Park et al. 2005a). Calcium accumulation was higher in tomatoes expressing *AtsCAX1* compared to *AtCAX4*, although increases in other minerals were also evident. Finally, *AtCAX1* containing the N-terminal auto-inhibitory domain that mitigates CAX1 activity *in vivo* (Mei et al. 2007) was expressed in rice plants, but the Ca content of the transgenic plants was not analyzed (Kim et al. 2005). In contrast to all the case studies reported for *AtsCAX1*, the heterologous expression of *AtsACA4* has not yet been used to increase Ca levels in plants.

The expression of *AtsCAX1* and *AtsACA4* in rice plants did not result in the anticipated increase in Ca^{2+} accumulation (Figure 1). *AtsCAX1* expression was low or absent in lines 11 and 35, whereas lines 2 and 39 expressed moderate to high levels of the transgene product. Line 11 expressed low levels of *AtsACA4* whereas lines 35 and 39 expressed this transgene at high levels (Figure 2B). Therefore, the actual population of transgenic lines in which Ca^{2+} accumulation was evaluated consisted of one line expressing *AtsCAX1* (line 2), one line expressing *AtsCAX1* and *AtsACA4* (line 39), and two lines expressing *AtsACA4* (lines 11 and 35).

Mineral accumulation was initially evaluated in plants growing under normal conditions (Figures 1A-D). Nipponbare wild-type plants accumulated more Ca^{2+} in leaves and seeds than EYI-105, a trend that was repeatedly observed during the mineral analysis experiments. Lines 11, 35 and 39 showed similar or lower Ca^{2+} levels when compared to the wild-type. In contrast, line 2 accumulated 40% more Ca^{2+} in leaves, although this was not observed in plants exposed to 2 mM $CaCl_2$. This concentration of $CaCl_2$ was used in previous studies in order to compensate the Ca deficiency symptoms that appeared in *AtsCAX1*-expressing plants, and mineral quantification in those studies were carried out with plants exposed to 2 mM $CaCl_2$ (Hirschi 1999; Park et al. 2004, 2005a, 2005b, 2009). In order to reproduce these conditions even though none of the transgenic rice lines appeared to suffer from Ca deficiency, I decided to grow the plants in the presence of 2 mM $CaCl_2$ before analyzing the mineral content. With the exception of a moderate (30%) increase in the Ca^{2+} content in seeds of line 11, no improvement in Ca^{2+} levels was observed in any tissue in any of the transgenic lines (Figures 1E-L). However, the seeds of lines 11 and 35 accumulated more Ca^{2+} than Nipponbare wild-type plants when grown in the presence of toxic levels (10 mM) of $CaCl_2$ (Figure 1M). It is remarkable that under these conditions, wild-type Nipponbare seeds accumulated less Ca^{2+} than wild-type EYI-105 seeds, thus reversing the trend observed thus far (Figure 1A-L). This suggests that the higher levels of Ca^{2+} in lines 11 and 35 may be artefacts caused by the abnormally low Ca^{2+} levels in wild-type Nipponbare seeds. Alternatively, the overexpression of *AtsACA4* in lines 11 and 35 may promote the storage of excess Ca^{2+} in the vacuoles, because previous studies suggested that ACA4 and its ortholog PCA1 may mediate the response to salt stress (Geisler et al. 2000; Qudeimat et al. 2008). However, if this is the case then increased Ca^{2+} accumulation should have been observed in the seeds of line 39, which also expressed *AtsACA4*.

The accumulation of Mg, Mn, Fe, Cu and Zn was measured because these minerals accumulated to higher levels in tobacco and tomato plants expressing *AtsCAX1*. Enhanced storage of other minerals was consistently observed in line 2 and to a lesser extent in lines 11 and 39 (Figure 1), which contrasted with the unaltered levels of Ca^{2+} in these transgenic lines. I speculate that these results were not the direct effect of *AtsCAX1* and *AtsACA4* expression, but instead they were a consequence of perturbed Ca homeostasis because of increased vacuolar Ca^{2+} transport. The *Arabidopsis cax1*

mutant was characterized by the increased expression and activity of other transporters (Cheng et al. 2003), which suggests that the overexpression of de-regulated CAX1 in rice cells could induce the downregulation of several endogenous Ca transporters, hence no overall change in Ca^{2+} accumulation. Perturbation of $[Ca^{2+}]_{cyt}$, which is predicted to occur when vacuolar Ca transport is enhanced/repressed, may affect a plethora of other nutrient transporters, altering the levels of such nutrients (Cheng et al. 2005). Accordingly, the accumulation of other minerals in transgenic rice plants would mainly depend on the particular alterations in Ca metabolism generated by the specific sCAX1 and sACA4 activity in each line. This would explain why transgenic lines expressing the same transgenes overaccumulate different minerals, as well as the differences in mineral accumulation between different crops overexpressing the same *AtsCAX1* gene (Table 3).

In order to test these hypotheses, the mRNA levels corresponding to several endogenous Ca transporters were measured in different organs of transgenic and wild-type plants (Figure 2). Rice plants express five CAX-family transporters: OsCAX1a, OsCAX1b, OsCAX1c, OsCAX2 and OsCAX3, which show different tissue-specific expression profiles (Kamiya et al. 2005). For each plant organ, I determined the mRNA levels of the CAX-family transporters known to be expressed in those organs. OsACA4 and OsACA11, the only two vacuolar Ca^{2+}-ATPases identified thus far (Pittman 2011), were measured in all organs. The CAX expression profile in each organ was consistent with previous reports, except for the lower-than expected expression of OsCAX1c in the leaves (Kamiya et al. 2005). As expected, each ACA had a distinct expression pattern, possibly reflecting different functions (Figure 2A).

In the leaves and seeds of plants grown in the presence of 2 mM $CaCl_2$, gene expression was mostly similar in the wild-type and transgenic lines (Figure 2C). Although some changes were observed, they were not correlated with mineral levels in each line. However, most transporters were overexpressed in the roots of line 2, including OsCAX2 and OsCAX3 (Figure 2C). These two transporters are grouped in the same cluster as AtCAX2, which has a low affinity for Ca^{2+} and has been reported to transport Mn^{2+} and other metals (Kamiya et al. 2005; Hirschi et al. 2000). OsCAX2 also showed low Ca transport activity, and OsCAX3 was proposed to transport Mn^{2+} *in planta* (Kamiya et al. 2005). Higher levels of several endogenous metal transporters

in the roots of line 2 could result in the enhanced accumulation of different minerals as observed (Figure 1).

The unchanged Ca^{2+} levels in the transgenic lines did not reflect a coordinated downregulation of endogenous Ca^{2+} transporter mRNA levels, but most CAXs and ACAs are heavily regulated at the post-translational level (Pittman 2011), so their activity could be downregulated in the transgenic lines despite the unchanged abundance of mRNA. Analyzing Ca^{2+}-ATPase and H^+/Ca^{2+}-exchange activities in transgenic and wild-type rice plants would therefore confirm whether endogenous Ca^{2+} transport is downregulated as a result of *AtsCAX1* and *AtsACA4* expression.

In Chapter 1, I described the difficulties encountered when generating transgenic rice plants overexpressing *AtsCAX1* and *AtsACA4*. I hypothesized that excessive CAX and ACA activity could disrupt $[Ca^{2+}]_{cyt}$ and cause negative selection pressure for the regeneration of transgenic lines expressing these transgenes. The higher number of rice lines expressing *AtAPS1* regenerated in parallel to the four lines expressing the two Ca transporters supports this hypothesis. None of the four transgenic lines evaluated in this study showed significant and consistent Ca^{2+} accumulation in any tissue. Taken together, these data suggest that Ca metabolism in rice is tightly regulated and recalcitrant to modulation through genetic engineering, so the overexpression of de-regulated Ca transporters should be replaced by or complemented with alternative approaches to attenuate excess Ca^{2+} transport activity.

Table 3. *Use of CAX1 and other antiporters for Ca biofortification in different crops.*

Species	Promoter-Gene	[Ca^{2+}] (Increase vs. wild-type)			Increase in other minerals	Fertilty	Reference
		Root/Tuber	Leaf	Fruit/Seed			
Tobacco (*Nicotiana tabacum*)	*35S:sCAX1*	35 mg/g DW (**2**)	20 mg/g DW (**1.3**)	ND	**P, K, Mg, Mn, Fe, Cu, Zn**	15%	Hirschi (1999) Mei et al. (2007)
Carrot (*Daucus carota*)	*35S:sCAX1* *cdc2a:sCAX1*	5 mg/g DW (**1.5**) 5 mg/g DW (**1.5**)	ND ND	ND ND	NO (*Mn, Fe, Cu, Zn analyzed*)	>90%	Park et al. (2004)
Potato (*Solanum tuberosum*)	*35S:sCAX1* *cdc2a:sCAX1* *35S:CAX2b*	1.5 mg/g DW (**3**) 1 mg/g DW (**2**) 1.5 mg/g (**1.7**)	10 mg/g DW (**1.5**) 10 mg/g DW (**1.5**) 1.5 mg/g (**1**)	ND ND ND	NO (*Mn, Fe, Cu, Zn analyzed*)	>90%	Park et al. (2005b)
Tomato (*Lycopersicon esculentum*)	*cdc2a:sCAX1* *35S:CAX4*	ND	(**1.2**) ND	4.5–5 mg/g DW (**2**) 1.8 mg/g DW (**1.5**)	**Mg, Mn, Fe, Cu, Zn** NO	17% >90%	Park et al. (2005a)
Rice (*Oryza sativa)*	*35S:CAX1*	ND	ND	ND	ND	>90%	Kim et al. (2005)
Lettuce (*Lactuca sativa*)	*35S:sCAX1* *cdc2a:sCAX1*	ND	19 mg/g (**1.3**)	ND	NO (*Mn, Fe, Cu, Zn analyzed*)	>90%	Park et al. (2009)

2.6.2. Effect of *AtAPS1* expression

AtAPS1 was previously overexpressed in Indian mustard, *Arabidopsis* and tobacco plants (Pilon-Smits et al. 1999; Sors et al. 2005a; McKenzie et al. 2009). Transgenic Indian mustard plants accumulated 2–3-fold more Se in shoots than wild-type plants, showed increased selenate reduction in shoots and were more tolerant to high Se concentrations (Pilon-Smits et al. 1999). In contrast, transgenic *Arabidopsis* plants accumulated less Se in the shoots and were less tolerant towards high environmental Se, although there was more selenate reduction in shoots compared to wild-type plants (Sors et al. 2005a). In between these two cases, transgenic tobacco plants exhibited the same Se tolerance and accumulation as wild-type plants, and Se speciation was not measured (McKenzie et al. 2009).

Selenate reduction was evaluated in transgenic rice plants sprayed with 230 μM Na_2SeO_4. The foliar application of Se fertilizers is a safe and effective method to increase the Se content of rice (Hu et al. 2002; Xu et al. 2004) and has already been used in biofortification programs in some Se-depleted regions of China (Chen et al. 2002). A recent study found that 40 mg/l Na_2SeO_3 (230 μM) optimized the Se enrichment of rice grains (Wang et al. 2013), so I used the same concentration of Na_2SeO_4 in this experiment. *AtAPS1* expression in rice increased selenate reduction, although not to the extent seen in transgenic Indian mustard and *Arabidopsis* plants (Pilon-Smits et al. 1999; Sors et al. 2005a). Selenate accumulation in leaves dropped from nearly 70% of total Se in wild-type plants to 50% in transgenic lines 2, 5, 28 and 39, and to less than 60% in line 37 (Figure 4B). Line 11 leaves accumulated the same percentage of selenate as wild-type leaves, indicating that ATP sulfurylase activity was not any higher in this line. The band pattern observed for line 11 in mRNA blots (see Section 1.5.2) showed that the full-length transcript was replaced by a larger transcript, which probably encoded a dysfunctional protein.

Se speciation in seeds did not differ significantly between the transgenic and wild-type plants (Figure 4B). SeMet accounted for most of the Se in polished grains, which also contained minute amounts of SeCys and selenate. This observation is consistent with previous studies that analyzed Se speciation in rice grains (Beilstein et al. 1991; Sun et al. 2010). In cereal crops, the accumulation of organic Se in the grain is influenced to only a minor extent by the selenate reduction capacity of seed cells. Like

most nutrients, Se accumulation in the grain depends primarily on phloem import from sources such as leaves (Zhang et al. 2007). Organic Se is loaded into the phloem much more efficiently than inorganic Se (Carey et al. 2012), which explains the high percentage of organic Se in the seeds of wild-type rice plants. This suggests that boosting selenate reduction in leaves will probably not affect Se speciation in seeds, but may increase the total Se content by enhancing the amount of organic Se species loaded into the phloem. This was observed in the seeds of transgenic lines 5 and 28, which accumulated 30–40% more Se than the wild-type seeds (Figure 4A). In this experiment, the leaf Se supply did not depend on Se uptake from the soil followed by xylem-mediated transport, but mainly on Se derived from the foliar spray. The Se content of the leaves therefore does not only reflect *AtAPS1* expression, as reported in previous studies (Pilion-Smits et al. 1999). The leaves of line 2 accumulated significantly less Se than wild-type leaves, which could reflect an enhanced translocation of Se into other plant organs, such as the seeds or roots (Carey et al. 2012). However, the total Se content of the seeds in line 2 did not increase (Figure 4A), which suggests that the lower Se content in the leaves of line 2 probably reflected the uneven supply of Se absorbed after foliar spraying.

AtAPS1 expression in rice plants resulted in less tolerance towards high Se concentrations. Seedlings of four transgenic lines grew more slowly in the presence of 50 μM Na_2SeO_4, although they accumulated less Se than wild-type plants (Figure 3). Similarly, *AtAPS1*-expressing *Arabidopsis* seedlings exposed to Se were also less tolerant towards Se despite of accumulating lower concentrations than wild-type seedlings (Sors et al. 2005a). However, the differences between transgenic and wild-type plants were more pronounced in *Arabidopsis* than in rice. *AtAPS1* expression has been shown to enhance ATP sulfurylase activity in transgenic seedlings (Pilon-Smits et al. 1999; Sors et al. 2005a), which increases the flux of the sulfate/selenate assimilation pathway in crops where selenate reduction to selenite is a rate-limiting step (Zayed et al. 1998). This may increase the generation of end-products such as SeMet or glutathione (GSH). The incorporation of SeMet (and SeCys) into proteins has been identified as the major mechanism of Se toxicity (Terry et al. 2000), and SeMet was the prevalent form of organic Se in the leaves of transgenic rice plants (Figure 4B). Therefore, the slower growth of transgenic seedlings exposed to 50 μM Na_2SeO_4 may reflect the incorporation of excess SeMet into proteins. Glutathione

levels were higher in *AtAPS1*-expressing *Arabidopsis* and Indian mustard plants (Sors et al. 2005a; Pilon-Smits et al. 1999). This molecule is translocated from shoots to roots through the phloem, acting as a transducing molecule that regulates the expression of sulfate transporters in roots (Lappartient et al. 1999). Substantially higher GSH production may therefore inhibit sulfate and selenate uptake, limiting the total sulfur and Se content of seedlings (Figures 3C-D).

As stated above, *AtAPS1* expression in tobacco plants did not alter Se tolerance or accumulation, suggesting that selenate reduction to selenite is not a rate-limiting step in tobacco plants (McKenzie et al. 2009). This was consistent with a previous study showing that tobacco cells overexpressing *AtAPS1* grew normally and were not more sensitive to selenate (Hatzfeld et al. 1998). In contrast to transgenic rice and *Arabidopsis* plants, Indian mustard seedlings overexpressing *AtAPS1* were more tolerant than wild-type seedlings towards high levels of Se, even though they accumulated more Se in their tissues (Pilon-Smits et al. 1999). Authors hypothesized that increased flux through the sulfate assimilation pathway may prevent Se-induced sulfur deficiency caused by competition between selenate and sulfate for uptake by sulfate transporters (Zayed and Terry 1994), thus enhancing the growth of transgenic plants more than wild-type plants. The absence of sulfate/selenate-mediated repression of sulfate transporters in roots could therefore increase the levels of Se and sulfur in transgenic plants (Hawkesford et al. 1993; Pilon-Smits et al. 1999). However, these hypotheses have not been tested experimentally and thus far there is no satisfactory explanation for the differences in Se tolerance and accumulation between *Arabidopsis* and Indian mustard transgenic plants.

The sulfate assimilation pathway in plants is tightly controlled by a complex network of regulatory elements that act at different steps (Saito 2004). The modulation of gene expression is one of the regulatory mechanisms operating along the pathway (Saito 2000). In this study, the transcript levels of several key enzymes of the pathway were analyzed in wild-type plants in response to foliar selenate (Figure 5). Under normal conditions, *Osapr* expression was strongly expressed compared to the other genes. APS reductase activity is essential in plants because the substrates of the reaction, i.e. APS and pyrophosphate, can be converted back to sulfate and ATP by ATP sulfurylase, and therefore they must be metabolized rapidly into selenite (Figure 6; Saito 2004). Under normal conditions, high levels of *Osapr* expression may be

required to ensure sufficient APS reductase activity in order for sulfate reduction to proceed. Previous studies showed that ATP sulfurylase expression was modulated coordinately with APS reductase (Leustek et al. 2000), but the expression of *Osaps* and *Osapr* followed the opposite trend (Figure 5A). I speculate that this could be a compensatory mechanism to avoid the massive accumulation of precursors in the pathway. In the presence of high concentrations of selenate, the strong downregulation of *Osapr* and upregulation of *Osaps* could result in APS reductase activity that is insufficient to convert all APSe to selenite. The APSe and pyrophosphate pools would therefore be converted back to selenate and ATP by ATP sulfurylase (Figure 6). Later, as *Osapr* expression increased and *Osaps* expression decreased, the imbalance between ATP sulfurylase and APS reductase activities would be progressively reverted, and APSe would be increasingly metabolized. Thus, selenate assimilation could be regulated at the entrance point in order to avoid disturbances in the downstream steps of the pathway.

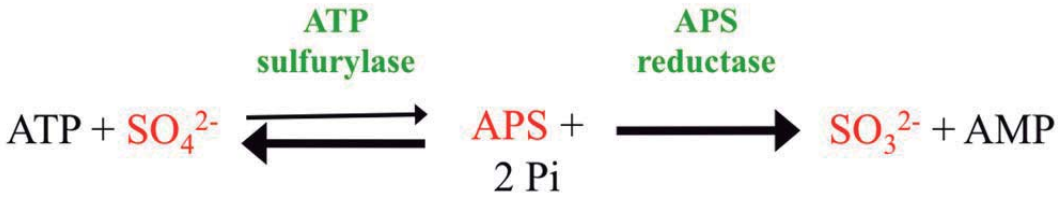


Figure 6. *Reduction of sulfate to sulfite. ATP sulfurylase activates sulfate (SO_4^{2-}) to APS by consuming ATP and the concomitant release of pyrophosphate (2Pi). The reaction equilibrium of ATP sulfurylase favors the formation of APT and sulfate, so APS reductase must metabolize APS rapidly to sulfite (SO_3^{2-}) and AMP.*

Sulfur assimilation follows a straight pathway until cysteine synthesis, from which the sulfur flux is diverted into the production of a variety of sulfur-containing metabolites, such as GSH and methionine (Sors et al. 2005b). Therefore the sulfur flux metabolized by methionine synthase must be less than that metabolized by upstream enzymes such as ATP sulfurylase, APS reductase and cysteine synthase. This may explain the low transcript levels of *Osmet* compared to *Osaps*, *Osapr* and *Oscys* before Se treatment (Figure 5A). Six hours after the application of selenate, *Osmet* expression had increased dramatically, probably reflecting selenate assimilation into SeMet, the predominant organic Se species produced in rice leaves (Figure 4B).

Cysteine is considered as the pivotal metabolite of the sulfur assimilation pathway. It is synthesized from O-acetylserine (OAS) and sulfide, thus linking sulfur and nitrogen metabolism (Saito 2004). In order to ensure an efficient cysteine production, a complex regulatory mechanism coordinates OAS synthesis and sulfate reduction through the formation of an enzyme complex involving cysteine synthase and serine acetyltransferase (SAT) as shown in Figure 7 (Rennenberg and Herschbach 2014). The concentration of cysteine synthase in chloroplasts greatly exceeds (nearly 300-fold) that of SAT, in order to ensure that free cysteine synthase remains available for cysteine synthesis (Droux et al. 1998). However, this was not reflected in the transgenic rice leaves, because *Oscys* mRNA levels were at most 20-fold higher than *OsSAT* mRNA levels (Figure 5A). Cysteine precursors are naturally abundant in rice plants (Yamaguchi et al. 2006), which suggests that the sudden decrease of *OsSAT* expression levels 6 h after the Se treatment may reflect the overaccumulation of OAS and selenide, and the subsequent need to increase the pool of free and active cysteine synthase to metabolize selenide (Saito 2004).

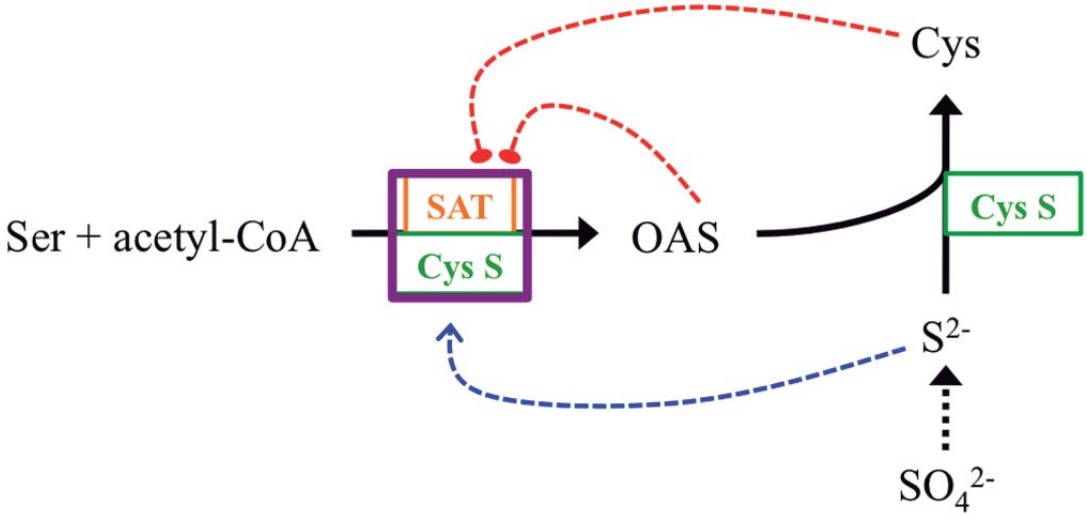


Figure 7. *Regulation of cysteine synthesis in plants (Saito 2004). Cysteine is synthesized by cysteine synthase (Cys S) from sulfide and O-acetylserine (OAS). The synthesis of OAS from serine and acetyl-CoA is catalyzed by the SAT/Cys S enzyme complex (purple), where Cys S loses most of its catalytic activity and acts as a regulatory subunit that increases SAT activity. Accumulation of sulfide promotes the formation of the enzyme complex (blue arrow), which results in OAS and cysteine synthesis. Conversely, cysteine or OAS accumulation triggers the dissociation of the enzyme complex (red arrows), thus inhibiting OAS and cysteine synthesis.*

AtAPS1 expression was analyzed in transgenic plants before Se treatment (Figure 5B).

Initially the transgene was expressed in all lines except Se2 and 84. The set of primers used to amplify *AtAPS1* (Set_1) produced a fragment representing the downstream part of the sequence. Therefore, I decided to use a second set of primers (Set_2) targeting an upstream fragment of the *AtAPS1* mRNA sequence, which allowed the detection of the transgene in line Se2 but not in line 84. Expression analysis by mRNA blotting (see Section 1.5.2) showed little or no *AtAPS1* expression in T_1 plants from line 84, and expression analysis by real-time RT-PCR confirmed that *AtAPS1* is not expressed in line 84. Detailed observation of the mRNA band pattern of line Se2 (Chapter 1) showed that the full-transcript was absent, but a shorter transcript was expressed that was absent in some other lines (Figure 3 in Chapter 1). This shorter transcript probably did not contain the fragment amplified by Set_1, and therefore could not be detected. It is intriguing, however, that Set_2 yielded lower *AtAPS1* expression levels than Set_1 in all lines, because the upstream fragment targeted by Set_2 should be present in the full-length transcript. Therefore Set_2 should yield the same or higher expression levels than Set_1, reflecting the amplification of the full-length and shorter transcripts.

The expression levels of *Osaps*, *Osapr*, *Oscys*, *Osmet* and *OsSAT* were analyzed in nine transgenic lines and compared to those in the corresponding wild-type plants (Figures 5C-F). The expression pattern of the 5 endogenous genes over time differed between the transgenic and wild-type plants, even though there was great variability between the transgenic lines and no obvious correlation with transgene expression levels (Figures 5B-F). However, the general trend was a progressive downregulation of the entire pathway compared to wild-type plants following Se treatment (Table 2). This may reflect a compensatory response to counter the higher selenate reduction capacity of the leaves in some of the transgenic lines (Figure 4B), to avoid the incorporation of excess Se into proteins.

To summarize, *AtAPS1* expression in rice plants causes a moderate increase in the capacity for selenate reduction, lower tolerance to high levels of Se in the environment, and lower Se and sulfur accumulation when Se is taken up through the roots as selenate. The endogenous selenate assimilation pathway in transgenic lines was downregulated compared to wild-type plants. These results are similar to those reported in transgenic *Arabidopsis* plants but not in Indian mustard plants, which may reflect the fact that rice and *Arabidopsis* are non-accumulator plants for Se whereas

Indian mustard is considered a secondary Se accumulator (McKenzie et al. 2009). However, the effects of *AtAPS1* expression in rice plants are less pronounced than in *Arabidopsis* plants. This may indicate that selenate activation by ATP sulfurylase is not such a rate-limiting step in rice plants as in *Arabidopsis*. If this is confirmed, other strategies to increase Se accumulation in rice should be considered, such as diverting Se assimilation into non-protein amino acids like MeSeCys by the overexpression of SMT, as explained in the General Introduction.

2.7. Conclusions

Four transgenic rice lines expressing *AtsCAX1* and *AtsACA4* were evaluated for their capacity to accumulate Ca^{2+}. None of the transgenic lines accumulated more Ca^{2+} than wild-type plants, although other minerals accumulated in some lines. The downregulation of endogenous Ca^{2+} transporters may limit Ca^{2+} accumulation, although this does not occur at the mRNA level. Calcium homeostasis seem to be more tightly regulated in rice than in other crops, where the overexpression of de-regulated Ca^{2+} transporters has been translated into enhanced Ca^{2+} levels.

Transgenic rice lines expressing *AtAPS1* were evaluated for their selenate assimilation capacity. The transgenic lines were able to reduce selenate more efficiently, as shown by the greater portion of organic Se in leaves and the lower Se tolerance. The endogenous selenate assimilation pathway was downregulated in the transgenic lines. These data indicate that selenate activation by ATP sulfurylase may not be such a rate-limiting step in the Se assimilation pathway in rice as it is in other crops, therefore other strategies to accumulate Se should be considered.

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Chapter 3

Towards the generation of *Striga*-resistant transgenic maize plants

Towards the generation of *Striga*-resistant transgenic maize plants

3.1. Abstract

The parasitic weed *Striga* is one of the major biotic constraints for crop production in sub-Saharan Africa. The development of *Striga*-resistant varieties by genetic engineering would represent a major step forward in the control of this weed. Strigolactones play a key role in the establishment of *Striga* infection, so reducing strigolactone production in crops could increase their resistance to colonization. In this chapter, two key genes involved in strigolactone biosynthesis were downregulated in transgenic maize plants by RNAi-mediated silencing. Lower levels of *Zmccd8* expression substantially reduced strigolactone production in the roots, altered the phenotype of the plants and reduced their capacity to stimulate the germination of *Striga* seeds, showing great potential for *Striga* control. In contrast, the downregulation of *Zmd27* did not affect strigolactone levels, although the capacity of the transgenic plants to stimulate *Striga* germination was moderately reduced, and the plants exhibited subtle phenotypic changes.

3.2. Introduction

The African continent is divided by the Sahara Desert into two zones with different primary crops. Wheat and barley are the main staples in the northern zone, whereas in sub-Saharan Africa most of the calories in the human diet come from maize, sorghum, millet and cassava (Gressel et al. 2004). The production of maize in sub-Saharan Africa has nearly doubled in the last 20 years (FAOSTAT 2014). Unfortunately, most regions in sub-Saharan Africa are infested with *Striga* spp. (see General Introduction), and this has resulted in a substantial loss of maize yields compared to the world average (Kanampiu et al. 2002). *Striga* control is a complex challenge that needs to be tackled using multiple strategies (Aly 2007). In an attempt to enlarge and diversify the range of available methods to combat *Striga*, new crop varieties with enhanced resistance to *Striga* infection are under development (Menkir et al. 2007). Genetic engineering has been used for more than a decade to introduce agronomic traits into crops (Christou and Twyman 2004), thus offering a suitable approach to generate weed-resistant maize plants.

Strigolactones are plant hormones that are secreted into the rhizosphere in order to control symbiosis with arbuscular mycorrhizal fungi (Besserer et al. 2006). Unfortunately, several parasitic weeds have evolved to recognize strigolactones as germination stimulants and chemical signals to infect their hosts (Cardoso et al. 2011). Therefore, one potential strategy to increase the resistance of crops to *Striga* infection would be to reduce the production of strigolactones. These plant hormones are structurally diverse and complex, and most of the late biosynthesis steps involve the conversion of one strigolactone into another and are not yet elucidated (Ruyter-Spira et al. 2013). Conversely, the upstream part of the pathway from β-carotene to carlactone was recently characterized (Alder et al. 2012). This takes place in the plastids, and consists of three sequential steps that are catalyzed by the enzymes β-carotene isomerase D27, and carotenoid cleavage dioxygenases (CCD) 7 and 8 (Waters et al. 2012). Mutants of the three corresponding genes produce much lower levels of strigolactones (Umehara et al. 2008; Gómez-Roldan 2008; Lin et al. 2009). Genetic engineering can be used to downregulate some of the key genes in the strigolactone biosynthesis pathway by RNA interference (RNAi). Tomato plants overexpressing RNAi constructs targeting *Slccd7* and *Slccd8* showed a substantial

decrease in strigolactone production that inversely correlated with an increase in resistance to *Striga* infection (Vogel et al. 2009; Kohlen et al. 2012).

In the work described in this chapter, transgenic maize lines expressing RNAi constructs for the inhibition of D27 and CCD8 were generated and the plants were analyzed for target gene expression, the production of strigolactones and the capacity to stimulate *Striga* germination.

3.3. Aims

The major aim of the work described in this chapter was to generate transgenic maize plants with enhanced resistance to *Striga* infection through RNAi-mediated silencing. The specific objectives were to:

1) Generate transgenic maize plants overexpressing RNAi constructs targeting different genes involved in the strigolactone biosynthesis pathway.

2) Confirm the downregulation of target genes in transgenic lines.

3) Determine strigolactone levels in root exudates of the transgenic lines.

4) Evaluate the resistance of transgenic lines to *Striga* infection.

3.4. Materials and methods

3.4.1. Plant material and growth conditions

Maize plants (*Zea mays* L. *cv*. M37W) were grown for 10 days in biodegradable pots inside a growth chamber at 26 ± 2°C, with a 12-h photoperiod (900 μmohn/m^2/s photosynthetically-active radiation) and 80% relative humidity. Seedlings from transgenic lines were first screened for phosphinothricin (PPT) tolerance by applying a 1.5 mg/ml PPT (Duchefa Biochemie, Haarlem, he Netherlands) to the leaves. They were then transferred to 15-l plastic pots (standard cultivation soil Traysubstract; Klasmann-Deilmann GmbH, Geeste, Germany), in a greenhouse with drip irrigation, at 28/20ºC day/night temperature with a 10-h photoperiod and 60–90% relative humidity, until harvest.

3.4.2. Gene cloning

Fragments from the *Zmd27*, *Zmccd7* and *Zmccd8* genes were cloned by RT-PCR

using sequences obtained from GenBank (*Zmd27* = NM_001151368.1; *Zmccd7* = NM_001196999.1; *Zmccd8* = NM_001197000.1), and the *Escherichia coli gusA* gene fragment was cloned from the plasmid pGZ63, which contains the entire gene. Total RNA was isolated from 120 mg of wild-type maize leaves as described in Section 1.4.3, and cDNA synthesis was carried out as described in Section 2.4.3. Gene fragments were amplified by PCR using specific primers containing terminal restriction sites to enable the construction of the RNAi cassettes (Table 1). Each 50-µl reaction comprised 25 ng cDNA, 10 µl 5x GoTaq Buffer (Promega, Wisconsin, US), 1 µl 10 mM dNTP mix (Promega), 1 µl 20 µM forward and reverse primers, 3 µl 25 mM $MgCl_2$, 2.5 µl DMSO, and 2.5 units of GoTaq DNA polymerase (Promega). Amplification started with a denaturation step at 95ºC for 3 min, followed by 35 cycles of 95ºC for 50 s, 55ºC for 50 s and 72ºC for 1 min, and a final elongation step at 72ºC for 10 min.

PCR products were separated by 1.2% TAE agarose gel electrophoresis and purified using the Geneclean II Kit (MP Biomedicals, LLC, France). They were inserted into the pGEM®-T Easy vector (Promega), and introduced into competent *E. coli* cells, which were plated on LB medium supplemented with 100 mg/l ampicillin, 28 mg/l X-gal and 0.05 mM IPTG, and incubated at 37ºC overnight. Plasmid DNA was isolated from white colonies using the Wizard® Plus SV Minipreps DNA Purification Kit (Promega) and sequenced (Servei de Seqüenciació, UdL, Lleida, Spain). The sequences were compared to GenBank database sequences using BLAST, and those showing 100% identity were selected for construction of RNAi cassettes.

3.4.3. RNAi cassettes

The *gusA* gene fragment was excised from pGEM®-T Easy by digesting with BamHI and HindIII, and was transferred to the pAL76 expression vector, containing the constitutive maize *ubiquitin-1* (*Ubi-1*) promoter, its first intron and the nopaline synthase (*nos*) transcriptional terminator. Positive clones were isolated and sequenced (Servei de seqüenciació, UdL) to confirm sequence identity. Gene fragments were isolated using appropriate restriction enzymes and ligated into the *sense* and *antisense* sites of pAL76 vectors containing the *gusA* gene fragment (Figure 1). Positive clones containing the whole RNAi construct were sequenced to confirm sequence identity, and those with 100% identity were selected for maize transformation.

Table 1. *Primers used to clone each gene fragment containing specific restriction sites to assemble the RNAi constructs.*

Gene	Primers		
	Restriction Sites (5'→3')	Sequence	Size (bp)
Zmd27	HindIII-BamHI	For: 5'-AAGCTTGGATCCGCAGTGGGACACTGTGGCCA-3'	**350**
	AgeI-SpeI	Rev: 5'-ACCGGTACTAGTTCATCTCGCAGCTCATGTCC-3'	
Zmccd7	HindIII-BamHI	For: 5'-AAGCTTGGATCCACAAGAAGCTGGACCCGTCC -3'	**288**
	AgeI-SpeI	Rev: 5'-ACCGGTACTAGTCTGATCCATCGGAGACGTCTAC -3'	
Zmccd8	HindIII-BamHI	For: 5'-AAGCTTGGATCCCATCGGCAAGTTCGAGTACA-3'	**421**
	AgeI-SpeI	Rev: 5'-ACCGGTACTAGTGTACGCGTTGATGAAGTGGA-3'	
EcgusA	BamHI-SpeI	For: 5'-GGATCCACTAGTATCCGCACCTCTGGCAACCGGGTGAAGGT-3'	**300**
	HindIII-AgeI	Rev: 5'-AAGCTTACCGGTAGTCGAGCATCTCTTCAGCGTAAGGGTAA-3'	

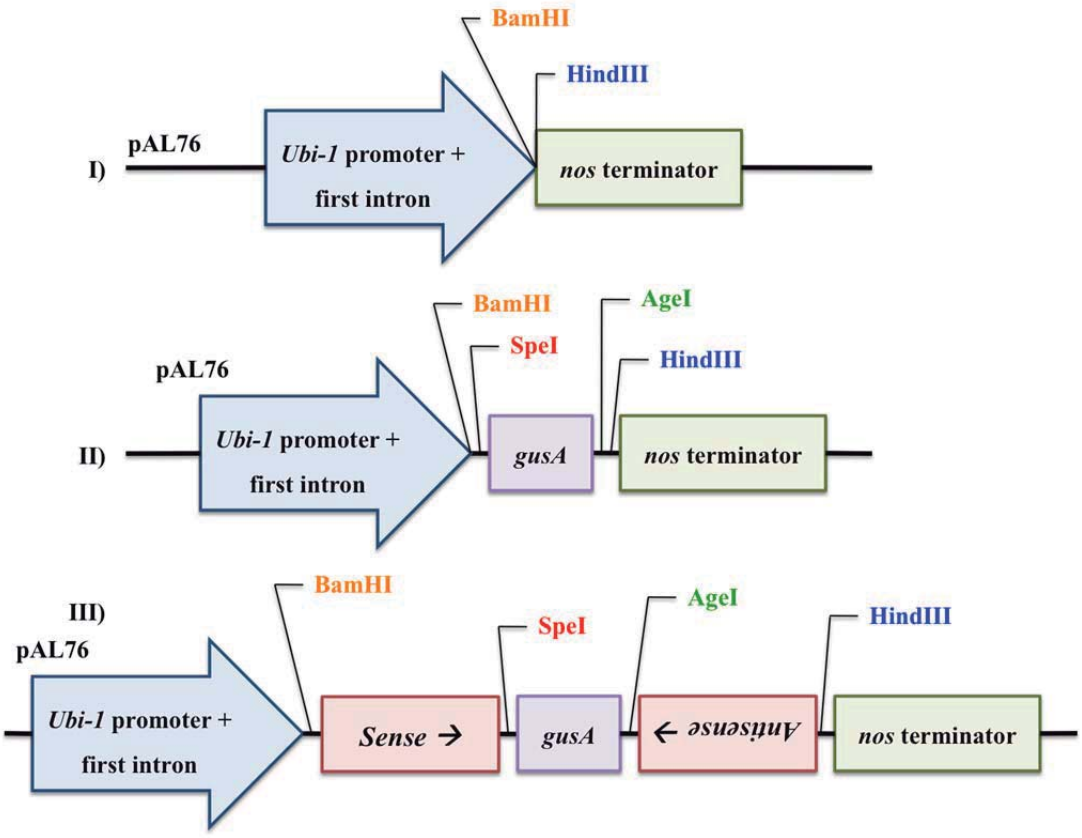

Figure 1. *Steps in the construction of RNAi cassettes. The pAL76 expression vector was digested with BamHI and HindIII restriction enzymes (I), and ligated with the* gusA *fragment flanked by appropriate restriction sites (II). BamHI/SpeI digestion allowed the insertion of the sense fragment, and AgeI/HindIII digestion enabled the complete formation of the RNAi cassette through the insertion of the antisense fragment (III).*

3.4.4. Maize transformation and regeneration

Maize (*Zea mays* L. *cv*. M37W) immature zygotic embryos were excised 10–14 days after pollination and cultured on N6 medium. After 4 days, they were transferred to high osmoticum N6 medium (0.2 M mannitol, 0.2 M sorbitol) 4 h before bombardment with gold particles (10 mg) coated with 40 μg of a DNA mixture containing one of the pAL76-derived plasmids and the *bar* selectable marker gene (Thompson et al. 1987) in a molar ratio of 3:1. Bombarded embryos were selected on N6 medium supplemented with 3 mg/l PPT in the dark, for 1 month with subculture every 2 weeks, as previously described (Drakakaki et al. 2005). Embryogenic callus was transferred to shoot-induction medium and subsequently to root-induction medium supplemented with PPT (3 mg/l) in the light. After regeneration, plantlets were transferred to pots (standard cultivation soil as above) and were grown in a growth chamber at 26 ± 2°C, with a 12-h photoperiod (900 μmohn/m^2/s photosynthetically-active radiation) and 80% relative humidity. Tables 2 and 3 show the media used in each step of the process, which is illustrated in Figure 2.

Table 2. *Media composition (1 l) used during maize transformation and regeneration.*

	Callus induction	Osmoticum medium	Selection medium	Shoot induction medium	Root induction medium
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
MS basal salts	--	--	--	4.4 g	4.4 g
Sucrose	20 g	20 g	20 g	30 g	30 g
Casein hydrolysate	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	--	--	--
Adjust pH to 5.8 using KOH					
Phytagel	4 g	--	4 g	4 g	4 g
Agarose	--	4 g	--	--	--
Autoclave at 121 ºC for 20 min					
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
BAP	--	--	--	10 mg	--
* From 20x or 200x stock solutions, which are described in Table 3.					

Table 3. *Stock solutions for N6 macronutrients, N6 micronutrients, N6 Fe-EDTA and N6 vitamins.*

20X N6 Macronutrients		**200X N6 Micronutrients**	
	Amount		Amount
$(NH_4)_2SO_4$	9.26 g	$MnSO_4 \cdot H_2O$	0.25 g
KNO_3	56.6 g	$ZnSO_4 \cdot 7H_2O$	0.15 g
$CaCl_2 \cdot 2H_2O$	3.32 g	H_3BO_3	0. 16 g
$MgSO_4 \cdot 7H_2O$	3.7 g	KI	0.08 g
KH_2PO_4	8 g	$Na_2MoO_4 \cdot 2H_2O$	0.025 g
		$CuSO_4 \cdot 5H_2O$	0.0025 g
200 X N6 Fe-EDTA		**200 X N6 Vitamins**	
	Amount		Amount
$FeSO_4 \cdot 7H_2O$	1.112 g	Nicotinic acid	0.02 g
EDTA-$Na_2 \cdot 2H_2O$	1.49 g	Pyridoxine-HCl	0.02 g
		Thiamine-HCl	0.04 g
		Glycine	0.08 g

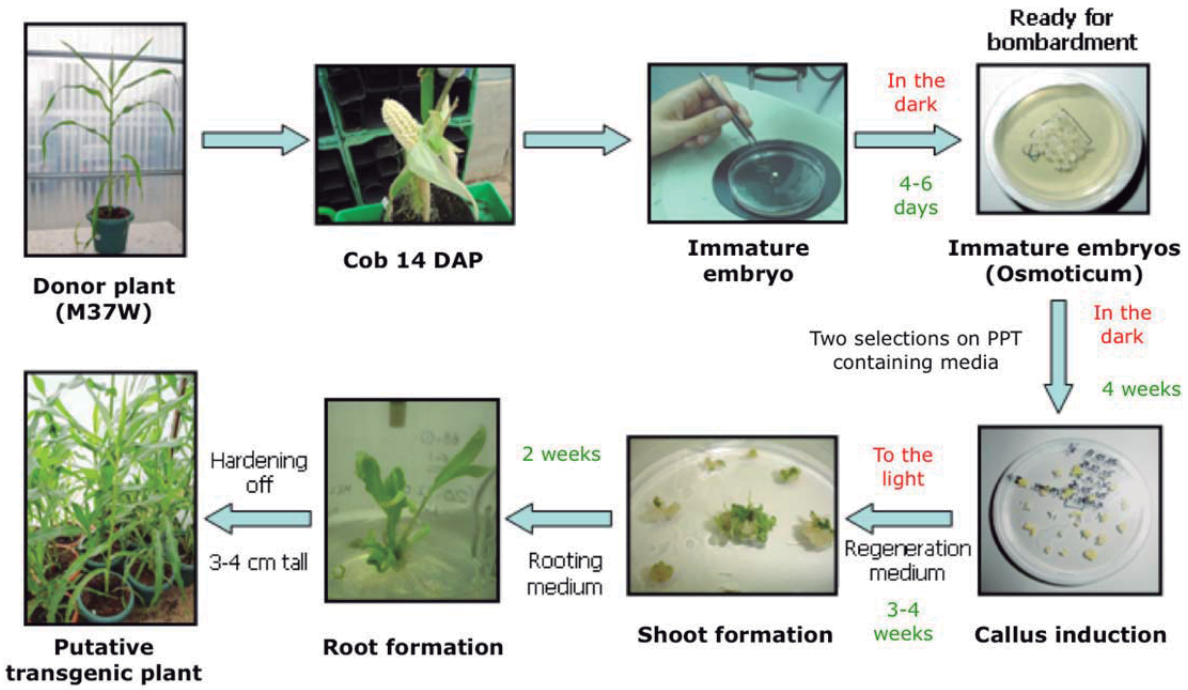

Figure 2. *Maize transformation process. DAP: days after pollination.*

3.4.5. mRNA blot analysis

Total RNA was extracted from 120 mg of leaf and root samples as described in Section 1.4.3, and mRNA blot analysis was carried out as described in Section 1.4.4.

DIG-labeled probes were synthesized using forward primer 5'-GCA CCT CTG GCA ACC GGG TGA AGG-3' and reverse primer 5'-CGA GCA TCT CTT CAG CGT AAG GG-3', amplifying the *gusA* fragment inside the RNAi construct (Figure 1).

3.4.6. Expression analysis of endogenous genes by real-time RT-PCR

DNase treatment and subsequent clean-up of samples, cDNA synthesis and real-time RT-PCR were carried out as described in Section 2.4.3. Specific primers targeting endogenous genes of the strigolactone biosynthesis pathway are listed in Table 4. Primers used for expression analysis targeted gene regions other than the fragment used for the RNAi cassettes.

Table 4. *Primers used to determine endogenous gene expression levels in the strigolactone biosynthesis pathway by real-time RT-PCR.*

Gene	Primers	PCR product
Zmd27	For: 5'-GCAAAGCAGAATCATCAAGTG-3' Rev: 5'-TCAGCAACCAGTTTCCTAACA-3'	214 bp
Zmccd7	For: 5'-GCGAGGATGACGGCTATGT-3' Rev: 5'-CCACAACTGCGTTCCTTTC-3'	108 bp
Zmccd8	For: 5'-CACGTCGGCAAGAAGTACC-3' Rev: 5'-AAGAAGGGCTCTGACGGCAC-3'	146 bp
Zmcullin	For: 5'-GAAGAGCCGCAAAGTTATGG-3' Rev: 5'-ATGGTAGAAGTGGACGCACC-3'	113 bp

3.4.7. Determination of strigolactone levels

Strigolactone levels were measured at the Laboratory of Plant Physiology, Wageningen University, Netherlands, as described in Kohlen et al. (2012). Maize seeds were pre-grown in perlite for 7 days. The perlite was then removed from the roots, and seedlings were transferred to an X-stream 20 aeroponics system (Nutriculture, Lancashire, UK) operating on 5 l half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950). In order to stimulate strigolactone production, phosphate starvation was applied for 7 days by supplying phosphorus-deficient half-strength Hoagland solution. The nutrient solution was replenished 24 h before exudate collection to remove all accumulated strigolactones. The exudates were collected, purified and concentrated (López-Ráez et al. 2008), and roots were stored for strigolactone extraction as described by López-Ráez et al. (2010).

Strigolactone analysis of root exudates was carried out by UPLC–MS/MS as described by López-Ráez et al. (2008), comparing retention times and mass transitions with those of available strigolactone standards (sorgolactone, strigol, 2-epistrigol, orobanchol, 2-epiorobanchol, 5-deoxystrigol, 2-epi-5-deoxystrigol, solanacol, orobanchyl acetate, sorgomol, 7-oxoorobanchol and 7-oxoorobanchyl acetate) using a Waters Xevo tandem mass spectrometer equipped with an electrospray ionization source and coupled to an Acquity UPLC system (Waters, Milford, MA, US). Data were acquired and analyzed using MassLynx (Waters).

3.4.8. *Striga* germination bioassay

The ability of root exudates to stimulate *Striga* germination was carried out as described by Jamil et al. (2012). *Striga* seeds were surface-sterilized in 2% sodium hypochlorite with 0.4% Tween-20 for 5 min, then rinsed three times with demineralized water for 10 min, and finally they were air-dried for 60 min. Fifty sterile *Striga* seeds were spread on 9-mm glass fiber filter paper discs (Sartorius, Goettingen, Germany), which were placed in 9-cm Petri dishes on filter paper (Whatman, Maidstone, UK) pre-wetted with 3 ml of sterile demineralized water. Petri dishes were sealed with Parafilm and placed in an incubator in darkness at 30ºC for 10 days to precondition the seeds. The discs were then dried for 50 min in a laminar flow cabinet. Root exudates were mixed with one volume of sterile miliQ water before vacuum centrifugation to evaporate acetone. The mixture was adjusted back to the original volume and then was diluted 10 times with sterile miliQ water before application to the *Striga* seeds. Dried discs containing *Striga* seeds were placed in another Petri dish containing a 1-cm filter paper ring pre-wetted with demineralized water. Diluted root exudates were applied (70 µl per disc) in five replicates. GR24 (0.2 µM) and demineralized water were used as positive and negative controls, respectively. Petri dishes were then incubated at 30ºC in darkness for 48 h and germination (seeds with radicle protruding through the seed coat) was scored using a binocular microscope.

3.4.9. Statistical analysis

Differences between transgenic and wild-type plants were tested by ANOVA and subsequent post-hoc comparison of means using the least significant difference (LSD) test ($P < 0.05$).

3.5. Results

3.5.1. Gene cloning and vector construction

RNAi cassettes were constructed for three genes with well-characterized functions in the strigolactone biosynthesis pathway: *Zmd27*, *Zmccd7* and *Zmccd8* (Ruyter-Spira et al. 2013). Although *Zmccd7* and *Zmccd8* are expressed at low levels in maize leaves (see Section 3.5.3), all three sequences could be isolated from the total leaf RNA of the wild-type variety M37W. The RNAi cassettes were constructed in the pAL76 expression vector as explained in Section 3.4.3, and their expression was driven by the maize *Ubi-1* constitutive promoter and its first intron (Figure 1).

3.5.2. Recovery of transgenic plants

M37W immature embryos were bombarded with each expression vector plus the *bar* selectable marker. Putative transgenic plants were recovered for each RNAi construct, and they were subsequently screened for transgene expression in leaves by mRNA blotting. Probes targeting the *gus*A fragment were used to detect the expression of all RNAi constructs. Three independent fertile transgenic lines were recovered expressing the *Zmd27* construct, and one expressing the *Zmccd8* construct, but *gus*A expression was not detected in any putative transgenic plants transformed with the *Zmccd7* construct. Lines C8-2 (for *Zmccd8*) and D27-13 (for *Zmd27*) showed high-level expression of the corresponding RNAi constructs, whereas lines D27-16 and D27-17 expressed low and very low levels, respectively (Figure 3). All lines showed a similar band pattern, potentially reflecting several cleavage products or different secondary structures of the RNAi transcript.

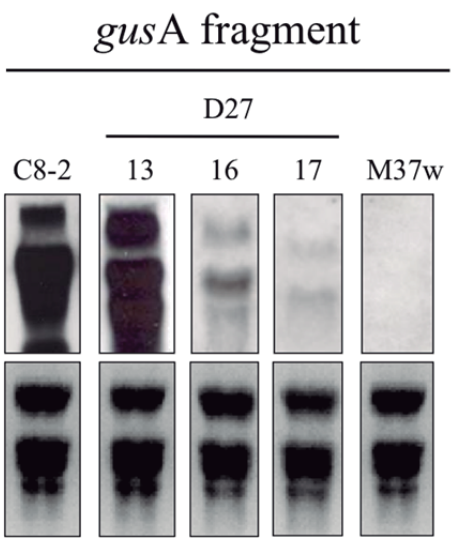

Figure 3. *mRNA blot analysis showing the expression of* gus*A in leaves from T_0 plants of transgenic lines C8-2, D27-13, D27-16 and D27-17. The wild-type variety M37W was used as a negative control. Ribosomal RNA stained with ethidium bromide was used as a loading control.*

3.5.3. Molecular characterization of transgenic plants

In order to characterize the upstream part of the strigolactone biosynthesis pathway in the M37W wild-type variety, real-time RT-PCR expression analysis of endogenous *Zmd27*, *Zmccd7* and *Zmccd8* was carried out in roots, shoots and leaves (Figure 4A). All three genes were expressed in roots although *Zmccd7* mRNA levels were six-fold higher than those of *Zmd27* and *Zmccd8.* Similarly, *Zmd27* expression levels were observed throughout the plant, whereas *Zmccd7* and *Zmccd8* transcripts accumulated at barely detectable levels in the shoots and leaves, as previously reported (Vallabhaneni et al. 2010; Guan et al. 2012). D27 transgenic lines were directly screened for *Zmd27* downregulation in the leaves of T_0 plants revealing 70–80% knockdown of *Zmd27* transcript levels compared to the wild-type, which did not reflect the differences in expression levels of the RNAi construct (Figure 4B). In contrast, line C8-2 showed a moderate (30%) knockdown of *Zmd27* in the leaves.

The expression of *Zmd27*, *Zmccd7* and *Zmccd8* was measured in the roots of T_1 plants from transgenic lines D27-13 and C8-2, and compared to wild-type. T_0 plants from lines D27-16 and D27-17 produced few seeds, which were kept for strigolactone analysis given that the knockdown of *Zmd27* had already been confirmed in leaves (Figure 4B). The knockdown of the target genes in roots was less pronounced than in

leaves. *Zmd27* levels in line D27-13 were only reduced by 50%, and *Zmccd8* levels in C8-2 line were only reduced by 30%. This did not reflect the weaker expression of the RNAi construct in the roots of lines D27-13 and C8-2 because high levels of the RNAi transcript were confirmed by real-time RT-PCR. The expression of non-target endogenous genes oscillated up and down compared to wild-type levels, although *Zmccd7* showed a 50% loss of expression in line C8-2 (Figure 4C).

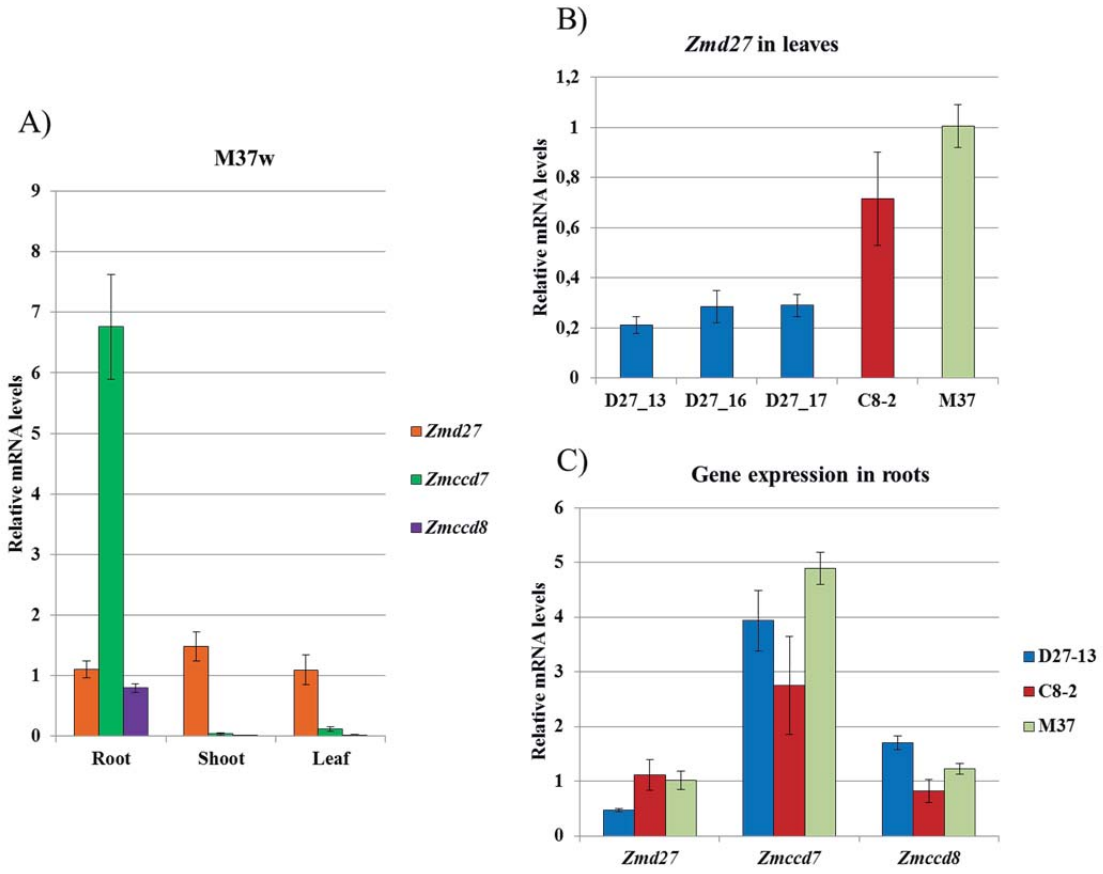

Figure 4. *Real-time RT-PCR analysis of genes involved in the strigolactone biosynthesis pathway. A) Expression levels of endogenous* d27, ccd7 *and* ccd8 *in the roots, shoots and leaves of 3-week-old M37W plants. B)* Zmd27 *expression in the leaves from transgenic* T_0 *plants compared to wild-type. C) Expression levels of* Zmd27, Zmccd7 *and* Zmccd8 *in roots from* T_1 *plants of transgenic lines C8-2 and D27-13 compared to wild-type. Cullin was used as a housekeeping gene. Values are the mean of three replicates ± SD.*

3.5.4. Strigolactone quantification

Germinating seedlings were first screened for PPT tolerance. None of the D27-16 seedlings was transgenic, and only one seedling from line D27-17 was PPT-tolerant. Therefore, line D27-16 was not included in the experiment and the single D27-17

transgenic plant was analyzed with the D27-13 plants. Strigolactone levels were measured in root exudates from nine independent plants from each transgenic line (D27-13 and C8-2) and eight wild-type plants. Exudates were collected at two different time points: 24 h and 3 days after the P-starvation treatment.

None of the known strigolactones was detected in any of the samples, but five major unidentified strigolactone-like compounds (SLCs) were consistently detected in all samples. Interestingly, the secretion of these five molecules declined by 75–95% in C8-2 plants compared to wild-type, whereas D27-13 plants secreted them at similar levels to wild-type plants (Figure 5). The D27-17 transgenic plant behaved like the D27-13 plants, thus accumulating wild-type levels of the SLCs. The SLCs were more abundant in exudates collected at the second time point, although the relative amounts of each compound were similar at both time points, with SLC 5 prevailing over the remaining compounds. The decline in the levels of SLCs in line C8-2 was accompanied by a slight shift in the relative amounts of the different compounds, favoring the accumulation of SLC 5 at the expense of SLC 2 (Table 5).

SLC levels in root exudates were not consistent with the real-time RT-PCR data. *Zmd27* knockdown in the leaves and roots of line D27-13 was not translated to a decline in the secretion of SLCs, but the potent decline in SLC secretion in line C8-2 did not reflect the moderate knockdown of *Zmccd8* in the roots. Therefore, we analyzed gene expression in some of the P-starved transgenic plants used for strigolactone quantification. *Zmd27* transcript level in the leaves were similar to the previous experiments, with a strong knockdown in D27-13 plants but near wild-type transcript levels in C8-2 plants (Figure 6D). In contrast, there was no consistent knockdown of target genes in the roots (Figure 6A-C). There was significant variability between independent plants of each line, and especially between wild-type plants, but the levels of *Zmd27* and *Zmccd8* mRNA were knocked down by an average of 50% in D27-13 and C8-2 plants, respectively. Non-targeted endogenous genes were expressed at wild-type levels in each transgenic line (Figure 6A-C).

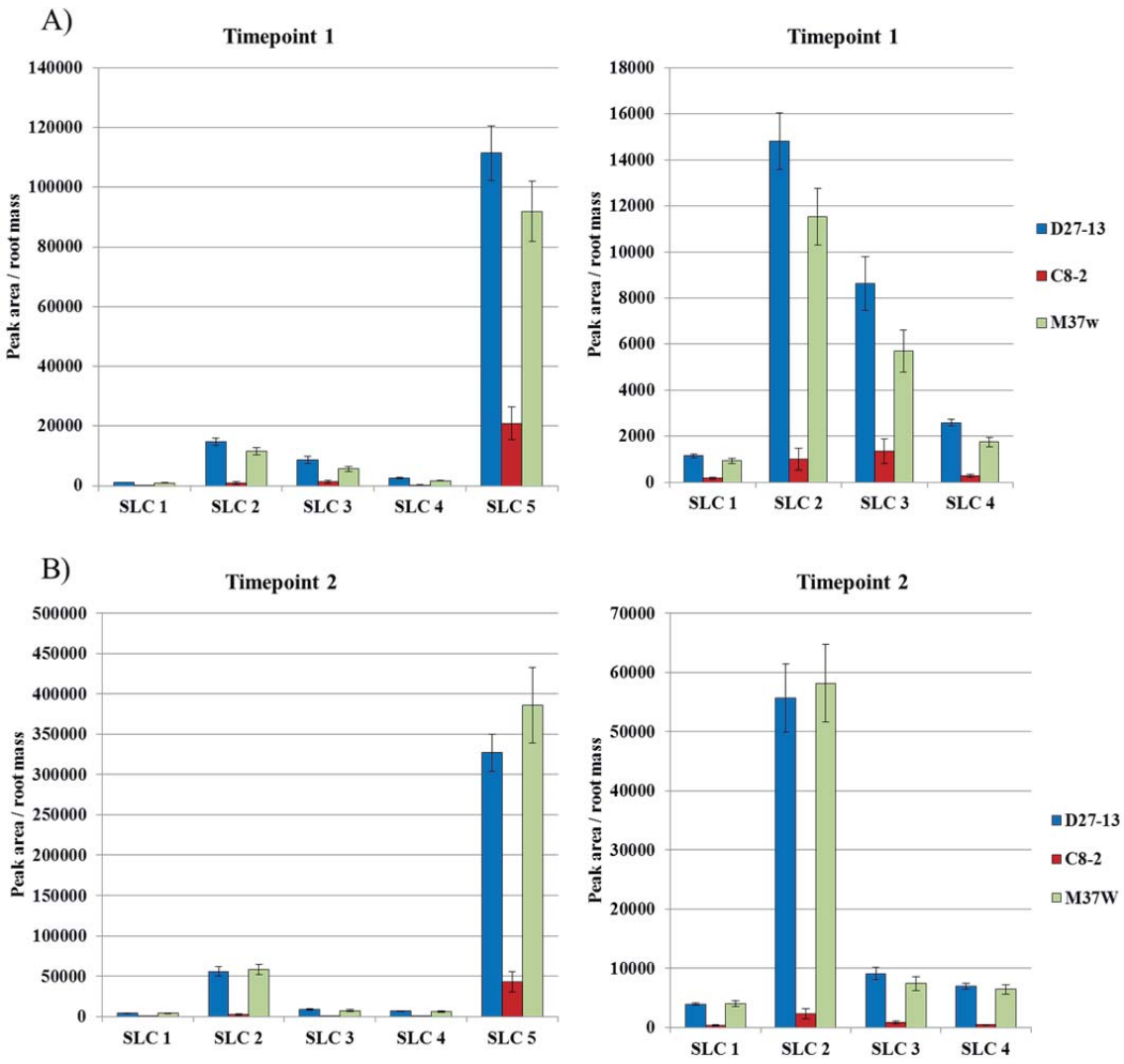

Figure 5. *UPLC-MS/MS analysis of the main strigolactone-like compounds (SLCs) present in root exudates from 4-week-old transgenic T_1 plants of lines C8-2 and D27-13 compared to wild-type. Exudates were collected 1 day (A) and 3 days (B) after the P-starvation treatment. No known strigolactones were detected, but five major unidentified SLCs (1–5) were revealed as peaks in all samples. Values are the mean of nine independent plants ± SE.*

Table 5. *Percentage of each strigolactone-like compound (SLC) in the root exudates of transgenic lines D27-13 and C8-2, compared to the wild-type roots.*

	Time point 1					Time point 2				
	SLC 1	SLC 2	SLC 3	SLC 4	SLC 5	SLC 1	SLC 2	SLC 3	SLC 4	SLC 5
D27-13	0.83	10.68	6.23	1.87	80.39	0.97	13.82	2.25	1.73	81.22
C8-2	0.69	4.16	5.69	1.15	88.31	0.67	4.85	1.73	0.83	91.92
M37W	0.83	10.31	5.10	1.56	82.21	0.87	12.60	1.61	1.39	83.53

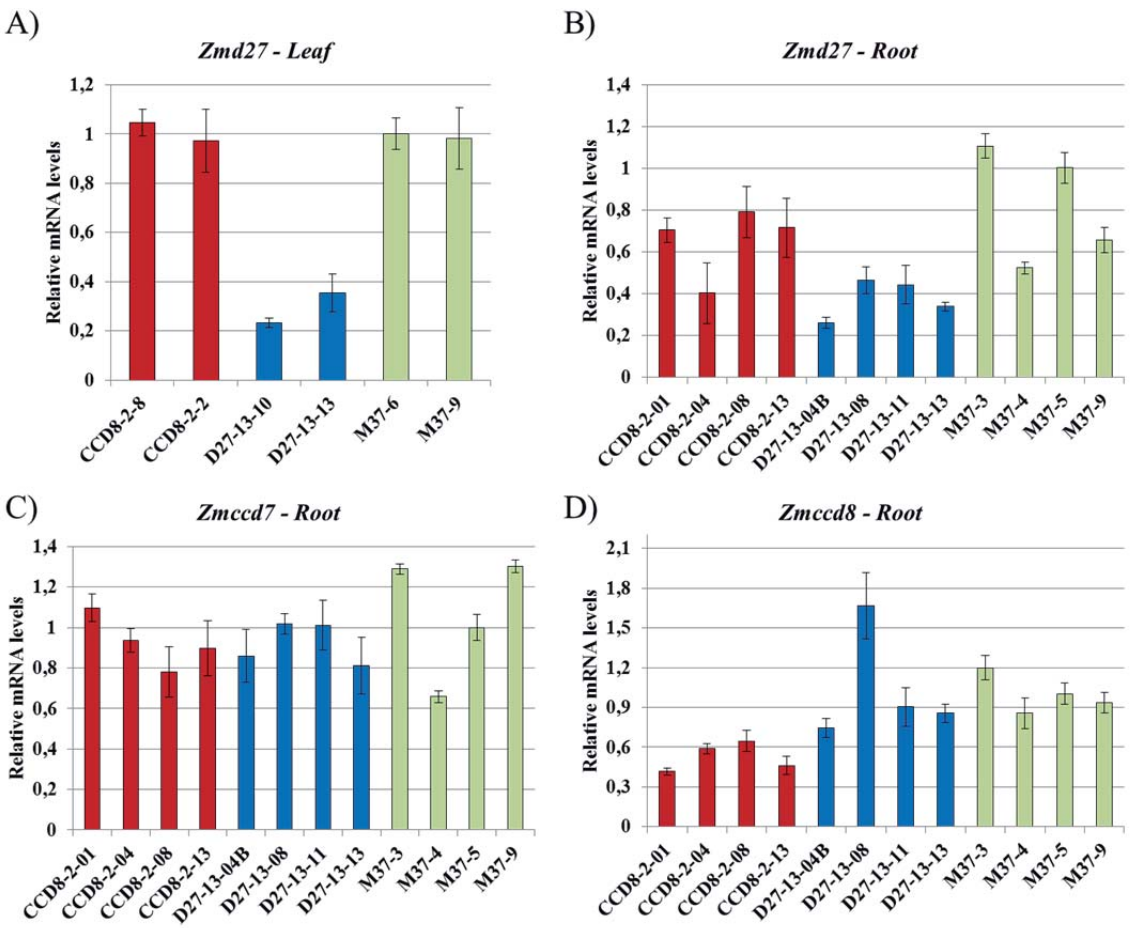

Figure 6. *Real-time RT-PCR analysis of* Zmd27 *(B),* Zmccd7 *(C) and* Zmccd8 *(D) in roots from individual 4-week-old T_1 plants of transgenic lines C8-2 and D27-13 compared to wild-type.* Zmd27 *expression was also measured in leaves (A). Samples were harvested after collecting exudates for strigolactone analysis. Cullin was used as a housekeeping gene. Values are the mean of three replicates ± SD.*

3.5.5. Phenotypic analysis of transgenic plants

A previous study characterizing a maize *ccd8* mutant reported several moderate phenotypic alterations, including reduced plant height, stunted root system and fewer branches (Guan et al. 2012). Therefore, I decided to analyze the phenotypic traits in the D27-13 and C8-2 transgenic lines. Plant height and root dry weight were measured in the 4-week-old plants used for strigolactone measurements, as well as in mature wild-type and T_1 transgenic plants grown in the greenhouse. In addition, the number of cobs per mature plant was also measured, because this is equivalent to the outgrowth of axillary branches (Guan et al. 2012). Both shoot length and root mass were significantly reduced in 4-week-old transgenic plants from lines C8-2 and

D27 13 compared to wild-type plants (Figure 7A), although this phenotype was not observed in mature plants. Root weight was greater in plants from both transgenic lines compared to wild-type plants but the differences were not statistically significant. The height of C8-2 transgenic plants was similar to the wild-type, whereas D27-13 transgenic plants were significantly shorter. Interestingly, the number of cobs per plant was significantly higher in both transgenic lines (Figure 7B).

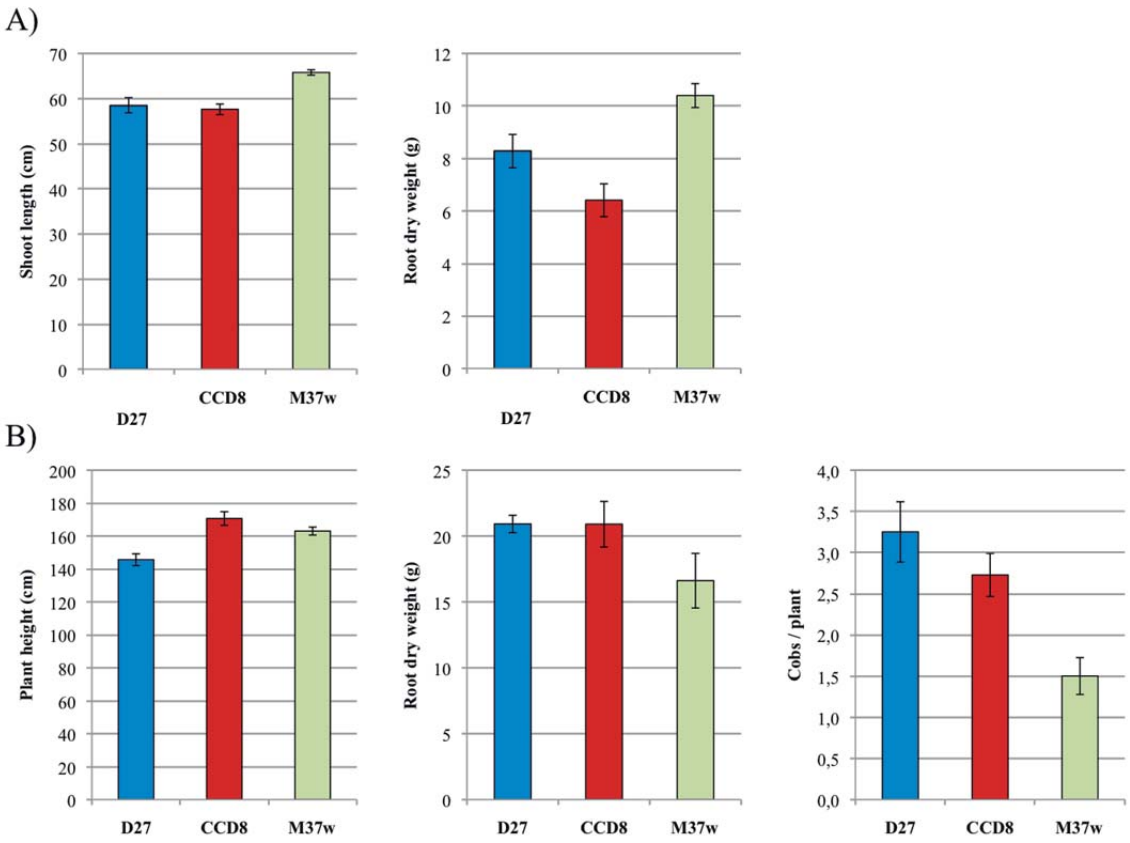

Figure 7. *Phenotypic analysis of transgenic lines C8-2 and D27-13 compared to wild-type. A) Shoot length and root dry weight of 4-week-old plants. Values are the mean of 10 plants ± SE. B) Plant height, root dry weight and number of cobs per 4-week-old plant. Values are the mean of eight plants ± SE for plant height and number of cobs per plant, and the mean of four plants ± SE for root dry weight.*

3.5.6. *Striga* germination bioassay

The capacity of the transgenic lines to stimulate *Striga* germination was evaluated in preliminary experiments. Root exudates from both transgenic lines and wild-type plants were collected, diluted and spotted onto discs containing *Striga* seeds, and after several days the number of germinating *Striga* seeds was counted. Compared to the

wild-type control, the capacity to induce germination of *Striga* seeds fell 50% in line C8-2 and 30% in line D27-13 (Figure 8).

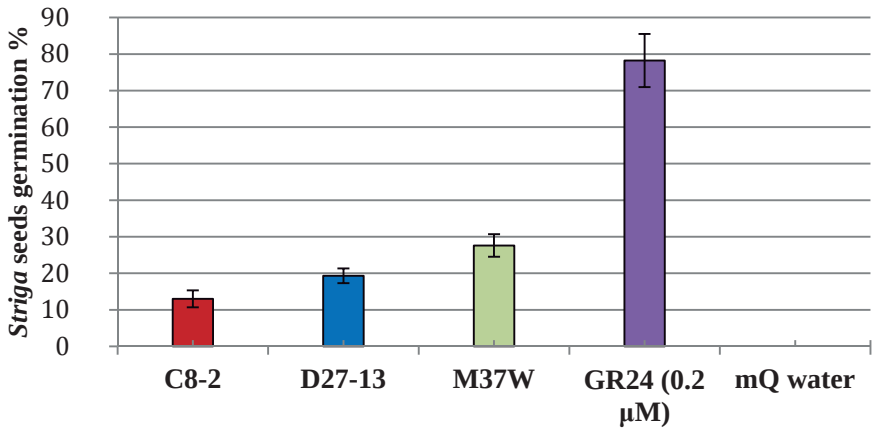

Figure 8. Striga *germination bioassay. Root exudates collected from C8-2, D27-13 and wild-type plants were spotted onto discs containing 50* Striga *seeds, and the percentage of germinating seeds was counted (0.2 µM GR24 and miliQ water were used as positive and negative controls, respectively). Values represent the mean of 15 discs ± SE (three plants per genotype and five discs per plant).*

3.6. Discussion

In the work described in this chapter, I aimed to generate transgenic maize plants with increased resistance to *Striga* infection by reducing the production of strigolactones. These plant hormones play a key role in host recognition by *Striga* plants (Cardoso et al. 2011), and the downregulation of key genes in the strigolactone biosynthesis pathway is an efficient strategy to induce *Striga* resistance in crops (Vogel et al. 2009; Kohlen et al. 2012). The entire strigolactone biosynthetic has not been fully elucidated, although the upstream component was recently characterized (Alder et al. 2012). Genetic studies of branching mutants from *Arabidopsis thaliana* (*more axillary growth* [*max*]), rice (*dwarf* [*d*]), pea (*ramosus* [*rms*]) and petunia (*decreased apical dominance* [*dad*]) indicated the existence of a novel branching hormone (Goulet and Klee 2010), and identified three genes involved in the synthesis of strigolactones: *max4*, *max3* and *max1*, encoding CCD7, CCD8 and a cytocrome P450, respectively (Sorefan et al. 2003; Booker et al. 2004; Matusova et al. 2005). The identification of strigolactones as the shoot-branching hormone confirmed an essential role of CCD7 and CCD8 in their synthesis (Gómez-Roldán et al. 2008; Umehara et al. 2008), and

the molecular characterization of the rice mutant *d27* revealed that the iron-containing enzyme D27 was also involved in the biosynthesis of strigolactones, although its exact role was unclear (Lin et al. 2009). An *in vitro* study described the sequential activity of D27, CCD7 and CCD8 in the conversion of β-carotene to carlactone, the first precursor with strigolactone-like activity (Alder et al. 2012). The cytochrome P450 encoded by *max1* has been postulated to act in the conversion of carlactone to 5-deoxystrigol (Ruyter-Spira et al. 2013).

In the wild-type variety M37W, *Zmccd7* and *Zmccd8* are expressed in roots at high and moderate levels, respectively, whereas their expression in leaves and shoots is much lower (Figure 4A). This matches with the expression profiles reported for other maize varieties in earlier studies (Vallabhaneni et al. 2010; Guan et al. 2012). *Zmd27* expression was characterized for the first time, and it is expressed in roots, stems and leaves at moderate levels (Figure 4A). The higher levels of *Zmd27* expression compared to the downstream genes *Zmccd7* and *Zmccd8* in the aerial parts of the plant may indicate an additional role for D27 in these organs. Similarly, the high levels of *Zmccd7* mRNA in roots may reflect its role not only in strigolactone biosynthesis but also in the formation of the C_{13} cyclohexenone and C_{14} mycorradicin apocarotenoids, which accumulate in large amounts late in the establishment of symbiosis with arbuscular mycorrhizal fungi and may be involved in the turnover of arbuscules (Vogel et al. 2009; Walter and Strack 2011).

The D27 transgenic maize lines showed a strong knockdown of *Zmd27* expression in leaves (Figure 4B). However, RNAi silencing seemed to be attenuated in roots, where the expression of target genes was reduced by 50% and 30% in the D27-13 and C8-2 plants, respectively (Figure 4C). Similar results were obtained with the P-starved plants used for strigolactone measurements, which showed on average a 50% knockdown of the target genes (Figure 6). In both transgenic lines, the RNAi construct was strongly expressed in the roots, so there is no obvious explanation why gene silencing was less effective than in leaves. Previous reports using RNAi-mediated silencing to inhibit *Slccd7* and *Slccd8* expression in tomato plants showed substantial (>80%) knockdown of the target genes in roots (Vogel et al. 2009; Kohlen et al. 2012). In those studies, the lower expression levels of the target genes strongly correlated with limited strigolactone production, the appearance of a distinctive phenotype, and increased resistance to *Striga* infection. Such correlations were

weaker in line C8-2, and were inconsistent in line D27-13. The moderate knockdown of *Zmccd8* in the roots of line C8-2 resulted in a severely limited production of SLCs, a moderately altered phenotype, and a reduced capacity to induce *Striga* germination (Figures 5, 7 and 8). Conversely, the knockdown of *Zmd27* in the roots of line D27-13 did not alter the production of SLCs. Even so, this line showed an altered phenotype similar to that observed in line C8-2, and a moderate loss of capacity to stimulate *Striga* germination (Figures 5, 7 and 8).

In contrast to the above, the major strigolactone produced in wild-type rice plants (2'-*epi*-5-deoxystrigol) was undetectable in root exudates of the rice *d27* mutant, which showed a greatly reduced capacity to stimulate the germination of *Orobanche* (Lin et al. 2009). However, *Osd27* expression was totally absent in the *d27* mutant, whereas *Zmd27* was still expressed at low levels in the transgenic maize line D27-13. Therefore, it is possible that D27 activity is not a rate-limiting step in strigolactone biosynthesis in maize, and that low levels of *Zmd27* expression are enough to produce the required amount of 9-*cis*-β-carotene for strigolactone production (Alder et al. 2012). Accordingly, the knockdown of CCD8, which has been proposed as a rate-limiting enzyme in rice and *Arabidopsis* (Lin et al. 2009; Auldridge et al. 2006), substantially reduced the production of SLCs in C8-2 plants. Nevertheless, this alone would not explain the altered phenotype of the D27-13 plants (Figure 7). As suggested above, D27 may have an additional function in the aerial part of the plant, perhaps in the final steps of strigolactone biosynthesis or even in the signaling pathways downstream of strigolactone production (Ruyter-Spira et al. 2013). In this case, the additional function could be affected by the potent knockdown of *Zmd27* in the leaves of line D27-13, resulting in the observed altered phenotype.

High-branching phenotypes are associated with strigolactone deficiency in a number of different plant species (Auldridge et al. 2006; Ruyter-Spira et al. 2013) and they were also observed in RNAi transgenic tomato lines with impaired strigolactone production (Vogel et al. 2009; Kohlen et al. 2012). However, the architecture of transgenic lines D27-13 and C8-2 was not altered substantially (e.g. no drastic increase in the number of tillers), as previously reported for the maize *ccd8* mutant (Guan et al. 2012). The architecture of maize plants is mainly determined by the transcription factor encoded by the *Teosinte branched 1* (*Tb1*) gene (Doebley et al. 1997), which inhibits the outgrowth of branches (tillers and ears) and initiates the

development of the female inflorescence (Clark et al. 2006; Doebley et al. 1997). Genetic studies with *Tb1* orthologs in *Arabidopsis*, rice and pea suggested that this gene acts downstream of the strigolactone signaling pathway to control branching in these species (Aguilar-Martínez et al. 2007; Minakuchi et al. 2010; Braun et al. 2012). However, the *Tb1* regulatory network in maize is uncoupled from strigolactone signaling, so these hormones only play a minor role in modeling plant architecture (Guan et al. 2012). The strigolactone-deficient maize *ccd8* mutant showed many subtle phenotypic alterations, such as narrow stems, a stunted root system, a slightly reduced plant height, and a modest increase in the number of cobs (Guan et al. 2012). D27-13 and C8-2 transgenic lines exhibited some of these characteristics, including an increased number of cobs per plant (Figure 7).

A previous study that aimed to characterize strigolactone production in maize, sorghum and pearl millet, showed that 5-deoxystrigol and sorghumol were detected in maize exudates (Awad et al. 2006). However, these two compounds are found at very low concentrations in maize exudates and they are not detected in every genotype (C Ruyter-Spira, Wageningen University, personal communication). All five unidentified SLCs detected in this study have been detected in other maize varieties. They stimulate *Striga* germination, are induced by P-starvation and are inhibited in the presence of fluridone (a carotenoid inhibitor). Although their structures have not been determined, preliminary UPLC-MS/MS analysis revealed the presence of the typical strigolactone D-ring in their structure (C Ruyter-Spira, Wageningen University, personal communication). These combined data strongly suggests that these five compounds are novel strigolactones.

The ultimate step in the characterization of these transgenic lines would be *Striga* infection assays to evaluate whether the reduced capacity to induce *Striga* germination is translated into a lower *Striga* infection rate in a realistic rhizosphere environment. In addition, the analysis of arbuscular mycorrhizal colonization of both transgenic lines would be needed to determine whether their capacity to establish symbiosis is affected.

3.7. Conclusions

RNAi-mediated silencing was used to knock down two endogenous genes involved in strigolactone biosynthesis in transgenic maize plants. *Zmccd8* knockdown in line C8-2

inhibited strigolactone production, caused modest phenotypic alterations and reduced the capacity of root exudates to stimulate the germination of *Striga* seeds, thus indicating that this strategy offers great potential to generate *Striga*-resistant maize varieties. Conversely, strigolactone levels in D27-13 plants were not affected by the knockdown of *Zmd27*, although phenotypic alterations were observed suggesting that D27 in maize plants may be required for additional roles other than strigolactone biosynthesis.

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Chapter 4

The contribution of transgenic plants to better health through improved nutrition: opportunities and constraints

The contribution of transgenic plants to better health through improved nutrition: opportunities and constraints

4.1. Abstract

Malnutrition is a prevalent and entrenched global socioeconomic challenge that reflects the combined impact of poverty, poor access to food, inefficient food distribution infrastructure and an over-reliance on subsistence mono-agriculture. The dependence on staple cereals lacking many essential nutrients means that malnutrition is endemic in developing countries. Most individuals lack diverse diets and are therefore exposed to nutrient deficiencies. Plant biotechnology could play a major role in combating malnutrition through the engineering of nutritionally-enhanced crops. In this article, we discuss different approaches that can enhance the nutritional content of staple crops by genetic engineering (GE) as well as the functionality and safety assessments required before nutritionally-enhanced GE crops can be deployed in the field. We also consider major constraints that hinder the adoption of GE technology at different levels, and suggest policies that could be adopted to accelerate the deployment of nutritionally-enhanced GE crops within a multicomponent strategy to combat malnutrition.

4.2. Introduction

Food security is one of the pillars of health and wellbeing in society because humans rely on food not only to supply energy but also for essential nutrients that maintain the immune system and keep the body in a good state of repair. Adequate nutrition therefore correlates with lower morbidity and mortality from both infectious and non-infectious diseases and is particularly important in children and pregnant women where the lack of essential nutrients can lead to irreversible physical and mental damage during development (Hoddinott et al. 2008).

Malnutrition is more prevalent in the developing world because it often reflects the lack of access to nutritious food. This in turn is frequently caused by poverty, which can occur due to ill-health and an inability to work, the typical consequences of malnutrition. Poverty, malnutrition and poor health therefore can form a self-reinforcing cycle from which many people (and in some cases entire populations) find it impossible to escape (Farré et al. 2011b).

Almost 50% of the world's population is currently affected by malnutrition (Christou and Twyman 2004). The majority are subsistence farmers who depend entirely on staple cereal crops such as maize or rice, which are deficient in several essential nutrients (Zhu et al. 2007). The poverty-malnutrition-disease cycle needs to be broken by multipoint interventions that provide direct, effective and sustainable approaches to increase the economic welfare of the world's poorest people, including the provision of drugs and vaccines that tackle poor health, and adequate access to nutritious food (Perseley 2000). Although there are many global initiatives promoting short and mid-term strategies that tackle poverty, food insecurity/malnutrition and disease, we need sustainable solutions that provide the means for the world's poor to build their own healthy societies, as envisaged by the Millennium Development Goals (Yuan et al. 2011).

Strategies to tackle malnutrition fall into three major categories (Gómez-Galera et al. 2010). The most desirable approach is to increase the diversity of food intake but this is impractical in many developing-country settings, particularly for low-income populations. In some cases it may be possible to change local food-processing conventions to prevent the loss of nutrients during preparation (Hotz and Gibson

2007) or to promote certain styles of preparation that maximize nutritional bioavailability (Yang and Tsou 2006).

The second approach is to add nutrients to the diet artificially (Supplementation), either by providing supplements or by the fortification of basic food products such as salt and flour, but it is unsustainable over the longer term because it relies on a robust distribution infrastructure and on consumer compliance (Hotz and Brown, 2004). Fortification is more sustainable and there have been remarkable successes, such as the provision of iodine-fortified salt, and wheat flour double-fortified with iron and folate (Rosenberg 2005). However, this strategy also requires government coordination and a distribution infrastructure and is therefore unsuitable for those parts of the developing world relying on subsistence agriculture.

The final strategy is known as biofortification, in which crops are modified or treated to accumulate additional nutrients at source (Zhu et al. 2007). In some cases biofortification can be achieved by adding nutrients to fertilizers, an approach that has been extraordinarily successful to increase the availability of zinc and selenium (Lyons et al. 2003). However, this is not suitable for iron, because it is immobilized in the soil (Gómez-Galera et al. 2012a), or for essential organic nutrients (vitamins and essential amino acids and fatty acids) which are the products of plant metabolism. It may be possible to increase the content of organic nutrients by conventional breeding if there is sufficient genetic diversity in existing germplasm, although it would take many generations to introgress these traits into local elite breeding varieties (Welch and Graham 2005). The alternative is genetic engineering (GE) technology, which can introduce genes from any source directly into the local varieties used by subsistence farmers, to increase the levels of organic and/or mineral nutrients. First-generation GE crops have already shown their potential to enhance agricultural productivity and reduce poverty in developing countries (Christou and Twyman 2004, Farré et al. 2010a, 2011b). A second generation of GE crops is now under development that can address nutritional requirements directly by contributing to multipoint intervention strategies (Yuan et al. 2011). As well as offering an unrestricted choice of genes for nutritional improvement, the GE approach allows nutritional traits to be targeted to specific organs (e.g. cereal seeds) and multiple traits can be combined in the same plants without complex breeding programs (Zhu et al. 2007; Naqvi et al. 2009a).

4.3. GE strategies to enhance the content of organic micronutrients

Humans can synthesize almost all the organic compounds needed for normal physiological activity but a small number of specific molecules known as essential nutrients are required in the diet (Table 1). Some of these essential nutrients are amino acids or fatty acids; the rest are chemically diverse but are grouped together as vitamins. All GE strategies focusing on essential organic micronutrients involve the modulation of plant metabolism.

Vitamin A

The reduced form of vitamin A (retinal) is required for the production of rhodopsin, which is essential for sight and also helps to maintain epithelial and immune cells. The acidic form (retinoic acid) is a morphogen in development. Humans can produce retinal and retinoic acid if provided with a source of retinol or one of its esters, which are abundant in meat and dairy products. However, retinal can also be synthesized directly from b-carotene (also known as pro-vitamin A), which is produced mainly by plants and photosynthetic microbes, but also some non-photosynthesizing organisms (Botella-Pavía and Rodríguez-Concepción 2006). Vitamin A deficiency (VAD) is one of the most prevalent deficiency diseases in developing countries, affecting more than 4 million children each year, up to 500,000 of whom become partially or totally blind (Harrison, 2005).

The amount of β-carotene produced by plants can be enhanced by increasing flux through the carotenoid pathway (Figure 1), e.g. by increasing the availability of carotenoid precursors, by expressing enzymes in the early part of the pathway between GGPP and lycopene, by increasing metabolic flux in the β-branch by favoring LYCB activity at the expense of LYCE, or by increasing the sink capacity for carotenoids to remove feedback limitations (Zhu et al. 2009; Farré et al. 2010b; 2011a; Bai et al. 2011). The overexpression of DXP synthase in tomato is an example of the first strategy, producing a carotenoid precursor that increases flux in the entire pathway and enhances the total carotenoid content (Enfissi et al. 2005). Cassava roots expressing the bacterial CrtB gene accumulated up to 21 mg/g of carotenoids, a 34-fold increase with respect to the wild-type (Welsch et al. 2010). The replacement of the daffodil gene with its maize ortholog is the basis of Golden Rice 2, which produces up to 37 mg of carotenoids per gram dry weight of grain, of which 31 mg/g

is b-carotene (Paine et al. 2005). The same genes expressed in maize have yielded kernels containing up to 57.3-60 mg/g dry weight of β-carotene (Zhu et al. 2008; Naqvi et al. 2009b; Table 2). The third strategy is exemplified by Golden Potato, where expression of three Erwinia genes encoding phytoene synthase (CrtB), phytoene desaturase (CrtI) and lycopene beta-cyclase (CrtY) lead to the diversion of carotenoid synthesis from the α- to the β- branch (Figure 1), resulting in the accumulation of 47 μg/g DW of β-carotene (Diretto et al 2007, Table 2). Potato also provides a good example of the fourth strategy. The expression of the cauliflower *Or* gene in tubers increased the storage capacity for carotenoids by promoting the formation of chromoplasts, generating transgenic potatoes with orange tuber flesh containing 10 times the normal amount of β-carotene (López et al. 2008, Table 2).

Vitamin C

Ascorbate (vitamin C) is an antioxidant and also cofactor of several enzymes, including those required for the synthesis of collagen, carnitine, cholesterol, and certain amino acid hormones. Vitamin C deficiency causes the ulceration disease scurvy, reflecting the breakdown of connective tissues (Bartholomew 2002).

There are several biosynthetic pathways that generate ascorbate in plants, and when ascorbate is oxidized it can be recycled through an additional pathway with glutathione as the reductant (Figure 2). The amount of ascorbate accumulating in plants can therefore be enhanced not only by increasing its biosynthesis but also the rate at which the molecule is recycled. In the first approach, the overexpression of L-gulono γ-lactone oxidase (GLOase) in lettuce resulted in the accumulation of up to 580 nmol/g fresh weight of ascorbate, a seven-fold improvement (Jain and Nessler 2000). A two-fold increase was achieved by expressing the same gene in potato tubers (Hemavathi et al. 2010). Multivitamin maize expressing the rice *dhar* gene from the ascorbate recycling pathway accumulated six times the normal level of ascorbate (Naqvi et al. 2009b, Table 2).

Vitamin B9

Folate (vitamin B9) is a tripartite molecule combining pterin, p-aminobenzoate (PABA) and one or more glutamate moieties, which are derived from three separate metabolic pathways in different subcellular compartments (Figure 3). Folate is the

source of tetrahydrofolate, which is essential for DNA synthesis and many other core metabolic reactions. In adults, folate deficiency causes macrocytic anemia and elevated levels of homocysteine, but the impact on pregnant women is much more severe, leading to the neural tube defect spina bifida in the fetus (Scholl and Johnson 2000).

Moderate increases in folate levels have been achieved by modifying the pterin and PABA pathways individually, but increasing the flux through one pathway only reveals bottlenecks in the other. Díaz de la Garza et al. (2007) crossed two transgenic tomato lines, one expressing GCH1, which enhanced the cytosolic (pterin) branch, and the other ADCS1 which enhanced the PABA branch. In the individual lines, the maximum enhancement was double the normal level of folate. However, combining the two transgenes in a single line released the bottlenecks in both branches simultaneously and achieved a 25-fold increase in folate levels. The same strategy in rice endosperm resulted in a 100-fold increase in folate levels because the base levels were lower than in tomato, indicating how powerful this strategy could be in developing country settings where rice is the staple diet (Storozhenko et al. 2007, Table 2).

Vitamin E

Vitamin E comprises eight related molecules known as tocochromanols. These are formed from a chromanol head decorated with methyl groups (to yield α, β, γ, and δ derivatives) plus either a phytyl tail to produce the tocopherols, or a geranylgeranyl tail to produce the tocotrienols (DellaPenna and Pogson 2006). The tocochromanols are powerful antioxidants that protect fatty acids, low-density lipoproteins (LDLs) and other components of cell membranes from oxidative stress.

Plants can be engineered to accumulate higher levels of vitamin E by overexpressing genes involved in tocochromanol synthesis (Figure 4). This can be achieved either by increasing the total tocochromanol content or skewing tocochromanol synthesis toward the more potent isomers, particularly α-tocopherol which is absorbed more efficiently by humans. For example, Cho et al. (2005) increased the α/γ tocopherol ratio in transgenic lettuce plants by expressing the Arabidopsis γ-tocopherol methyltransferase (γ-TMT), and achieved near complete conversion to a-tocopherol in

the best-performing transgenic lines. Similarly, Tavya et al. (2007) reported a 10.4-fold increase in α-tocopherol levels and a 14.9-fold increase in β-tocopherol levels in soybean seeds expressing *Perilla frutescens* γ-TMT. The constitutive expression of two Arabidopsis cDNA clones encoding r-hydroxyphenylpyruvate dioxygenase (HPPD) and 2-methyl-6-phytylplastoquinol methyltransferase (MPBQ MT) increased the tocopherol content by threefold in transgenic maize (Naqvi et al. 2011). Overexpression of Arabidopsis HPPD in rice grains shifted tocopherol synthesis from the γ to the α form with no increase in absolute tocopherol levels (Farré et al. 2012).

Essential amino acids

Nine of the 20 standard amino acids are constitutive essential nutrients because they cannot be synthesized de novo by humans, and others are essential under certain circumstances, such as child development or in the context of metabolic disorders. The most relevant examples are lysine, threonine, tryptophan, methionine and cysteine because staple cereals are poor sources of lysine and threonine, and staple legumes are poor sources of tryptophan, methionine and/or cysteine (Zhu et al. 2007).

Two GE strategies have been used to tackle amino acid deficiency: (i) engineering plants to produce proteins containing essential amino acids; and (ii) engineering amino acid metabolism to increase the availability of essential amino acids in the free amino acid pool. Lysine was the first target in both strategies. One of the earliest attempts to overcome the poor nutritional value of cereal proteins was the expression of lysine-rich pea legumin in cereal endosperm, which resulted in transgenic rice and wheat grain protein containing up to 4.2% lysine (Sindhu et al. 1997; Stöger et al. 2001). More significant improvements were achieved by adding lysine residues to endogenous cereal storage proteins e.g. 12 residues added to barley hordothionine to produce HT12, and eight added to barley high lysine protein to produce HL8 (Jung and Carl, 2000). When expressed in maize along with the bacterial enzyme dihydrodipicolinate synthase (DHPS) the total lysine content was four times the normal amount, accounting for 0.8% of total protein composition. Another lysine-rich storage protein (sb401) increased the lysine content in maize seeds by 55% (Yu et al. 2004). Similar improvements have been achieved by expressing lysine-rich animal proteins such as porcine α-lactalbumin, which increased the lysine content in maize by 47% (Bicar et al. 2008). Maize seeds containing up to 26% lysine have also been

produced by expressing a heterotypical Arabidopsis lysyl tRNA synthetase, which inserts lysine residues in place of other amino acids during the synthesis of seed-storage proteins (Wu et al. 2007). Finally, the lysine content of maize has been increased by using RNA interference to silence one of the zein storage protein genes allowing the protein complement to be filled with lysine-rich storage proteins (Segal et al. 2003).

One of the success stories in terms of storage protein engineering involves the expression in staple crops of *Amaranthus hypochondriacus* seed storage protein, which is rich in all the essential amino acids and has a composition close to ideal for humans. Transgenic maize seeds expressing the AH protein contained up to 32% more protein than wild-type seeds and contained higher levels of lysine, tryptophan and isoleucine (Rascón-Cruz et al. 2004). Similarly transgenic potato tubers expressing AH contained 45% more protein than normal (Chakraborty et al. 2000) and transgenic wheat seeds contained nearly 2.5% AH as a proportion of total seed protein, increasing the levels of lysine to 6.4% and tyrosine to 3.8% (Tamás et al. 2009).

Expression of feedback-insensitive dihydrodipicolinate synthase (DHPS) in maize increased lysine levels from <2% to ~30% of the free amino acid pool, with concomitant increases in threonine (Frizzi et al. 2008). The key rate-limiting enzyme in tryptophan synthesis is anthranilate synthase, which catalyzes the conversion of chorismate to anthranilate, and tryptophan levels in rice have been increased more than 400-fold by expressing a feedback insensitive version (Wakasa et al. 2006). Tryptophan levels also increased 30-fold in potato tubers (Yamada et al. 2004) and 20-fold in soybean seeds (Ishimoto et al. 2010) expressing feedback-insensitive AS.

Essential fatty acids

Humans can synthesize most fatty acids de novo but the health-promoting ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) are exceptions that need to be sourced from the diet (Djoussé et al. 2011). Once acquired, simple ω-3 PUFAs such as α-linolenic acid can be converted into more complex very-long-chain polyunsaturated fatty acids (VLC-PUFAs) such as arachidonic acid (ARA), and these can be converted back to the simpler species. This interconversion means the class rather than individual PUFAs is regarded as essential. The same principles apply to ω-6 PUFAs. The

essential fatty acids are abundant in fish, shellfish, nuts and leafy vegetables but they are not present in cereals (Farré et al. 2011b).

The fatty acid biosynthesis pathway in plants was modulated to produce ω-3 and ω-6 PUFAs by introducing the microbial enzymes responsible for a sequence of fatty acid desaturation and elongation reactions (Domergue et al. 2005). The key targets are linoleic acid and α-linolenic acid, and the VLC-PUFAs ARA, eicosapentenoic acid (EPA) and docosahexenoic acid (DHA) (Zhu et al. 2007). The biosynthesis of VLC-PUFAs has been achieved by expressing microbial desaturases and elongases in linseed, soybean and mustard (Abbadi et al. 2004; Kinney et al. 2004; Wu et al. 2005) with the experiments in mustard demonstrating the feasibility of a stepwise approach to first optimize the accumulation of ω-3 fatty acids (i.e. EPA rather than ARA) and then to convert this to DHA, resulting in the accumulation of ARA (4%), EPA (8%) and DHA (0.2%). These successes have led to the long-term goal of producing such fatty acids in transgenic oilseed crops (Abbadi et al. 2004; Domergue 2005).

4.4. GE strategies to enhance the content of mineral micronutrients

Unlike organic nutrients, which are the product of plant metabolism, inorganic nutrients (minerals) must be taken up by plants from the environment. GE approaches to enhance mineral nutrients are therefore diverse, focusing on strategies such as increasing the solubility of these nutrients in the rhizosphere, mobilizing them in the plants, transporting them to storage organs, increasing the storage capacity of the plant, and maximizing bioavailability (Gómez-Galera et al. 2010). The four most significant minerals in terms of nutrient deficiency are iron, zinc, selenium and calcium. Other minerals, while no less essential, are so abundant and ubiquitous that deficiency disorders are very rare. It is not uncommon for populations to be deficient for several of the above minerals at the same time so there has been recent interest in the development of crops tailored to provide all these limiting nutrients simultaneously (Zhu et al. 2008; Naqvi et al. 2009a).

Iron

Iron deficiency is the most prevalent form of mineral malnutrition in the world, with more than 2 billion people at risk. The primary clinical manifestation is anemia, and it

is estimated that more than half of all cases of anemia worldwide are caused by a lack of iron (Benoist et al. 2008).

Biofortification offers a more sustainable approach for subsistence farmers and many different strategies have been investigated (Drakakaki et al. 2005; Wirth et al. 2009). One of the major challenges with iron is that its mobility in the rhizosphere is dependent on the soil conditions, because only the ferrous form (Fe^{2+}) is soluble and bioavailable to plants whereas the ferric form (Fe^{3+}) is sequestered into insoluble complexes with soil particles (Gómez-Galera et al. 2012a). Plants have evolved two counterstrategies, one of which is to secrete reductases into the soil to convert ferric iron into the soluble ferrous form, and the other is to release chelating agents known as phytosiderophores (PS) that can be reabsorbed by the roots as PS-Fe^{3+} complexes. Iron levels in plants can therefore be improved by increasing the export of both reductases and phytosiderophores, e.g. by overexpressing the enzymes nicotianamine synthase (NAS) or nicotianamine aminotransferase (NAAT) which are involved in phytosiderophore synthesis (Zheng et al. 2010; Johnson et al. 2011). For example, transgenic rice plants expressing the NAS genes OsNAS1, OsNAS2 or OsNAS3 accumulated up to 19 mg/g of iron in the endosperm (Johnson et al. 2011, Table 2). Additional strategies include the overexpression of iron transporter proteins (many of which also co-transport zinc, see below), the overexpression of ferritin (which stores large amounts of iron in a bioavailable form) and the expression of phytase, which breaks down phytate and makes the stored iron easier to absorb in the human digestive system (see section on antinutritional factors, below).

Zinc

Zinc is required as a cofactor in many different enzymes and is also a coordinating ion in the DNA-binding domains of transcription factors. Zinc deficiency affects more than 2 billion people worldwide predominantly in developing countries and manifests as a spectrum of symptoms including hair loss, skin lesions, fluid imbalance (inducing diarrhea), and eventually wasting of body tissues (Hambidge and Krebs 2007).

Cereal grains are poor sources of zinc, but because this mineral is more soluble than iron and easier to take up from the soil, GE strategies to increase the zinc content of plants have concentrated on transport and accumulation (Palmgren et al. 2008). Many phytosiderophores and transporters can interact with both iron and zinc so the

expression of NAS/NAAT and transporters such as *OsYSL15* and *OsIRT1* in rice can increase the levels of both minerals (e.g. Lee et al. 2011, Table 2).

Selenium

Selenium is a component of enzymes and other proteins that contain the amino acids selenocysteine and selenomethionine, which are required for the interconversion of thyroid hormones; therefore selenium and iodine deficiency can have similar symptoms (Khalili et al. 2008). Selenium deficiency is rare at a population level because the mineral is taken up efficiently from the soil, and fertilizers with selenium have proven successful to prevent deficiency in areas where the soil is depleted (Lyons et al. 2003). GE strategies to increase selenium levels therefore focus on storage and accumulation. For example, the expression of Arabidopsis ATP sulfurylase in mustard increased the selenite content by up to threefold in roots and shoots (Pilion-Smits et al. 1999, Table 2).

Calcium

Soluble calcium is an electrolyte and signaling molecule, but most of the calcium in the human body is present in its mineralized form as a component of bones and teeth. The replenishment of serum calcium by bone resorption is slow, so dietary calcium deficiency in the short term can lead to electrolyte imbalance and over the long term can cause osteoporosis. The risk is higher in children as the rapid bone growth that occurs during childhood results in a high demand for calcium. Calcium-rich dairy products tend to be inaccessible in developing country settings so many children and adults are malnourished (Dayod et al. 2010). Root vegetables and leafy crops such as lettuce are good sources of calcium although in some vegetables the high content of phytate and oxalate makes the calcium difficult to absorb (Jeong and Gourinet 2008). GE strategies to increase the calcium content of plants include the expression of calcium transporters such as AtCAX1, which increased the calcium content of carrots and potatoes by up to threefold (Connolly 2008; Park et al. 2005, Table 2).

Nutrient enhancers and anti-nutrients

A number of compounds are known to enhance or inhibit the absorption of plant minerals by the human digestive system, and mineral bioavailability can therefore be increased by promoting the accumulation of enhancers or eliminating anti-nutrients

(Gibson 2007). Some key nutrients act as enhancers and therefore provide dual benefits, e.g. ascorbate and b-carotene promote iron uptake by chelating and/or reducing Fe^{3+} and preventing interactions with phytate and polyphenols (García-Casal et al. 2000).

Phytic acid is a key anti-nutrient because it is abundant in cereals, legumes and oil seeds where it binds all the principal mineral nutrients and sequesters them into stable complexes that cannot be absorbed (Lopez et al. 2002). The amount of phytic acid in seeds can be reduced by silencing genes involves in its biosynthesis, such as *myo*-inositol-1-phosphate synthase (Nunes et al. 2006) or 1D-*myo*-inositol 3-phosphate synthase (Kuwano et al. 2009). Expression of a thermostable recombinant fungal phytase increased iron bioavailability in wheat (Brinch-Pedersen et al. 2006) and maize (Chen et al. 2008). Transgenic maize plants expressing phytase may well be the first second-generation GE crop to reach the market (Chen et al. 2008).

Cassava is an important staple crop in sub-Saharan Africa but the high content of the cyanogenic glycosides linamarin and lotaustralin means it must be processed before consumption (Jørgensen et al. 2005). Two genes encoding cytochrome P450s (CYP79D1 and CYP79D2) catalyze the first step in linamarin and lotaustralin synthesis), and suppression of these genes has reduced linamarin by 94% (Siritunga and Sayre 2003). RNA interference targeting the same genes reduced the cyanogenic glucoside content by 99% (Jørgensen et al. 2005).

4.5. Risk assessment and regulation of GE crops

Genetically engineered (GE) crops must undergo a risk assessment to assess their potential impact on human health and the environment before they receive market authorization (EFSA 2010a). This involves detailed molecular characterization, comparison to conventional crops of the same species, assessment of potential toxicity/allergenicity and nutritional analysis (USFDA 1992; EFSA 2011). In most parts of the world, authorization follows automatically from a positive evaluation, but the decision-making process in the European Union (EU) has become politicized and it is now almost inevitable that regulatory approvals are ignored and overruled (Sabalza et al. 2011; Ramessar et al. 2010).

There are two key differences between the regulatory systems adopted in the EU (where few GE crops are approved) and in the US, where GE agriculture is strongly established and has an unblemished 15-year safety record (Ramessar et al. 2009). The first is that the US system focuses on the safety of the product compared to a conventional counterpart, whereas the EU system focuses instead on the process. This leads to two major conflicts of logic: (i) identical GE and conventional crops are subject to different tiers of regulation; and (ii) imported GE products are regulated differently because they are not grown in the EU and are not under regulations applied to cultivation, i.e. identical GE products are subject to different tiers of regulation according to origin. The second difference is that the US system is based on substantial equivalence, i.e. a GE crop is approved if it is largely the same as its conventional counterpart and that the differences do not introduce clearly defined risks that can be demonstrated experimentally. In contrast, the EU system is based on the precautionary approach, which essentially means that a GE crop is not approved unless the absence of risk can be demonstrated. Since it is impossible to prove that a risk is zero (as opposed to vanishingly small) this creates an effective moratorium on GE agriculture in Europe.

The risk assessment of GE crops focuses on two main aspects – the potential effect on the environment and the potential effect on human health through consumption. Ostensibly the assessment criteria should be based on rational scientific premises but there remain several key areas where arbitrary factors are included, e.g. the risk of gene transfer from plants to microbes in the environment, which is too low to quantify because there is no evidence for this process throughout millions of years of evolution yet it cannot be proven to be zero (Ramessar et al. 2007; EFSA 2010a). Even EFSA itself acknowledges this inconsistency yet the requirement persists (EFSA 2009). It is also necessary to determine the risk posed by GM plants, their pollen and residues to agricultural workers when no adverse effects have been reported after 15 years of GE agriculture in the US, Argentina, Brazil, China and India, whereas similar risk assessments are not required for conventional crops, even those known to produce potentially harmful products such as alkaloids in tobacco and erucic acid in rapeseed and mustard oil (EFSA 2010a).

GE crops must also undergo toxicological analysis to prove the absence of unintended effects using animal models (usually rodents) and this may be extended from acute

tests to multigenerational chronic toxicity/reproductive tests if deemed appropriate (EFSA 2011).

The best known example of a nutritionally enhanced crop is Golden Rice (Ye et al. 2000), which was developed in 1999 but has been mired in regulatory bureaucracy ever since despite the successful completion of multiple safety assessments *in silico*, *in vitro*, in animal models and in human trials (Tang et al. 2009). Golden Rice therefore provides an excellent example of the major deficiency in current regulatory processes, i.e. that they focus on the risks rather than a risk-benefit analysis (Kowalski 2007). Genetic engineering reduces the time and cost required for developing plants enhanced with one or several traits, but these savings are compromised by the long and costly regulatory process that is not applied to conventional varieties even if they incorporate exactly the same traits and use similarly "unnatural" processes such as forced hybridization or chemical mutagenesis (Sabalza et al. 2011).

4.6. Functionality testing

The ultimate success of biofortification can only be judged by its impact on human health and nutrition after deployment (König et al. 2004). The anti-nutritional factors discussed earlier prevent the digestion, absorption and/or utilization of nutrients, so GE crops must undergo bioavailability studies to predict their impact at the population level, and this is also an important part of the regulatory process (Hirschi 2008)

The bioavailability of micronutrients in a staple crop depends on overlapping diet and host-related factors such as co-consumed foods, the geographical region, and the efficiency with which individual consumers absorb certain nutrients (Gibson 2007). Diet-related factors, which predominantly reflect interactions among micronutrients and other organic compounds, have a profound effect on the efficiency of a nutritionally-enhanced crop (Graham et al. 2001). Such factors include the chemical form (speciation) of the nutrient, e.g. selenomethionine is a more bioavailable form of selenium than any inorganic source (Combs 2001) and heme-iron from meat is much more bioavailable than the non-heme iron from cereals and legumes (Monsen and Balintfy 1982).

4.7. Constraints preventing adoption

Nutritionally enhanced GE crops have an enormous potential to tackle poverty, malnutrition and ill-health particularly in the developing world, but there are several constraints preventing their adoption, cultivation and use. The development of crops with higher levels of key nutrients requires the simultaneous transfer and expression of multiple transgenes in local varieties, which may be recalcitrant to GE approaches. This requirement becomes even more complex if several nutritional components are addressed at the same time (Naqvi et al. 2009a, 2010; Zhu et al. 2008; Farré et al. 2011a).

As well as technical limitations, a further constraint to the adoption of nutritionally enhanced GE crops is their potential socioeconomic impact. The first generation of GE crops provided substantial economic benefits for farmers in developed and developing countries (Qaim 2009; Brookes and Barfoot 2006) and second-generation crops are expected to provide similar socio-economic benefits although favoring the consumer as well as the producer (Qaim et al. 2007). Even so, the costs of development (predominantly regulatory compliance) can exceed $15 million (Kalaitzandonakes et al. 2007) which blocks the approval pathway de facto for public sector institutions and small and medium companies that lack the financial resources of the agro-industry. Only few major companies have the economic muscle to complete the regulatory process, and R&D investment will not be recouped from humanitarian projects (Lemaux 2009). This limits the incentive to invest in GE products tailored for developing countries (Qaim et al. 2007; Qaim 2009).

EU legislation for the approval of GE crops (Directive 2001/18/EC and Regulation EC 1829/2003) also acts as a constraint because it is highly politicized and driven by single-interest groups such as the organic farming lobby and environmental activists rather than rational scientific discourse (Apel 2010). The regulatory pipeline requires years to negotiate even if the applicant has unlimited funds and precise legal knowledge, and acts as a barrier for testing, scientific investigations and commercial production (Kalaitzandonakes et al. 2007; Gómez-Galera et al. 2012b).

Perhaps the most insidious problem with the adoption of nutritionally enhanced GE crops is that this scientific breakthrough has become the flagship campaign for activists, who appear to have garnered public opinion and thus have a disproportionate

effect on politicians, who in turn determine the rules followed by the regulators. The unfortunate consequence of this negative cycle is that the regulations are changing under political influence to make any kind of GE agriculture almost impossible in Europe. For example, the European Parliament recently published a proposal to allow EU member states the opportunity to opt out of GE agriculture without any scientific justification, ostensibly to prevent single member states vetoing cultivation throughout Europe. However, the politicians have overlooked the negative impact this decision would have on the acceptability of GE crops to the public (Sabalza et al. 2011; Morris and Spillane 2010). The EU already labors under scientifically unjustified coexistence measures, including excessive isolation distances for GE crops and unmanageable adventitious presence thresholds that cause trade conflicts with other countries (Ramessar et al. 2009, 2010). Within this hostile environment there is no voice for scientists and indeed no voice for science, a worrying glimpse of a future dark age where superstition and hearsay are more important than facts (Farré et al. 2010a, 2011b).

4.8. Future prospects

The world population will reach 9 billion in 2050, with most of the population increase in developing countries. Global food production will need to increase by 70%, including 3 billion tons of cereals, which means global rice and maize production must double even as the land available for agriculture shrinks in the face of urbanization, land degradation and dwindling access to water (FAO 2009). Future food security therefore depends on our ability to improve the quality as well as the quantity of food and to reduce the global health burden of micronutrient deficiency disease. This will require multidisciplinary collaboration between stakeholders such as farmers, health providers, nutrition experts, plant breeders, the food and agrochemical industries, the biotechnology industry, governments and NGOs to reduce the impact of malnutrition on human populations (Martin et al. 2011). In addition, scientific and technological advances will need to be accompanied by a plethora of policy reforms targeting the current inequalities in global agricultural trade as well as the establishment of mechanisms to effectively guarantee the right-to-food, as stated in the General Introduction.

We also need to increase awareness of the environmental and health benefits of GE crops, which are rarely discussed in the media, such as reduced pesticide and fossil fuel usage in agriculture, reduced mycotoxin levels, and reduced exposure to toxic agrochemicals (Raybould and Quemada 2010; Sanahuja et al. 2011). For example, the planting of Bt cotton has reduced pesticide applications by 50% in India, avoiding up to 9 million poisoning incidents and saving $51 million (Kouser and Qiam 2011). Bt corn also has lower levels of mycotoxins (e.g. fumonisin), saving $23 million annually in crop losses (Wu 2006). Nutritional traits therefore need to be combined with first-generation input traits for maximum benefit (Sun et al. 2004).

Another key future development will be the provision of incentives to develop nutritionally enhanced orphan crops, i.e. crops such as finger millet, cassava and fonio that are grown on a small scale in niche geographical areas. These crops have not been studied in detail and generally produce lower yields and nutritionally poorer or more toxic products than staple crops (Tadele 2009). With sufficient targeted resources they could yet be developed into alternative staples for niche geographical areas (Naylor et al. 2004).

Many developing countries have followed EU protectionism and banned GE products due to consumer and environmental concerns, e.g. the moratorium on GE eggplant in India (Chong 2011), putting millions of lives at risk from malnutrition and the continuous indiscriminate use of toxic pesticides (Ramaswamy 2007). There needs to be a shift from risk assessment to risk-benefit analysis, which is applied in all other technology areas (Ramessar et al. 2009). We need to establish a globally harmonized risk-benefit system that is based on traits rather than events (the product not the process). Furthermore, the Cartagena Protocol on Biosafety needs to be redesigned to reflect the reality of global food security challenges and to address the issue of GE crops specifically, as the current protocol does not consider GE foods that are outside the definition of a living modified organism. We need to remove the precautionary approach and other non-science based doctrines in the context of nutritionally enhanced crops to ensure they can be deployed where they are needed the most and where they can get on with the important task of saving lives.

Table 1. *Food sources of organic and inorganic micronutrients*

Nutrient	Sources	RDA
Vitamin A	Carrot, spinach, kale, apricots, papaya, mango, liver[1]	700 – 1300[*1]
Vitamin C	Peppers, orange, kiwifruit, berries, brussels sprouts[1]	65-120 mg/day[1]
Vitamin B9	Spinach, broccoli, asparagus, beans, peas, citrus fruits, liver[1]	400-600 μg/day[1]
Vitamin E	Nuts, seeds, vegetable oils, spinach, broccoli, kiwifruit[1]	15-19 mg/day[1]
Iron	Red meat, fish, poultry, soybeans, lentils, beans[1]	8-27 mg/day[1]
Zinc	Seafood, meat, poultry, beans, nuts, whole grains[1]	8-13 mg/day[1]
Selenium	Nuts and peanuts, seafood, some meats and cereals[1]	55-70 μg/day[1]
Calcium	Dairy products, nuts, chinese cabbage, kale, broccoli[1]	1000-1300 mg/day[1]
Lysine	Casein, beef, fish, egg, field pea, dry bean, carrot, apple[3]	1734 – 3621 mg/day[2]
Threonine	Casein, fish, chicken, egg, mushroom, potato, corn, fieldpea[3]	918 – 1917 mg/day[2]
Tryptophan	Casein, egg, milk, cheese, cabbage, drybean, mushroom, sorghum[3]	238 - 497 mg/day[2]
Methionine	Cheese, egg, beef, casein, rice, corn, banana, carrot[3]	850 - 1775 mg/day[2]
Cysteine	Egg, beef, chicken, wheat, banana, sorghum, rice, corn[3]	
Linoleic acid	Fish, shellfish, seeds, nuts, leafy vegetables[4]	11-17 g/day[2]
Linolenic acid		1.0 – 1.6 g/day[2]

[*] Recomended Dietary Allowances (RDA) for vitamin A are listed as micrograms (μg) of Retinol Activity Equivalents (RAE) to account for the different biological activities of retinol and provitamin A carotenoids.

[1]Office of Dietary Supplements (2011) National Institutes of Health, Bethesda; [2] Trumbo et al. (2002) J Am Diet Assoc 102:1621-1630; [3] Sosulski and Imafidon (1990) J Agric Food Chem 38:1351-1356; [4] Calder and Deckelbaum (2011) Curr Opin Nutr Metab Care 14:113-114

Table 2. *Transgenic crops engineered for enhanced vitamin and mineral content (continues in the following pages).*

Nutrient	Species	Genes used	Total amount (increase vs. wild-type)	Reference
Vitamin A	Maize *(Zea mays)*	*PacrtB, PacrtI*	33.6 µg/g DW (34)	Aluru et al. (2008)
		Zmpsy1, PacrtI, PcrtW, Gllycb,	146.7 µg/g DW (133)	Zhu et al. (2008)
		Zmpsy1, PacrtI	163.2 µg/g DW (112)	Naqvi et al. (2009)
	Wheat *(Triticum aestivum)*	*Zmpsy1, PacrtI*	4.96 µg/g DW (10.8)	Cong et al. (2009)
	Potato *(Solanum tuberosam)*	*EuCrtB, EuCrtI, EuCrtY*	114 µg/g DW (20)	Diretto et al. (2007)
		BoOr	28.22 µg/g DW (6)	López et al. (2008)
		AtZEP	60.8 µg/g DW (5.7)	Romer et al. (2002)
		PacrtB	35.5 µg/g DW (6.3)	Ducreux et al. (2005)
	Cassava *(Manihot esculenta)*	*PacrtB*	21.84 µg/g DW (33.6)	Welsch et al. (2010)
	Rice *(Oryza sativa)*	*Nppsy1, EucrtI*	1.6 µg/g	Ye et al. (2000)
		Zmppsy1, EucrtI	37 µg/g (23)	Paine et al. (2005)
Vitamin C	Maize *(Zea mays)*	*Osdhar*	110 µg/g DW (6)	Naqvi et al. (2009)
	Tomato *(Lycopersicum esculentam)*	*AcGGP*	46-111 mg/100 g FW (3-6)	Bulley et al. (2011)
	Potato *(Solanum tuberosum)*	*StVTC2A*	1.65 mg/g FW (3)	Bulley et al. (2011)

Nutrient	Species	Genes used	Total amount (increase vs. wild-type)	Reference
Folic acid	Rice (*Oryza sativa*)	*AtGTPCHI, AtADCS*	38.3 nmol/g (100)	Storozhenko et al. (2007)
	Tomato *(Lycopersicum esculentum)*	*AtGCH, AtADCS1*	25 nmol/g (25)	Storozhenko et al. (2007)
Fe	Rice (*Oryza sativa*)	*Osnas2*	19 µg/g DW in polished seeds (4.2)	Johnson et al. (2011)
		Gmferritin, Afphytase, Osnas1	7 µg/g DW in polished seeds (4-6.3)	Wirth et al. (2009)
		Activation tagging of *Osnas3*	32 µg/g DW in dehusked (2.9)	Lee et al. (2009)
	Corn (*Zea mays)*	*Gmferritin, Af phytase*	30 µg/g DW in whole seed (2)	Drakakaki et al. (2005)
	Cassava *(Manihot esculenta)*	*Crfea1*	40 µg/g DW in tuber	Sayre et al. (2011)
Zn	Rice (*Oryza sativa*)	Activation tagging of *Osnas2*	40-45 µg/g DW in polished seeds (2.9)	Lee et al. (2011)
		Osnas2	52-76 µg/g DW in polished seeds (2.2)	Johnson et al. (2011)
		Gmferritin, Afphytase, Osnas1	35 µg/g DW in polished seeds (1.6)	Wirth et al. (2009)
	Cassava *(Manihot esculenta)*	*AtZAT1*	In tuber (4)	Sayre et al. (2011)
		AtZIP	In tuber (2-10)	Sayre et al. (2011)

Nutrient	Species	Genes used	Total amount (increase vs. wild-type)	Reference
Se	Indian Mustard (*Brassica juncea*)	*AtAPS1*	In leaves (2-3)	Pilon-Smits et al. (1999)
		Absmt1	In leaves (2-4)	LeDuc et al. (2004)
Ca	Carrot (*Daucus carota*)	*AtsCAX1*	3.9 mg/g DW (1.6)	Park et al. (2004)
	Lettuce (*Lactuca sativa*)	*AtsCAX1*	18.9 mg/g DW (1.3)	Park et al. (2009)
	Potato (*Solanum tuberosum*)	*AtsCAX1*	1.7 mg/g DW (3)	Park et al. (2005)
		AtsCAX2B	2.5 mg/g DW (3)	Kim et al. (2006)
	Tomato *(Lycopersicum esculentum)*	*AtsCAX1*	4.5-5 mg/g DW (2)	Park et al. (2005)
		AtCAX4	1.8 mg/g DW (1.5)	

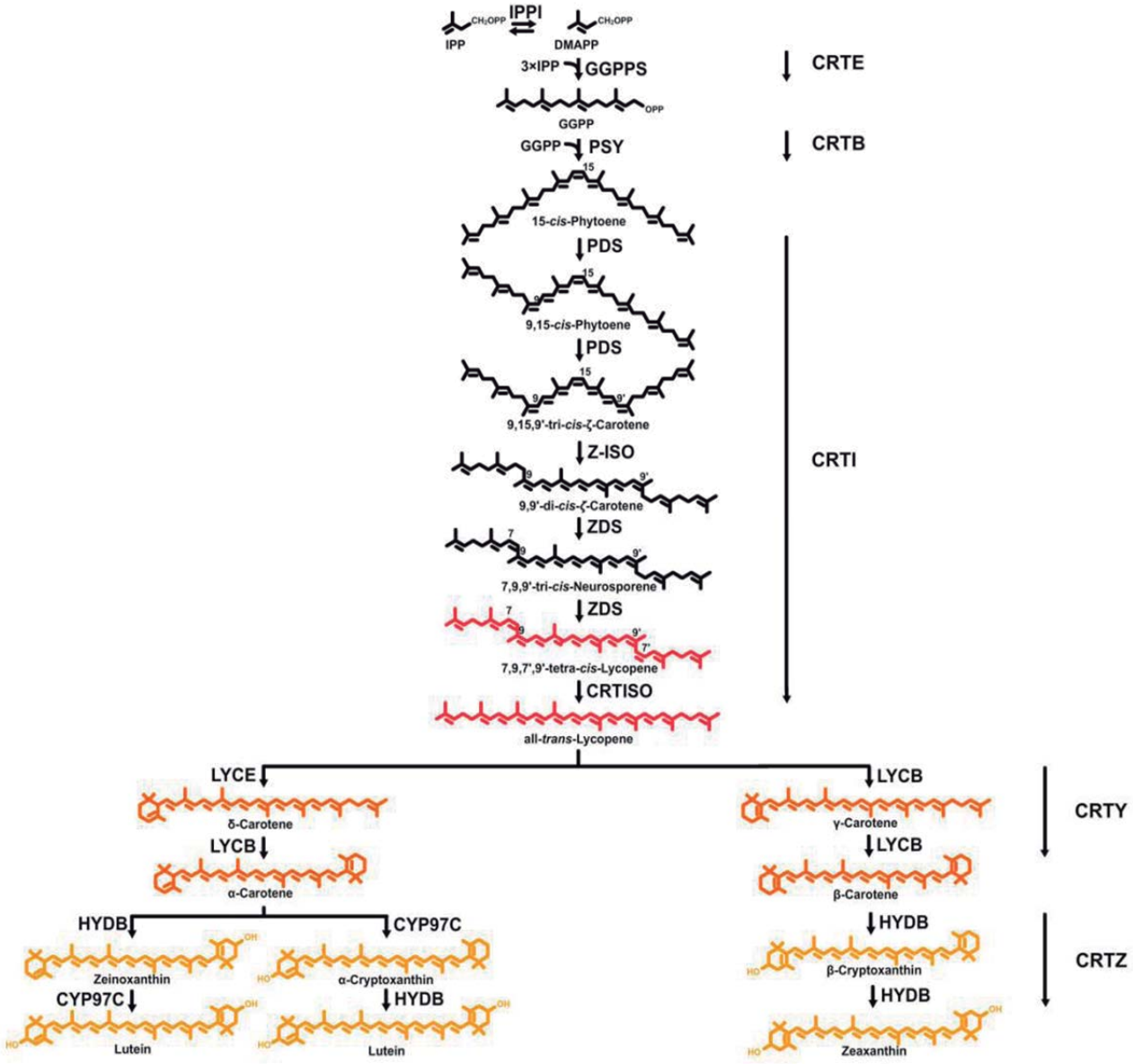

Figure 1. *Carotenoid biosynthesis pathway in plants and its equivalent steps in bacteria on the left (Farré et al. 2011a). IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; CYP97C, carotene ε-ring hydroxylase; HYDB, β-carotene hydroxylase; CRTE, bacterial geranylgeranyl diphosphate synthase; CRTB, bacterial phytoene synthase; CRTI, bacterial phytoene desaturase/isomerase; CRTY, bacterial lycopene cyclase; CRTZ, bacterial β-carotene hydroxylase.*

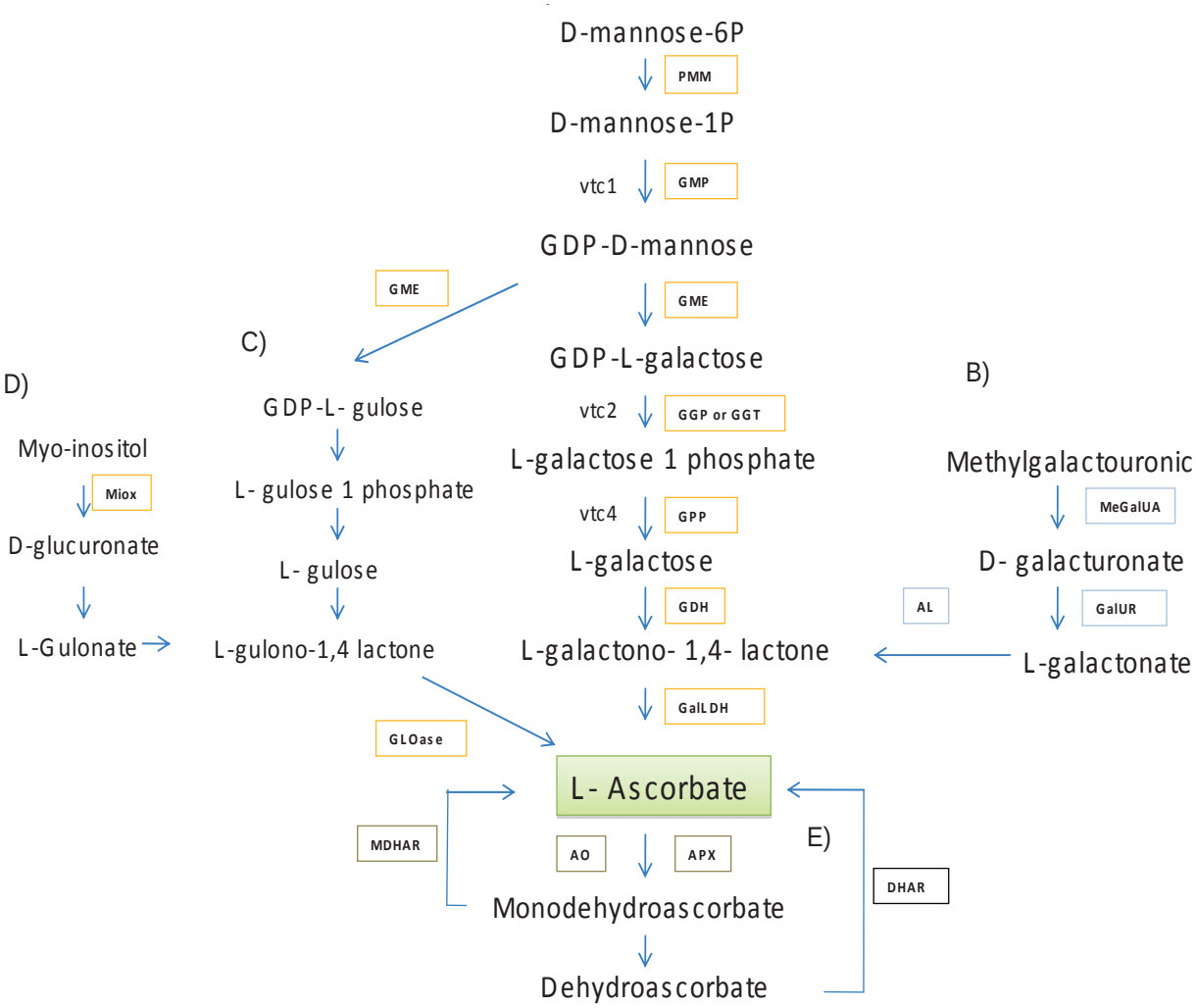

Figure 2. *Ascorbate biosynthetic (A, B, C, D), catabolic and recycling (E) pathways in plants (modified from Naqvi et al. 2009b). PMM, Phosphomannomutase; GDP, Guanosine diphosphate; GMP, GTP Mannose Pyrophosphorolase; GME, GTP-Mannose-3,5-epimerase; GGT, L-Galactose Guanyltransferase; GGP, GDP-L-Galactose phosphorylase; GPP, L-Galactose -1-P-Phosphatase; GDH, L-Galactose Dehydrogenase; GalDH, L-Galactono-1,4-lactone Dehydrogenase; MiOX, myo-Inositol Oxidase; AO, Ascorbate oxidase; APX, Ascorbate peroxidase; DHAR, Dehydroascorbate reductase; MDHAR, Monodehydroascorbate reductase; GLOase, L-gulono γ-lactone oxidase; AL, aldonolactonase; MegalUA, D-galacturonate reductase; GalUR, D-galacturonate reductase;vtc1, Arabidopsis thaliana GTP Mannose Pyrophosphorolase; vtc2, Arabidopsis thaliana GDP-L-Galactose phosphorylase; vtc 4, Arabidopsis thaliana L-Galactose -1-P-Phosphatase.*

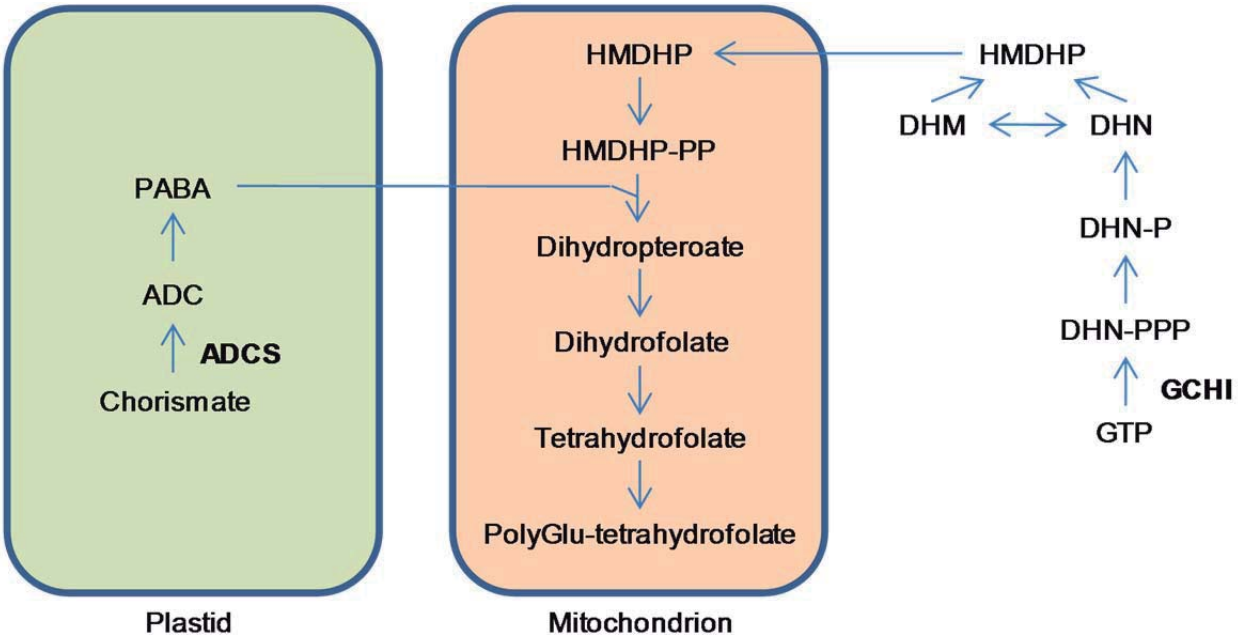

Figure 3. *Simplified scheme of the folate biosynthesis pathway in plants (Naqvi et al.2009b). GTP, Guanosine triphosphate; DHN, dihydroneopterin; GCHI, guanosine triphosphate cyclohydrolase I; -P/-PP/-PPP, mono/di/triphosphate; DHM, dihydromonapterin; HMDHP, hydroxymethyldihydropterin; ADC, aminodeoxychorismate; ADCS, aminodeoxychorismate synthase; PABA, p-aminobenzoate.*

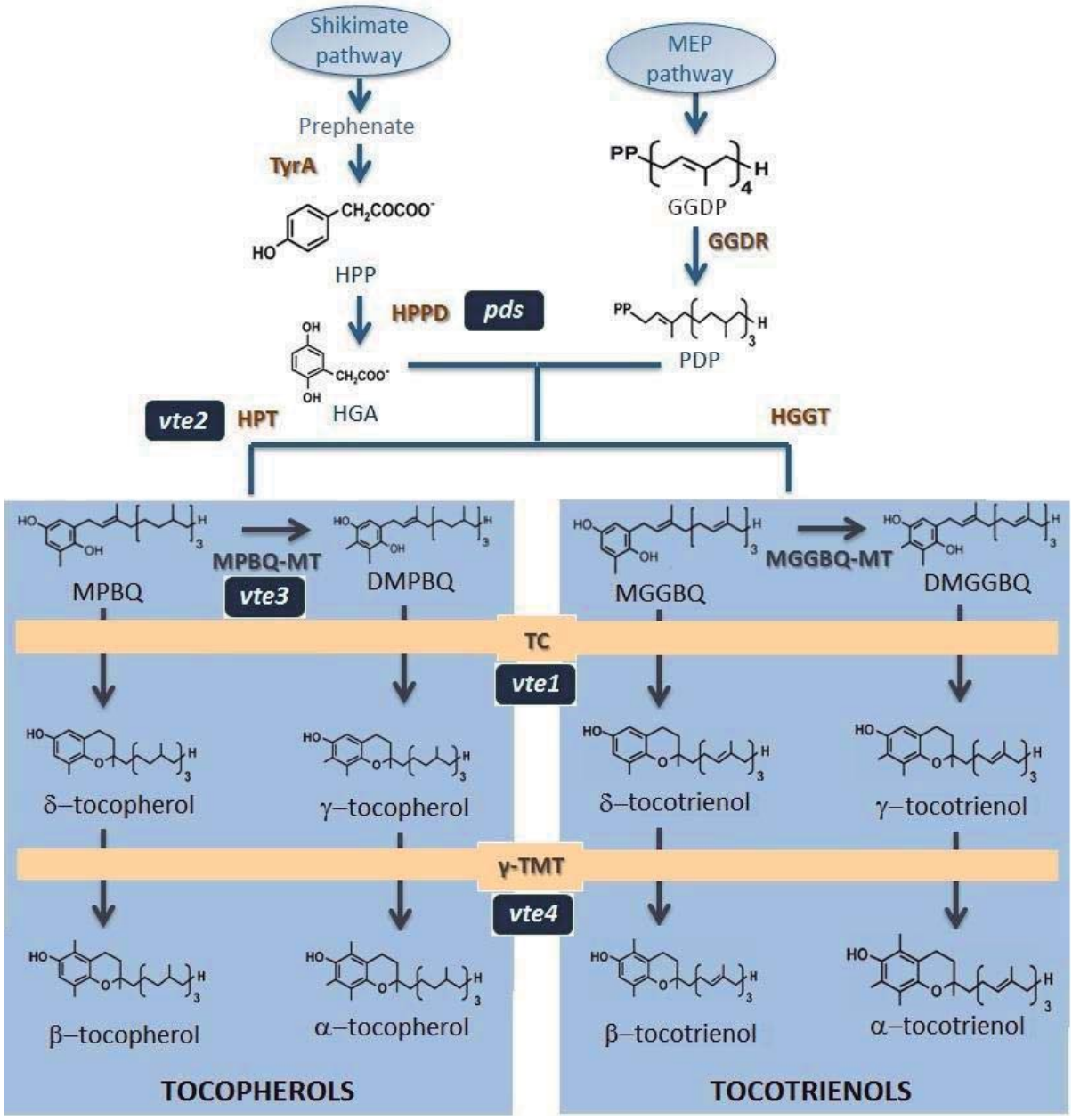

Figure 4. *Tocopherol biosynthesis in plants (modified from Farré et al. 2012). HGA, homogentisic acid; PDP, phytyldiphosphate; MEP, methylerythritol phosphate; HPT, homogentisate phytyltransferase; HGGT, homogentisate geranylgeranyl transferase; GGDP, Geranylgeranyl diphosphate; HPP, ρ-hydroxyphenylpyruvic acid; MPBQ, 2-methyl-6-phytylbenzoquinol; DMPBQ, 2,3-dimethyl-5-phytylbenzoquinol; MGGBQ, 2-methyl-6-geranylgeranylplastoquinol; DMGGBQ, 2,3-dimethyl-5-geranylgeranylplastoquinol; HPPD, HPP dioxygenase; GGDR, Geranylgeranyl diphosphate reductase; MPBQ-MT, MPBQ methyltransferase; MGGBQ-MT, MGGBQ methyltransferase; TC, tocopherol cyclase; γ–TMT, γ-tocopherol methyltransferase; TyrA, Prephanate dehydrogenase; pds1, Arabidopsis thaliana HPPD; Vte2, Arabidopsis thaliana HPT; Vte3, Arabidopsis thaliana MPBQ-MT; Vte1, Arabidopsis thaliana TC; Vte4, Arabidopsis thaliana γ–TMT.*

4.9. References

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General Conclusions

General Conclusions

1. Twelve fertile transgenic rice lines overexpressing genes involved in the accumulation of Ca and Se were generated. The recovery of plants expressing *AtAPS1* was more efficient than the recovery of plants expressing the two Ca-related genes.

2. None of the four transgenic lines expressing *AtsCAX1* and *AtsACA4* showed superior Ca^{2+} accumulation than wild-type plants, although other minerals were overaccumulated in some tissues. Expression of endogenous Ca^{2+} transporters was not affected in transgenic lines.

3. Our results, together with previous reports, suggest that Ca homeostasis is more tightly regulated in rice than in other crops where overexpression de-regulated Ca^{2+} transporters resulted in increased Ca^{2+} levels. Therefore, strategies to attenuate excessive CAX and ACA activity in transgenic plants should be approached.

4. Expression of *AtAPS1* in transgenic rice lines resulted in enhanced selenate reduction capacity and reduced tolerance to high Se concentrations. Endogenous selenate assimilation pathway was down-regulated at mRNA level in transgenic lines. Two transgenic lines accumulated 40% more Se in the grain than wild-type plants.

5. Effects of *AtAPS1* expression in transgenic rice lines were less pronounced than in other plant species. This suggests that selenate activation by ATP sulfurylase in rice may not be a rate-limiting step in the Se assimilation pathway; therefore alternative strategies to accumulate Se should be approached.

6. RNAi-mediated silencing is an effective strategy to reduce strigolactone biosynthesis in transgenic maize plants. *Zmccd8* knockdown resulted in 75-95% decreased production of strigolactone-like compounds, modest phenotypic alterations and reduced capacity to stimulate germination of *Striga* seeds. Performing a *Striga* infection assay will be the ultimate step in the characterization of the C8-2 transgenic line.

7. Knockdown of *Zmd27* did not result in the expected reduced strigolactone content, although phenotype alterations were observed, which suggests the existence of particular roles of D27 in maize plants other than in strigolactone biosynthesis.

8. Genetic engineering (GE) has been used to generate crops with enriched levels of key nutrients, including several vitamins and minerals. These could be used to combat malnutrition in the short term, although major non-scientific constraints hinder the adoption of GE technology at different levels.

Outputs

Publications

- **Pérez-Massot E**, Banakar R, Gómez-Galera S, Zorrilla U, Sanahuja G, Arjó G, Miralpeix B, Vamvaka E, Farré G, Rivera SM, Dashevskaya S, Berman J, Sabalza M, Yuan D, Bai C, Zhu C, Bassie L, Twyman RM, Capell T, Christou P (2013) The contribution of transgenic plants to better health through improved nutrition: opportunities and constraints. Genes Nutr 8:29-41

- Berman J, Zhu C, **Pérez-Massot E**, Arjó G, Zorrilla-López U, Masip G, Banakar R, Sanahuja G, Farré G, Miralpeix B, Bai C, Vamvaka E, Sabalza M, Twyman RM, Bassié L, Capell T, Christou P (2013) Can the world afford to ignore biotechnology solutions that address food insecurity? Plant Mol Biol 83:5-19

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- Zhu C, Sanahuja G, Yuan D, Farré G, Arjó G, Berman J, Zorrilla-López U, Banakar R, Bai C, **Pérez-Massot E**, Bassié L, Capell T, Christou P (2013) Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies. Plant Biotechnol J 11:129-141

- Masip G, Sabalza M, **Pérez-Massot E**, Banakar R, Cebrián D, Twyman RM, Capell T, Albajes R, Christou P (2013) Paradoxical EU agricultural policies on genetically engineered crops. Trends Plant Sci 18:312-324

Annex

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REVIEW

The contribution of transgenic plants to better health through improved nutrition: opportunities and constraints

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Abstract Malnutrition is a prevalent and entrenched global socioeconomic challenge that reflects the combined impact of poverty, poor access to food, inefficient food distribution infrastructure, and an over-reliance on subsistence mono-agriculture. The dependence on staple cereals lacking many essential nutrients means that malnutrition is endemic in developing countries. Most individuals lack diverse diets and are therefore exposed to nutrient deficiencies. Plant biotechnology could play a major role in combating malnutrition through the engineering of nutritionally enhanced crops. In this article, we discuss different approaches that can enhance the nutritional content of staple crops by genetic engineering (GE) as well as the functionality and safety assessments required before nutritionally enhanced GE crops can be deployed in the field. We also consider major constraints that hinder the adoption of GE technology at different levels and suggest policies that could be adopted to accelerate the deployment of nutritionally enhanced GE crops within a multicomponent strategy to combat malnutrition.

Keywords Transgenic crops · Micronutrients · Food security · Vitamins · Minerals · Genetic engineering

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Introduction

Food security is one of the pillars of health and well-being in society because humans rely on food not only to supply energy but also for essential nutrients that maintain the immune system and keep the body in a good state of repair. Adequate nutrition therefore correlates with lower morbidity and mortality from both infectious and non-infectious diseases and is particularly important in children and pregnant women where the lack of essential nutrients can lead to irreversible physical and mental damage during development (Hoddinott et al. 2008).

Malnutrition is more prevalent in the developing world because it often reflects the lack of access to nutritious food. This in turn is frequently caused by poverty, which often occurs due to ill health and an inability to work, the typical consequences of malnutrition. Poverty, malnutrition and poor health therefore form a self-reinforcing cycle from which many people (and in some cases entire populations) find it impossible to escape (Farré et al. 2011b).

Almost 50 % of the world's population is currently affected by malnutrition (Christou and Twyman 2004). The majority are subsistence farmers who depend entirely on staple cereal crops such as maize or rice, which are deficient in several essential nutrients (Zhu et al. 2007). The poverty-malnutrition-disease cycle needs to be broken by multipoint interventions that provide direct, effective and sustainable approaches to increase the economic welfare of the world's poorest people, including the provision of drugs and vaccines that tackle poor health, and adequate access to nutritious food (Perseley 2000). Although there are many global initiatives promoting short and mid-term strategies that tackle poverty, food insecurity/malnutrition and disease, we need sustainable solutions that provide the means for the world's poor to build their own healthy societies, as envisaged by the Millennium Development Goals (Yuan et al. 2011).

Strategies to tackle malnutrition fall into three major categories (Gómez-Galera et al. 2010). The most desirable approach is to increase the diversity of food intake but this is impractical in many developing-country settings, particularly for low-income populations. In some cases it may be possible to change local food-processing conventions to prevent the loss of nutrients during preparation (Hotz and Gibson 2007) or to promote certain styles of preparation that maximize nutritional bioavailability (Yang and Tsou 2006).

The second approach is to add nutrients to the diet artificially, either by providing supplements (Supplementation) or by the fortification of basic food products such as salt and flour, but it is unsustainable over the longer term because it relies on a robust distribution infrastructure and on consumer compliance (Hotz and Brown 2004). Fortification is more sustainable, and there have been remarkable successes, such as the provision of iodine-fortified salt, and wheat flour double-fortified with iron and folate (Rosenberg 2005). However, this strategy also requires government coordination and a distribution infrastructure and is therefore unsuitable for those parts of the developing world relying on subsistence agriculture.

The final strategy is known as biofortification, in which crops are modified or treated to accumulate additional nutrients at source (Zhu et al. 2007). In some cases biofortification can be achieved by adding nutrients to fertilizers, an approach that has been extraordinarily successful to increase the availability of zinc and selenium (Lyons et al. 2003). However, this is not suitable for iron, because it is immobilized in the soil (Gómez-Galera et al. 2012a), or for essential organic nutrients (vitamins and essential amino acids and fatty acids) which are the products of plant metabolism. It may be possible to increase the content of organic nutrients by conventional breeding if there is sufficient genetic diversity in existing germplasm, although it would take many generations to introgress these traits into local elite breeding varieties (Welch and Graham 2005). The alternative is genetic engineering (GE) technology, which can introduce genes from any source directly into the local varieties used by subsistence farmers, to increase the levels of organic and/or mineral nutrients. First-generation GE crops have already shown their potential to enhance agricultural productivity and reduce poverty in developing countries (Christou and Twyman 2004, Farré et al. 2010a, 2011b). A second generation of GE crops is now under development that can address nutritional requirements directly by contributing to multipoint intervention strategies (Yuan et al. 2011). As well as offering an unrestricted choice of genes for nutritional improvement, the GE approach allows nutritional traits to be targeted to specific organs (e.g., cereal seeds) and multiple traits can be combined in the same plants without complex breeding programs (Zhu et al. 2007; Naqvi et al. 2009a).

GE strategies to enhance the content of organic micronutrients

Humans can synthesize almost all the organic compounds needed for normal physiological activity but a small number of specific molecules known as essential nutrients are required in the diet (Online Resource 1). Some of these essential nutrients are amino acids or fatty acids; the rest are chemically diverse but are grouped together as vitamins. All GE strategies focusing on essential organic micronutrients involve the modulation of plant metabolism.

Vitamin A

The reduced form of vitamin A (retinal) is required for the production of rhodopsin, which is essential for sight and also helps to maintain epithelial and immune cells. The acidic form (retinoic acid) is a morphogen in development. Humans can produce retinal and retinoic acid if provided with a source of retinol or one of its esters, which are abundant in meat and dairy products. However, retinal can also be synthesized directly from β-carotene (also known as pro-vitamin A) which is produced mainly by plants and photosynthetic microbes, but also some non-photosynthesizing organisms (Botella-Pavía and Rodríguez-Concepción

2006). Vitamin A deficiency (VAD) is one of the most prevalent deficiency diseases in developing countries, affecting more than 4 million children each year, up to 500,000 of whom become partially or totally blind (Harrison 2005).

The amount of β-carotene produced by plants can be enhanced by increasing flux through the carotenoid pathway (Online Resource 2), for example, by increasing the availability of carotenoid precursors, by expressing enzymes in the early part of the pathway between GGPP and lycopene, by increasing metabolic flux in the β-branch by favoring LYCB activity at the expense of LYCE, or by increasing the sink capacity for carotenoids to remove feedback limitations (Zhu et al. 2009; Farré et al. 2010b; 2011a; Bai et al. 2011). The overexpression of DXP synthase in tomato is an example of the first strategy, producing a carotenoid precursor that increases flux in the entire pathway and enhances the total carotenoid content (Enfissi et al. 2005). Cassava roots expressing the bacterial CrtB gene accumulated up to 21 μg/g of carotenoids, a 34-fold increase with respect to the wild type (Welsch et al. 2010). The replacement of the daffodil gene with its maize ortholog is the basis of Golden Rice 2, which produces up to 37 μg of carotenoids per gram dry weight (DW) of grain, of which 31 μg/g is β-carotene (Paine et al. 2005). The same genes expressed in maize have yielded kernels containing up to 57.3–60 μg/g dry weight of β-carotene (Zhu et al. 2008; Naqvi et al. 2009b; Table 1). The third strategy is exemplified by Golden Potato, where expression of three Erwinia genes encoding phytoene synthase (CrtB), phytoene desaturase (CrtI) and lycopene beta-cyclase (CrtY) lead to the diversion of carotenoid synthesis from the α- to the β-branch (Online Resource 2), resulting in the accumulation of 47 μg/g DW of β-carotene (Diretto et al. 2007, Table 1). Potato also provides a good example of the fourth strategy. The expression of the cauliflower *Or* gene in tubers increased the storage capacity for carotenoids by promoting the formation of chromoplasts, generating transgenic potatoes with orange tuber flesh containing 10 times the normal amount of β-carotene (Lopez et al. 2008, Table 1).

Vitamin C

Ascorbate (vitamin C) is an antioxidant and also cofactor of several enzymes, including those required for the synthesis of collagen, carnitine, cholesterol, and certain amino acid hormones. Vitamin C deficiency causes the ulceration disease scurvy, reflecting the breakdown of connective tissues (Bartholomew 2002).

There are several biosynthetic pathways that generate ascorbate in plants, and when ascorbate is oxidized it can be recycled through an additional pathway with glutathione as the reductant (Online Resource 3). The amount of ascorbate accumulating in plants can therefore be enhanced not only by increasing its biosynthesis but also the rate at which the molecule is recycled. In the first approach, the overexpression of L-gulono γ-lactone oxidase (GLOase) in lettuce resulted in the accumulation of up to 580 nmol/g fresh weight of ascorbate, a sevenfold improvement (Jain and Nessler 2000). A twofold increase was achieved by expressing the same gene in potato tubers (Hemavathi et al. 2010). Multivitamin maize expressing the rice *dhar* gene from the ascorbate recycling pathway accumulated six times the normal level of ascorbate (Naqvi et al. 2009b, Table 1).

Vitamin B9

Folate (vitamin B9) is a tripartite molecule combining pterin, p-aminobenzoate (PABA) and one or more glutamate moieties, which are derived from three separate metabolic pathways in different subcellular compartments (Online Resource 4). Folate is the source of tetrahydrofolate, which is essential for DNA synthesis and many other core metabolic reactions. In adults, folate deficiency causes macrocytic anemia and elevated levels of homocysteine, but the impact on pregnant women is much more severe, leading to the neural tube defect spina bifida in the fetus (Scholl and Johnson 2000).

Moderate increases in folate levels have been achieved by modifying the pterin and PABA pathways individually, but increasing the flux through one pathway only reveals bottlenecks in the other. Díaz de la Garza et al. (2007) crossed two transgenic tomato lines, one expressing GCH1 which enhanced the cytosolic (pterin) branch, and the other ADCS1 which enhanced the PABA branch. In the individual lines, the maximum enhancement was double the normal level of folate. However, combining the two transgenes in a single line released the bottlenecks in both branches simultaneously and achieved a 25-fold increase in folate levels. The same strategy in rice endosperm resulted in a 100-fold increase in folate levels because the base levels were lower than in tomato, indicating how powerful this strategy could be in developing-country settings where rice is the staple diet (Storozhenko et al. 2007, Table 1).

Vitamin E

Vitamin E comprises eight related molecules known as tocochromanols. These are formed from a chromanol head decorated with methyl groups (to yield α, β, γ, and δ derivatives) plus either a phytyl tail to produce the tocopherols, or a geranylgeranyl tail to produce the tocotrienols (DellaPenna and Pogson 2006). The tocochromanols are powerful antioxidants that protect fatty acids, low-

Table 1 Transgenic crops enhanced for mineral and vitamin content

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

density lipoproteins (LDLs) and other components of cell membranes from oxidative stress.

Plants can be engineered to accumulate higher levels of vitamin E by overexpressing genes involved in tocochromanol synthesis (Online Resource 5). This can be achieved either by increasing the total tocochromanol content or skewing tocochromanol synthesis toward the more potent isomers, particularly α-tocopherol which is absorbed more efficiently by humans. For example, Cho et al. (2005) increased the α/γ tocopherol ratio in transgenic lettuce plants by expressing the Arabidopsis γ-tocopherol methyltransferase (γ-TMT), and achieved near complete

conversion to α-tocopherol in the best-performing transgenic lines. Similarly, Tavya et al. (2007) reported a 10.4-fold increase in α-tocopherol levels and a 14.9-fold increase in β-tocopherol levels in soybean seeds expressing *Perilla frutescens* γ-TMT. The constitutive expression of two Arabidopsis cDNA clones encoding ρ-hydroxyphenylpyruvate dioxygenase (HPPD) and 2-methyl-6-phytylplastoquinol methyltransferase (MPBQ MT) increased the tocopherol content by threefold in transgenic maize (Naqvi et al. 2011). Overexpression of Arabidopsis HPPD in rice grains shifted tocopherol synthesis from the γ to the α form with no increase in absolute tocopherol levels (Farré et al. in press).

Essential amino acids

Nine of the 20 standard amino acids are constitutive essential nutrients because they cannot be synthesized de novo by humans, and others are essential under certain circumstances, such as child development or in the context of metabolic disorders. The most relevant examples are lysine, threonine, tryptophan, methionine and cysteine because staple cereals are poor sources of lysine and threonine, and staple legumes are poor sources of tryptophan, methionine and/or cysteine (Zhu et al. 2007).

Two GE strategies have been used to tackle amino acid deficiency: (1) engineering plants to produce proteins containing essential amino acids; and (2) engineering amino acid metabolism to increase the availability of essential amino acids in the free amino acid pool. Lysine was the first target in both strategies. One of the earliest attempts to overcome the poor nutritional value of cereal proteins was the expression of lysine-rich pea legumin in cereal endosperm, which resulted in transgenic rice and wheat grain protein containing up to 4.2 % lysine (Sindhu et al. 1997; Stöger et al. 2001). More significant improvements were achieved by adding lysine residues to endogenous cereal storage proteins for example, 12 residues added to barley hordothionine to produce HT12, and eight added to barley high lysine protein to produce HL8 (Jung and Carl 2000). When expressed in maize along with the bacterial enzyme dihydrodipicolinate synthase (DHPS) the total lysine content was four times the normal amount, accounting for 0.8 % of total protein composition. Another lysine-rich storage protein (sb401) increased the lysine content in maize seeds by 55 % (Yu et al. 2004). Similar improvements have been achieved by expressing lysine-rich animal proteins such as porcine α-lactalbumin, which increased the lysine content in maize by 47 % (Bicar et al. 2008). Maize seeds containing up to 26 % lysine have also been produced by expressing a heterotypical Arabidopsis lysyl tRNA synthetase, which inserts lysine residues in place of other amino acids during the synthesis of seed storage proteins (Wu et al. 2007). Finally, the lysine content of maize has been increased by using RNA interference to silence one of the zein storage protein genes allowing the protein complement to be filled with lysine-rich storage proteins (Segal et al. 2003).

One of the success stories in terms of storage protein engineering involves the expression in staple crops of *Amaranthus hypochondriacus* seed storage protein, which is rich in all the essential amino acids and has a composition close to ideal for humans. Transgenic maize seeds expressing the AH protein contained up to 32 % more protein than wild-type seeds and contained higher levels of lysine, tryptophan and isoleucine (Rascón-Cruz et al. 2004). Similarly transgenic potato tubers expressing AH contained 45 % more protein than normal (Chakraborty et al. 2000) and transgenic wheat seeds contained nearly 2.5 % AH as a proportion of total seed protein, increasing the levels of lysine to 6.4 % and tyrosine to 3.8 % (Tamás et al. 2009).

Expression of feedback-insensitive dihydrodipicolinate synthase (DHPS) in maize increased lysine levels from <2 % to ~30 % of the free amino acid pool, with concomitant increases in threonine (Frizzi et al. 2008). The key rate-limiting enzyme in tryptophan synthesis is anthranilate synthase, which catalyzes the conversion of chorismate to anthranilate, and tryptophan levels in rice have been increased more than 400-fold by expressing a feedback-insensitive version (Wakasa et al. 2006). Tryptophan levels also increased 30-fold in potato tubers (Yamada et al. 2004) and 20-fold in soybean seeds (Ishimoto et al. 2010) expressing feedback-insensitive AS.

Essential fatty acids

Humans can synthesize most fatty acids de novo but the health-promoting ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) are exceptions that need to be sourced from the diet (Djoussé et al. 2011). Once acquired, simple ω-3 PUFAs such as α-linolenic acid can be converted into more complex very-long-chain polyunsaturated fatty acids (VLC-PUFAs) such as arachidonic acid (ARA), and these can be converted back to the simpler species. This interconversion means the class rather than individual PUFAs is regarded as essential. The same principles apply to ω-6 PUFAs. The essential fatty acids are abundant in fish, shellfish, nuts and leafy vegetables but they are not present in cereals (Farré et al. 2011b).

The fatty acid biosynthesis pathway in plants was modulated to produce ω-3 and ω-6 PUFAs by introducing the microbial enzymes responsible for a sequence of fatty acid desaturation and elongation reactions (Domergue et al. 2005). The key targets are linoleic acid and α-linolenic acid, and the VLC-PUFAs ARA, eicosapentenoic acid

(EPA) and docosahexenoic acid (DHA; Zhu et al. 2007). The biosynthesis of VLC-PUFAs has been achieved by expressing microbial desaturases and elongases in linseed, soybean and mustard (Abbadi et al. 2004; Kinney et al. 2004; Wu et al. 2005) with the experiments in mustard demonstrating the feasibility of a stepwise approach to first optimize the accumulation of ω-3 fatty acids (i.e., EPA rather than ARA) and then to convert this to DHA, resulting in the accumulation of ARA (4 %), EPA (8 %) and DHA (0.2 %). These successes have led to the long-term goal of producing such fatty acids in transgenic oilseed crops (Abbadi et al. 2004; Domergue et al. 2005).

GE strategies to enhance the content of mineral micronutrients

Unlike organic nutrients, which are the product of plant metabolism, inorganic nutrients (minerals) must be taken up by plants from the environment. GE approaches to enhance mineral nutrients are therefore diverse, focusing on strategies such as increasing the solubility of these nutrients in the rhizosphere, mobilizing them in the plants, transporting them to storage organs, increasing the storage capacity of the plant, and maximizing bioavailability (Gómez-Galera et al. 2010). The four most significant minerals in terms of nutrient deficiency are iron, zinc, selenium and calcium. Other minerals, while no less essential, are so abundant and ubiquitous that deficiency disorders are very rare. It is not uncommon for populations to be deficient for several of the above minerals at the same time so there has been recent interest in the development of crops tailored to provide all these limiting nutrients simultaneously (Zhu et al. 2008; Naqvi et al. 2009a).

Iron

Iron deficiency is the most prevalent form of mineral malnutrition in the world, with more than 2 billion people at risk. The primary clinical manifestation is anemia, and it is estimated that more than half of all cases of anemia worldwide are caused by a lack of iron (Benoist et al. 2008).

Biofortification offers a more sustainable approach for subsistence farmers and many different strategies have been investigated (Drakakaki et al. 2005; Wirth et al. 2009). One of the major challenges with iron is that its mobility in the rhizosphere is dependent on the soil conditions, because only the ferrous form (Fe^{2+}) is soluble and bioavailable to plants whereas the ferric form (Fe^{3+}) is sequestered into insoluble complexes with soil particles (Gómez-Galera et al. 2012a). Plants have evolved two counterstrategies, one of which is to secrete reductases into the soil to convert ferric iron into the soluble ferrous form, and the other is to release chelating agents known as phytosiderophores (PS) that can be reabsorbed by the roots as PS-Fe^{3+} complexes. Iron levels in plants can therefore be improved by increasing the export of both reductases and phytosiderophores, for example, by overexpressing the enzymes nicotianamine synthase (NAS) and/or nicotianamine aminotransferase (NAAT) which are involved in phytosiderophore synthesis (Zheng et al. 2010; Johnson et al. 2011). For example, transgenic rice plants expressing the NAS genes *Osnas1*, *Osnas2* or *Osnas3* accumulated up to 19 μg/g of iron in the endosperm (Johnson et al. 2011, Table 1). Additional strategies include the overexpression of iron transporter proteins (many of which also co-transport zinc, see below), the overexpression of ferritin (which stores large amounts of iron in a bioavailable form) and the expression of phytase, which breaks down phytate and makes the stored iron easier to absorb in the human digestive system (see section on antinutritional factors, below).

Zinc

Zinc is required as a cofactor in many different enzymes and is also a coordinating ion in the DNA-binding domains of transcription factors. Zinc deficiency affects more than 2 billion people worldwide predominantly in developing countries and manifests as a spectrum of symptoms including hair loss, skin lesions, fluid imbalance (inducing diarrhea), and eventually wasting of body tissues (Hambidge and Krebs 2007).

Cereal grains are poor sources of zinc, but because this mineral is more soluble than iron and easier to take up from the soil, GE strategies to increase the zinc content of plants have concentrated on transport and accumulation (Palmgren et al. 2008). Many phytosiderophores and transporters can interact with both iron and zinc so the expression of NAS/NAAT and transporters such as *Oysl15* and *Osirt1* in rice can increase the levels of both minerals (e.g., Lee et al. 2011, Table 1).

Selenium

Selenium is a component of enzymes and other proteins that contain the amino acids selenocysteine and selenomethionine, which are required for the interconversion of thyroid hormones; therefore selenium and iodine deficiency can have similar symptoms (Khalili et al. 2008). Selenium deficiency is rare at a population level because the mineral is taken up efficiently from the soil, and fertilizers with selenium have proven successful to prevent deficiency in areas where the soil is depleted (Lyons et al. 2003). GE strategies to increase selenium levels therefore focus on storage and accumulation. For example, the

expression of Arabidopsis ATP sulfurylase in mustard increased the selenite content by up to threefold in roots and shoots (Pilion-Smits et al. 1999, Table 1).

Calcium

Soluble calcium is an electrolyte and signaling molecule, but most of the calcium in the human body is present in its mineralized form as a component of bones and teeth. The replenishment of serum calcium by bone resorption is slow, so dietary calcium deficiency in the short term can lead to electrolyte imbalance and over the long term can cause osteoporosis. The risk is higher in children as the rapid bone growth that occurs during childhood results in a high demand for calcium. Calcium-rich dairy products tend to be inaccessible in developing-country settings so many children and adults are malnourished (Dayod et al. 2010). Root vegetables and leafy crops such as lettuce are good sources of calcium although in some vegetables the high content of phytate and oxalate makes the calcium difficult to absorb (Jeong and Guerinot 2008). GE strategies to increase the calcium content of plants include the expression of calcium transporters such as AtCAX1, which increased the calcium content of carrots and potatoes by up to threefold (Connolly 2008; Park et al. 2005, Table 1).

Nutrient enhancers and anti-nutrients

A number of compounds are known to enhance or inhibit the absorption of plant minerals by the human digestive system, and mineral bioavailability can therefore be increased by promoting the accumulation of enhancers or eliminating anti-nutrients (Gibson 2007). Some key nutrients act as enhancers and therefore provide dual benefits, for example, ascorbate and β-carotene promote iron uptake by chelating and/or reducing Fe^{3+} and preventing interactions with phytate and polyphenols (García-Casal et al. 2000).

Phytic acid is a key anti-nutrient because it is abundant in cereals, legumes and oil seeds where it binds all the principal mineral nutrients and sequesters them into stable complexes that cannot be absorbed (Lopez et al. 2002). The amount of phytic acid in seeds can be reduced by silencing genes involved in its biosynthesis, such as *myo*-inositol-1-phosphate synthase (Nunes et al. 2006) or 1D-*myo*-inositol 3-phosphate synthase (Kuwano et al. 2009). Expression of a thermostable recombinant fungal phytase increased iron bioavailability in wheat (Brinch-Pedersen et al. 2006) and maize (Chen et al. 2008). Transgenic maize plants expressing phytase may well be the first second-generation GE crop to reach the market (Chen et al. 2008).

Cassava is an important staple crop in sub-Saharan Africa but the high content of the cyanogenic glycosides linamarin and lotaustralin means it must be processed before consumption (Jørgensen et al. 2005). Two genes encoding cytochrome P450s (CYP79D1 and CYP79D2) catalyze the first step in linamarin and lotaustralin synthesis, and suppression of these genes has reduced linamarin by 94 % (Siritunga and Sayre 2003). RNA interference targeting the same genes reduced the cyanogenic glucoside content by 99 % (Jørgensen et al. 2005).

Risk assessment and regulation of GE crops

Genetically engineered (GE) crops must undergo a risk assessment to assess their potential impact on human health and the environment before they receive market authorization (EFSA 2010). This involves detailed molecular characterization, comparison to conventional crops of the same species, assessment of potential toxicity/allergenicity and nutritional analysis (USFDA 1992; EFSA 2011). In most parts of the world, authorization follows automatically from a positive evaluation, but the decision-making process in the European Union (EU) has become politicized and it is now almost inevitable that regulatory approvals are ignored and overruled (Sabalza et al. 2011; Ramessar et al. 2010).

There are two key differences between the regulatory systems adopted in the EU (where few GE crops are approved) and in the US, where GE agriculture is strongly established and has an unblemished 15-year safety record (Ramessar et al. 2009). The first is that the US system focuses on the safety of the product compared to a conventional counterpart, whereas the EU system focuses instead on the process. This leads to two major conflicts of logic: (1) identical GE and conventional crops are subject to different tiers of regulation; and (2) imported GE products are regulated differently because they are not grown in the EU and are not under regulations applied to cultivation, that is, identical GE products are subject to different tiers of regulation according to origin. The second difference is that the US system is based on substantial equivalence, that is, a GE crop is approved if it is largely the same as its conventional counterpart and that the differences do not introduce clearly defined risks that can be demonstrated experimentally. In contrast, the EU system is based on the precautionary approach, which essentially means that a GE crop is not approved unless the absence of risk can be demonstrated. Since it is impossible to prove that a risk is zero (as opposed to vanishingly small) this creates an effective moratorium on GE agriculture in Europe.

The risk assessment of GE crops focuses on two main aspects—the potential effect on the environment and the potential effect on human health through consumption.

Ostensibly the assessment criteria should be based on rational scientific premises but there remain several key areas where arbitrary factors are included, for example, the risk of gene transfer from plants to microbes in the environment, which is too low to quantify because there is no evidence for this process throughout millions of years of evolution yet it cannot be proven to be zero (Ramessar et al. 2007; EFSA 2010). Even EFSA itself acknowledges this inconsistency yet the requirement persists (EFSA 2009). It is also necessary to determine the risk posed by GE plants, their pollen and residues to agricultural workers when no adverse effects have been reported after 15 years of GE agriculture in the US, Argentina, Brazil, China and India, whereas similar risk assessments are not required for conventional crops, even those known to produce potentially harmful products such as alkaloids in tobacco and erucic acid in rapeseed and mustard oil (EFSA 2010).

GE crops must also undergo toxicological analysis to prove the absence of unintended effects using animal models (usually rodents) and this may be extended from acute tests to multigenerational chronic toxicity/reproductive tests if deemed appropriate (EFSA 2011).

The best known example of a nutritionally enhanced crop is Golden Rice (Ye et al. 2000), which was developed in 1999 but has been mired in regulatory bureaucracy ever since despite the successful completion of multiple safety assessments in silico, in vitro, in animal models and in human trials (Tang et al. 2009). Golden Rice therefore provides an excellent example of the major deficiency in current regulatory processes, that is, that they focus on the risks rather than a risk–benefit analysis (Kowalski 2007). Genetic engineering reduces the time and cost required for developing plants enhanced with one or several traits, but these savings are compromised by the long and costly regulatory process that is not applied to conventional varieties even if they incorporate exactly the same traits and use similarly "unnatural" processes such as forced hybridization or chemical mutagenesis (Sabalza et al. 2011).

Functionality testing

The ultimate success of biofortification can only be judged by its impact on human health and nutrition after deployment (König et al. 2004). The anti-nutritional factors discussed earlier prevent the digestion, absorption and/or utilization of nutrients, so GE crops must undergo bioavailability studies to predict their impact at the population level, and this is also an important part of the regulatory process (Hirschi 2008).

The bioavailability of micronutrients in a staple crop depends on overlapping diet and host-related factors such as co-consumed foods, the geographical region, and the efficiency with which individual consumers absorb certain nutrients (Gibson 2007). Diet-related factors, which predominantly reflect interactions among micronutrients and other organic compounds, have a profound effect on the efficiency of a nutritionally enhanced crop (Graham et al. 2001). Such factors include the chemical form (speciation) of the nutrient, for example, selenomethionine is a more bioavailable form of selenium than any inorganic source (Combs 2001) and heme–iron from meat is much more bioavailable than the non-heme iron from cereals and legumes (Monsen and Balintfy 1982).

Constraints preventing adoption

Nutritionally enhanced GE crops have an enormous potential to tackle poverty, malnutrition and ill health particularly in the developing world, but there are several constraints preventing their adoption, cultivation and use. The development of crops with higher levels of key nutrients requires the simultaneous transfer and expression of multiple transgenes in local varieties, which may be recalcitrant to GE approaches. This requirement becomes even more complex if several nutritional components are addressed at the same time (Naqvi et al. 2009a, 2010; Zhu et al. 2008; Farré et al. 2011a).

As well as technical limitations, a further constraint to the adoption of nutritionally enhanced GE crops is their potential socioeconomic impact. The first generation of GE crops provided substantial economic benefits for farmers in developed and developing countries (Qaim 2009; Brookes and Barfoot 2006) and second-generation crops are expected to provide similar socio-economic benefits although favoring the consumer as well as the producer (Qaim et al. 2007). Even so, the costs of development (predominantly regulatory compliance) can exceed $15 million (Kalaitzandonakes et al. 2007) which blocks the approval pathway de facto for public sector institutions and small and medium companies that lack the financial resources of the agro-industry. Only few major companies have the economic muscle to complete the regulatory process, and R&D investment will not be recouped from humanitarian projects (Lemaux 2009). This limits the incentive to invest in GE products tailored for developing countries (Qaim et al. 2007; Qaim 2009).

EU legislation for the approval of GE crops (Directive 2001/18/EC and Regulation EC 1829/2003) also acts as a constraint because it is highly politicized and driven by single-interest groups such as the organic farming lobby and environmental activists rather than rational scientific discourse (Apel 2010). The regulatory pipeline requires years to negotiate even if the applicant has unlimited funds

and precise legal knowledge, and acts as a barrier for testing, scientific investigations and commercial production (Kalaitzandonakes et al. 2007; Gómez-Galera et al. 2012b).

Perhaps the most insidious problem with the adoption of nutritionally enhanced GE crops is that this potentially life-saving scientific breakthrough has become the flagship campaign for activists, who appear to have garnered public opinion and thus have a disproportionate effect on politicians, who in turn determine the rules followed by the regulators. The unfortunate consequence of this negative cycle is that the regulations are changing under political influence to make any kind of GE agriculture almost impossible in Europe. For example, the European Parliament recently published a proposal to allow EU member states the opportunity to opt out of GE agriculture without any scientific justification, ostensibly to prevent single member states vetoing cultivation throughout Europe. However, the politicians have overlooked the negative impact this decision would have on the acceptability of GE crops to the public (Sabalza et al. 2011; Morris and Spillane 2010). The EU already labors under scientifically unjustified coexistence measures, including excessive isolation distances for GE crops and unmanageable adventitious presence thresholds that cause trade conflicts with other countries (Ramessar et al. 2009, 2010). Within this hostile environment there is no voice for scientists and indeed no voice for science, a worrying glimpse of a future dark age where superstition and hearsay are more important than facts, and those that bear the consequences are the world's poor who do not have the luxury of choice and will continue to suffer from malnutrition for the foreseeable future (Farré et al. 2010a, 2011b).

Future prospects

The world population will reach 10.5 billion in 2050, with most of the population increase in developing countries. Global food production will need to increase by 70 %, including 3 billion tons of cereals, which means global rice and maize production must double even as the land available for agriculture shrinks in the face of urbanization, land degradation and dwindling access to water (FAO 2009). Future food security therefore depends on our ability to improve the quality as well as the quantity of food and to reduce the global health burden of micronutrient deficiency disease. This will require multidisciplinary collaboration between stakeholders such as farmers, health providers, nutrition experts, plant breeders, the food and agrochemical industries, the biotechnology industry, governments and NGOs to reduce the impact of malnutrition on human populations (Martin et al. 2011).

We also need to increase awareness of the environmental and health benefits of GE crops, which are rarely discussed in the media, such as reduced pesticide and fossil fuel usage in agriculture, reduced mycotoxin levels, and reduced exposure to toxic agrochemicals (Raybould and Quemada 2010; Sanahuja et al. 2011). For example, the planting of Bt cotton has reduced pesticide applications by 50 % in India, avoiding up to 9 million poisoning incidents and saving $51 million (Kouser and Qiam 2011). Bt corn also has lower levels of mycotoxins (e.g., fumonisin), saving $23 million annually in crop losses (Wu 2006). Nutritional traits therefore need to be combined with first-generation input traits for maximum benefit (Sun et al. 2004).

Another key future development will be the provision of incentives to develop nutritionally enhanced orphan crops, that is, crops such as finger millet, cassava and fonio that are grown on a small scale in niche geographical areas (Online Resource 6). These crops have not been studied in detail and generally produce lower yields and nutritionally poorer or more toxic products than staple crops (Tadele 2009). With sufficient targeted resources they could yet be developed into alternative staples for niche geographical areas (Naylor et al. 2004).

Many developing countries have followed EU protectionism and banned GE products due to consumer and environmental concerns, for example, the moratorium on GE eggplant in India (Chong 2011), putting millions of lives at risk from malnutrition and the continuous indiscriminate use of toxic pesticides (Ramaswami 2007). There needs to be a shift from risk assessment to risk–benefit analysis, which is applied in all other technology areas (Ramessar et al. 2009). We need to establish a globally harmonized risk–benefit system that is based on traits rather than events (the product not the process). Furthermore, the Cartagena Protocol on Biosafety needs to be redesigned to reflect the reality of global food security challenges and to address the issue of GE crops specifically, as the current protocol does not consider GE foods that are outside the definition of a living modified organism. We need to remove the precautionary approach and other non-science-based doctrines in the context of nutritionally enhanced crops to ensure they can be deployed where they are needed the most and where they can get on with the important task of saving lives.

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Can the world afford to ignore biotechnology solutions that address food insecurity?

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Abstract Genetically engineered (GE) crops can be used as part of a combined strategy to address food insecurity, which is defined as a lack of sustainable access to safe and nutritious food. In this article, we discuss the causes and consequences of food insecurity in the developing world, and the indirect economic impact on industrialized countries. We dissect the healthcare costs and lost productivity caused by food insecurity, and evaluate the relative merits of different intervention programs including supplementation, fortification and the deployment of GE crops with higher yields and enhanced nutritional properties. We provide clear evidence for the numerous potential benefits of GE crops, particularly for small-scale and subsistence farmers. GE crops with enhanced yields and nutritional properties constitute a vital component of any comprehensive strategy to tackle poverty, hunger and malnutrition in developing countries and thus reduce the global negative economic effects of food insecurity.

Keywords Genetically engineered crops · Food insecurity · Nutritionally enriched crops · Economic impact

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Introduction

Food security exists when all people, at all times, have physical and economic access to sufficient amounts of safe and nutritious food meeting their dietary needs and preferences (World Food Summit 1996; Farré et al. 2010, 2011). Everyone should therefore have access to food which is available on a sustainable basis (FAO 2006). The nutritional quality of food is just as important as the calorific value (Pérez-Massot et al. 2013). Food insecurity therefore not only affects the 870 million hungry people in the world (FAO 2012a) but also the additional 3 billion people who achieve the minimum dietary energy requirement (MDER) but nevertheless suffer from diseases caused by inadequate nutrition (Christou and Twyman 2004; Gómez-Galera et al. 2010; Farré et al. 2010). Food insecurity is prevalent in developing countries, particularly in sub-Saharan Africa and South Asia, which account for 98 % of the world's hungry and the largest proportion of undernourished people (FAO 2012a).

Despite isolated episodes of sudden food insecurity caused by unpredictable production deficits (FAO 2011a), global food production at present is generally sufficient to feed everyone in the world with an average 2,790 calories per person per day (FAO 2012a). Therefore today's food insecurity has more to do with limited access to food in poverty-stricken regions than limited availability (Smith et al. 2000).

Many factors prevent access to food in developing countries, including natural disasters (caused by biotic and abiotic stress), conflict, civil strife, lack of infrastructure, land ownership disputes, unsafe water and poorly-developed health and education systems (POST 2006). However, poverty is considered the main underlying cause of chronic food insecurity in the developing world (Van Wijk 2002; Christou and Twyman 2004; Yuan et al. 2011). More than a quarter of the population in developing countries subsists on less than $US 1 per day, increasing to at least half the population in sub-Saharan Africa and in the least developed countries on each continent (UN 2011). Therefore, poverty must be tackled to address food insecurity in the long term, through increasing rural employment-based income and boosting the agricultural productivity of subsistence farmers (Christou and Twyman 2004). Currently, most global initiatives addressing global food insecurity and malnutrition embrace short- and middle-term strategies. For example, the Micronutrient Initiative (MI 2010) along with the Global Alliance for Improved Nutrition (GAIN 2012) and the Sprinkles Global Health Initiative (SGHI 2009) strive to reduce malnutrition through supplementation and food fortification programs.

The most obvious consequences of food insecurity are undernourishment and malnutrition, leading to illness, disability, impaired cognitive development and premature death (FAO 2012a). In addition, children can suffer behavioral and psychosocial problems as well as impaired learning (CHP 2002). The lack of access to food often means that the poor are unable to work, or even excluded from income-generating opportunities, thus perpetuating this status. Poverty, illness and food insecurity thus form a self-reinforcing negative cycle from which many people (sometimes entire communities) find it impossible to escape (Farré et al. 2011). On a larger scale, this translates into productivity losses that can account for 2–4 % of gross domestic product (GDP) as demonstrated for several countries in South Asia (FAO 2012a).

This review considers the economic consequences of food insecurity in developing countries by measuring direct and indirect costs and dissecting them into their main components. We also examine the cost of today's unsustainable food insecurity solutions, as well as the potential cost benefits of more sustainable solutions, such as the development of nutritionally-enhanced genetically engineered (GE) crops.

Food insecurity in developing countries: consequences for industrialized countries

Food insecurity and poverty have led to mass immigration from developing to industrialized countries, which has resulted in social problems caused by the mismanagement of immigrant populations (de Haan and Yaqub 2009). Immigrants are often blamed for displacing the native population from the employment market and thus increasing unemployment rates, and for overburdening public services such as healthcare and education (IPC 2009; MAC 2012). Despite the inaccuracy of such claims, they are often used as propaganda by extremist parties to gain public support (van Spanje 2010).

Food insecurity and poverty also perpetuate unacceptable labor conditions in developing countries by forcing people to carry out menial work for low wages as an alternative to starvation (Meyers 2004). Large companies in industrialized countries often take advantage of this situation to save labor costs and avoid more rigorous (and costly) regulatory scrutiny (Hippert 2010). Therefore, tens of thousands of jobs have been lost through relocation, and working conditions in industrial countries have deteriorated as workers are forced to compete with a less expensive labor force in developing countries (Levine 2011; Pedersini 2006).

Most of the rural population in developing countries is made up of subsistence farmers, aiming to grow enough food to feed their families (Christou and Twyman 2004). They sometimes cultivate export-oriented crops for additional income, and in some cases these can be illicit cash crops such as opium (Afghanistan) or coca (Colombia) with local chiefs and warlords taking a cut of the profits (Díaz and Sánchez 2004; Goodhand 2005). Drug trafficking has become an extraordinary income-generating activity for many criminal groups worldwide, which benefit from demand in the industrial world while exploiting developing country farmers (UNODC 2012). Although governments in the industrialized world have tried with limited success to block the import of drugs (GCDP 2011), a more sensible approach would be to simultaneously improve living standards in developing countries by tackling poverty and ensuring food security, so farmers are less likely to turn to drug production to supplement their incomes (GCDP 2011).

Food insecurity and poverty are also perpetuated by poor governance and corruption, exacerbated by the exploitation of valuable natural resources such as oil, minerals and timber by some governments, criminal organizations and some transnational companies (TNCs; Ascher 1999). Far from benefiting the industrialized countries where TNCs are located, such arrangements are harmful both to the developing country (where land is depleted and becomes unsuitable for agriculture) and the industrialized country (because the natural resources are exploited in a non-sustainable manner; Giljum et al. 2008). Addressing the basic needs of the population would contribute to a more equitable society with the ability to

control its own resources in a sustainable manner (Baland and Platteau 1996).

The economic cost of food insecurity

The current FAO estimate of 870 million hungry people in the world (FAO 2010, 2012a) is 150 million higher than 10 years ago, reflecting the consequences of two crises that were different in nature and origins but had a similar impact on food security (FAO 2011b). The first was the food price crisis that peaked in 2008, reflecting the slow increase in food prices between 2003 and 2006 followed by a surge between 2006 and 2008 before declining in the second half of that year (Mittal 2009). These increases took many by surprise, increasing concerns that the world food economy was unable to adequately feed billions of people (FAO 2011b) (Fig. 1). Although opinions varied as to the relative importance of different contributory factors, there is a strong consensus that multiple factors sparked the price increases that began in 2003 (Mittal 2009; Wiebe et al. 2011), including the slowing of agricultural production reflecting lower investment and adverse weather conditions (Zeigler and Mohanty 2010; Zhao and Running 2010), declining global grain stocks (FAO 2012a) (Fig. 2), higher energy prices which increased production costs and thus the export prices of major food commodities (Mitchell 2008), the increased food demand from emerging economies such as India and China (Mittal 2009), speculation in financial markets causing the hyperinflation of basic food staples (Mittal 2009), and the increased use of land for biofuel production. However, further investigation revealed that the record grain prices in 2008 were not caused by higher biofuel production, but were based on a speculative bubble concerning high petroleum prices, a weak US dollar, and increased volatility due to commodity index fund investments (Mueller et al. 2011) (Fig. 3).

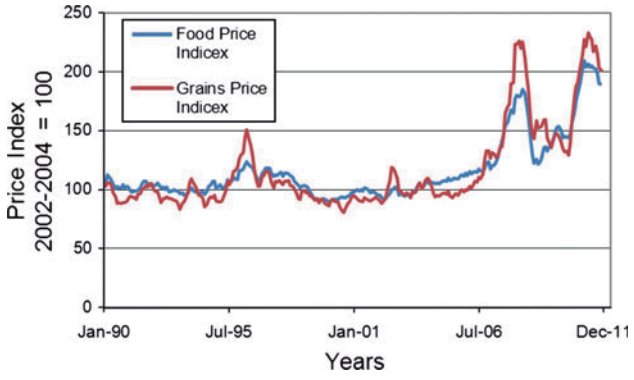

Fig. 1 Global monthly grains price index (*blue*) and total food price index (*red*), January 1990–November 2011. The values for 2002 to 2004 are set at 100. *Source*: FAO (2012b)

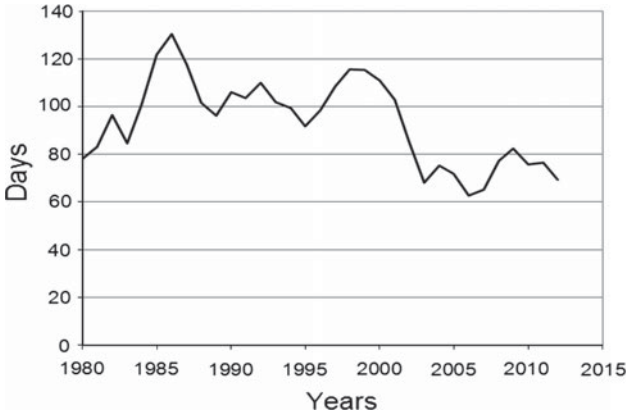

Fig. 2 World grain stocks shown as days of consumption between 1980 and 2012. This shows strong decline since 2000 reflecting policy shifts and greater dependence on trade. *Source*: EPI from USDA (2012a)

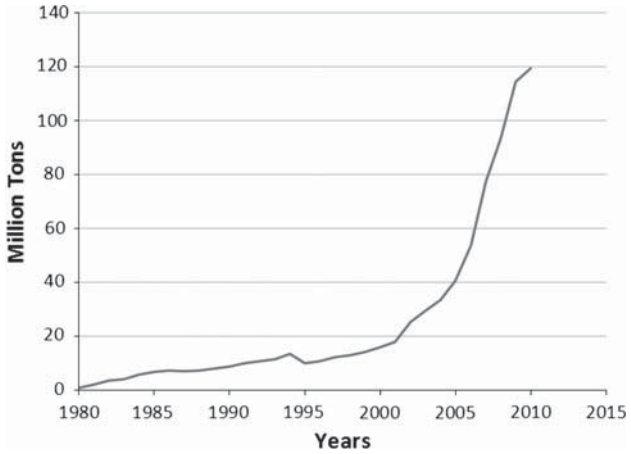

Fig. 3 Corn used for fuel ethanol in the US between 1980 and 2010 in millions of tons. The corn used for fuel is derived from the previous year's harvest, thus the 119 million tons of corn used for fuel ethanol in 2010 represents 28.7 % of the 2009 grain crop (416 million tons). *Source*: EPI from USDA (2012b)

The second crisis is the ongoing severe global financial and economic decline which in 2011 saw international food prices rise to levels not seen in decades, coinciding with the global population reaching 7 billion people. The export-weighted FAO food price index climbed to a record 238 points in February 2011 (Fig. 1), and the price of wheat and corn doubled reflecting the drought and subsequent wheat export ban in the Russian Federation and the poor yield of corn in United States as well as the weakening dollar (FAO 2011b). The global food system is becoming more vulnerable to episodes of high prices and volatility (The Economist 2011). Specialization in a few export commodities such as coffee or cocoa has increased the dependence of developing countries on food imports converting them from net exporters, with an overall agricultural surplus of US$7 billion in the 1960s, to predominantly

net importers in the 1990s and 2000s, with a deficit of US$11 billion in 2001 (FAO 2004; Action Aid International 2008).

In light of these crises, earlier progress towards food security based on the 1996 World Food Summit goal (reducing the number of hungry people in the world) and Millennium Development Goal 1 (halving the hungry population by 2015) has been reversed (Wiebe et al. 2011; Yuan et al. 2011). Multilateral investments in developing country agriculture by industrialized governments and global institutions such as the World Bank have steadily declined (Jomo 2008). USAID, the United States International Development Agency, has cut agricultural aid by 75 % over the last 20 years (USAID 2004). Only 4 % of current development aid to Africa is spent on agriculture, and agricultural research grants were cut by more than 80 % between 1980 and 2006, with the United States alone reducing its contribution from US$ 2.3 billion to US$ 624 million (Jomo 2008).

In many parts of the world, agricultural growth is needed to address the current world food crisis by contributing to overall economic growth and helping to achieve MDG1 (Yuan et al. 2011). There have been numerous attempts to estimate the cost of achieving MDG1, mostly at the global or regional levels, including the United Nations Zedillo Report, studies by the World Bank and the United Nations Development Program, and the International Food Policy Research Institute (IFPRI). These estimates have varied widely, mostly because of different methodologies, assumptions, coverage, measures and interpretations. The Zedillo report contains some rough estimates of the additional aid required to achieve the MDGs, with US$ 20 billion of the US$ 50 billion total required to halve poverty and hunger (UN 2001). Using two different approaches, the World Bank estimated that the additional foreign aid required to achieve MDG1 by 2015 is US$ 40–60 billion per year (Devarajan et al. 2002). IFPRI estimates that a total global annual investment in agriculture of US$ 14.3 billion per year is necessary, although under a high-investment scenario, these requirements would double to US$ 28.5 billion per year (Fan and Rosegrant 2008).

Direct costs of the causes and consequences of food insecurity

The direct costs of food insecurity have been estimated by the US Department of Agriculture (USDA) based on food assistance and nutrient supplementation programs. Such programs promote food security in developing countries by providing food aid to save lives and help low-income families (World Bank 2012). Additional direct costs reflecting the burden on healthcare systems dealing with hunger and malnutrition are included, although these are expressed as disability adjusted life years (DALYs) and are considered indirect costs in the discussion below.

Food assistance and nutrient supplementation programs

Malnutrition has been addressed directly by supplementation (short-term micronutrient delivery), industrial fortification, biofortication, dietary diversification and the support of public health measures (Stein et al. 2006). The United States has led international efforts to combat malnutrition and hunger for more than 60 years. Through food aid and assistance programs, the USDA provides support to the agricultural development sector, as well as food security and humanitarian help following natural or manmade disasters in developing countries, with average annual donations of $US 2.2 billion. In 2012, USDA food assistance benefited more than 9.7 million people through the Food for Progress, Food for Peace, Local and Regional Procurement Pilot Project, and McGovern-Dole International Food for Education and Child Nutrition Program initiatives (Table 1). The major participants are nonprofit charitable organizations, governments, intergovernmental organizations and academic institutions (Ho and Hanrahan 2010).

Food for Peace (also known as Public Law 480) encompasses four sections: Trade and Economic Development Assistance; Emergency and Development Assistance; Food for Development; and Farmer-to-Farmer Assistance. This program aims to improve global food security and nutrition, promote agricultural development, expand international trade and foster private enterprise. Food for Progress donates US commodities to developing countries in order to initiate and expand free enterprise in the agricultural sector. The objective is to improve agricultural productivity and commercialization by training farmers, developing agricultural infrastructure and improving farming methods (e.g. irrigation systems, cooperatives and microcredit support). The Local Regional Food Aid Procurement Pilot Project provides food aid following price fluctuations and thus promotes food security in developing countries. The McGovern-Dole International Food for Education and Child Nutrition Program contributes to educational programs, as well as maternal and child nutrition in developing countries, by providing technical and financial assistance and agricultural commodities such as dairy, cotton, fruits/vegetables, poultry and livestock. This promotes primary school attendance, maternal health during pregnancy and breastfeeding, and children's health and hygiene at school. Under this program, the Micronutrient-Fortified Food Aid Products Pilot

Table 1 The social and economic benefits of the Food for Progress Program and the McGovern-Dole International Food for Education and Child Nutrition Program in 2012

Country	Participant	Potential beneficiaries	Estimated value ($)
Food for progress program			
El Salvador	FINCA International	270,000	11,000,000
Guatemala	Counterpart International	70,000	7,500,000
Honduras	TechnoServe	550,000	12,000,000
Mali	Aga Khan Foundation	710,000	24,000,000
Mongolia	Mercy Corps	140,000	6,800,000
Mozambique	Land O'Lakes	970,000	22,000,000
Nicaragua	TechnoServe	270,000	9,500,000
Nicaragua	Catholic Relief Services	1,490,000	20,300,000
Senegal	International Relief and Development	1,560,000	14,800,000
Senegal	Shelter for Life	20,000	14,800,000
Tanzania	Catholic Relief Services	900,000	15,300,000
Total		6,950,000	158,000,000
McGovern-Dole program			
Afghanistan	World Vision	80,000	16,600,000
Cambodia	International Relief and Development	30,000	6,900,000
Cameroon	Counterpart International	120,000	16,400,000
Ethiopia	World Food Program	240,000	26,500,000
Haiti	World Food Program	300,000	8,000,000
Honduras	Catholic Relief Services	50,000	16,000,000
Kenya	World Food Program	700,000	9,700,000
Kyrgyzstan	Mercy Corps	70,000	10,900,000
Laos	Catholic Relief Services	30,000	12,000,000
Liberia	World Food Program	340,000	6,400,000
Malawi	World Food Program	340,000	8,300,000
Nepal	World Food Program	210,000	6,000,000
Mozambique	Planet Aid	70,000	20,000,000
Mozambique	World Vision	110,000	20,000,000
Senegal	Counterpart International	30,000	11,200,000
Sierra Leone	Catholic Relief Services	30,000	10,800,000
Total		2,750,000	205,700,000

In this program, the USDA buys US products such as dehydrated potato flakes, lentils, corn-soy blend, cornmeal, rice, sorghum, soybeans and wheat, and donates them to participants (private voluntary organizations and government agencies) in developing countries. Food for Progress beneficiaries sell the products and use the funds to introduce and develop free enterprise in the agricultural market. The McGovern-Dole Program participants use or sell the products to support food security, child development and education. Nearly 10 million people benefited from these programs in 2012, with estimated total costs of US$ 360 million

(MFFAPP) explores the potential to fight micronutrient deficiencies through the distribution of micronutrient-fortified food aid (Ho and Hanrahan 2010). USDA supports the development of micronutrient-fortified foods by investing more than $US 8.5 million in nutrient-enhanced food to address micronutrient deficiencies in women and children. Furthermore, private companies such as Heinz offer support for the distribution of micronutrient powders to children in developing countries, e.g. Heinz has provided $US 5 million thus far to support the Micronutrient Campaign.

Burden on healthcare systems dealing with hunger and malnutrition

The global impact of hunger and malnutrition on healthcare systems includes the costs of mortality, morbidity and disability, but also longer-term consequences on physical and mental health (Black et al. 2008). Cost-effectiveness analysis (CEA) has been used to investigate the efficiency of healthcare resources by comparing the relative costs and health gains of different interventions. Current data are predominantly derived from high-income countries

although the results can be extrapolated to developing countries (Hutubessy et al. 2003). For example, the additional costs of managing malnutrition in Dutch nursing homes are up to US$ 366 million per year (US$ 10,494 per patient at risk of malnutrition and US$ 13,117 per malnourished patient) based on the extra costs of nutritional screening, monitoring and treatment (Meijers et al. 2011). In Brazilian hospitals, malnourishment results in an average daily cost of US$ 228.00/patient, compared to US$ 138.00/patient for well-nourished individuals, an increase of 60.5 % (Correia and Waitzberg 2003). But the actual costs are even higher because malnutrition increases the length of hospital stays by an average of 43 % (Pirlich et al. 2006). More recent data from the UK suggest that the healthcare costs of malnourished patients over a 6-month timeframe (US$ 2,829) are more than twice those of well-nourished patients (US$ 1,210) (Guest et al. 2012). Nutrient supplements in hospital can result in substantial savings because well-nourished patients recover better and faster and have fewer complications (Russell 2007) (Table 2).

Indirect costs

Among the 26 major global health burdens, iron deficiency anemia, zinc deficiency and vitamin A deficiency (VAD) rank 9, 11 and 13, respectively (Fig. 4). This means that 30 % of the global population suffers from one or more of these diseases (WHO 2009). Furthermore, large numbers of people also suffer from diseases caused by a lack of selenium, folate, calcium and iodine (Stein et al. 2007; Stein 2010).

Women and children are the most vulnerable groups because pregnancy, breast-feeding and menstruation, as well as rapid body growth in children, increase micronutrient requirements and make it even more difficult to achieve adequate intakes (Benoist et al. 2008). More than one third of child deaths are attributed to malnutrition. If a child is undernourished or malnourished during pregnancy and/or the first 2 years of life, this affects physical and mental health for life. For example, children suffering from iron deficiency show poor cognitive development, psychomotor development and socio-emotional activity (Lozoff et al. 2006, Beard 2008). Iron deficiency anemia also affects reproductive performance and increases the risk of death during pregnancy (Hunt 2002). Malnutrition directly affects school enrolment and class performance, reducing the likelihood of a complete education (Khanam et al. 2011; Liu and Raine 2006). Micronutrient malnutrition also reduces the aggregate productivity and economic development of communities and countries (World Bank 1994; Qaim et al. 2007).

The accurate measurement of losses caused by micronutrient deficiency is difficult but the usual approach is to calculate DALYs representing the sum of years of life lost (YLL) plus the years lived with disability (YLD). One DALY is equivalent to 1 year of healthy life lost. Although human life cannot be measured in monetary terms, in this case a value for human illness and loss of life can be calculated based on the annual average per capita income of people in a particular country (Stein 2010). The WHO has reported that 28 million DALYs were lost due to zinc deficiency in 2002 and 35.1 million DALYs were lost to iron deficiency anemia. All mineral deficiencies combined result in 65.6 million DALYs lost and this figure increased to 92 million DALYs when vitamin A deficiency was included (WHO 2002, 2004). In an average year India loses up to 4 million DALYs to iron deficiency anemia, and 2.8 million to zinc deficiency (Stein 2010). In 1994, the World Bank reported that DALY losses due to protein energy malnutrition, vitamin A, iodine and iron deficiency was 5 % of GDP in Sub-saharan Africa, China, India, Latin American and Middle Eastern countries (Stein 2010), which can be easily overcome by investing 0.3 % of the GDP into malnutrition alleviation programs in these countries (Adamson 2004). In South Asia, where iron deficiency anemia is most prevalent, annual economic losses of US$ 5 billion are estimated, with productivity

Table 2 The economic benefits of commercial oral nutritional supplements for hospital patients (adapted from Russell 2007)

	Supplementation	Cost of stay (US$)	Cost of complication (US$)	Total costs of malnutrition (US$)
Abdominal surgery	Ensure Plus, Ross Laboratories, UK (vitamin C, vitamin E and selenium)	1,135	324.4	1,459.4
Orthopedic surgery	Milk-based drinks (Fortisip, Nutricia Clinical Care, Trowbridge, Wiltshire) or juice-based drinks (Enlive, Abbott Laboratories Limited, Maidenhead, Berkshire, UK)	721.8	783.4	1,505.2
Non-surgical patients	Clinutren soup (Nestlé Clinical Nutrition, Brussels, Belgium)	535.3	181.2	716.5

All costs are shown in US dollars per day

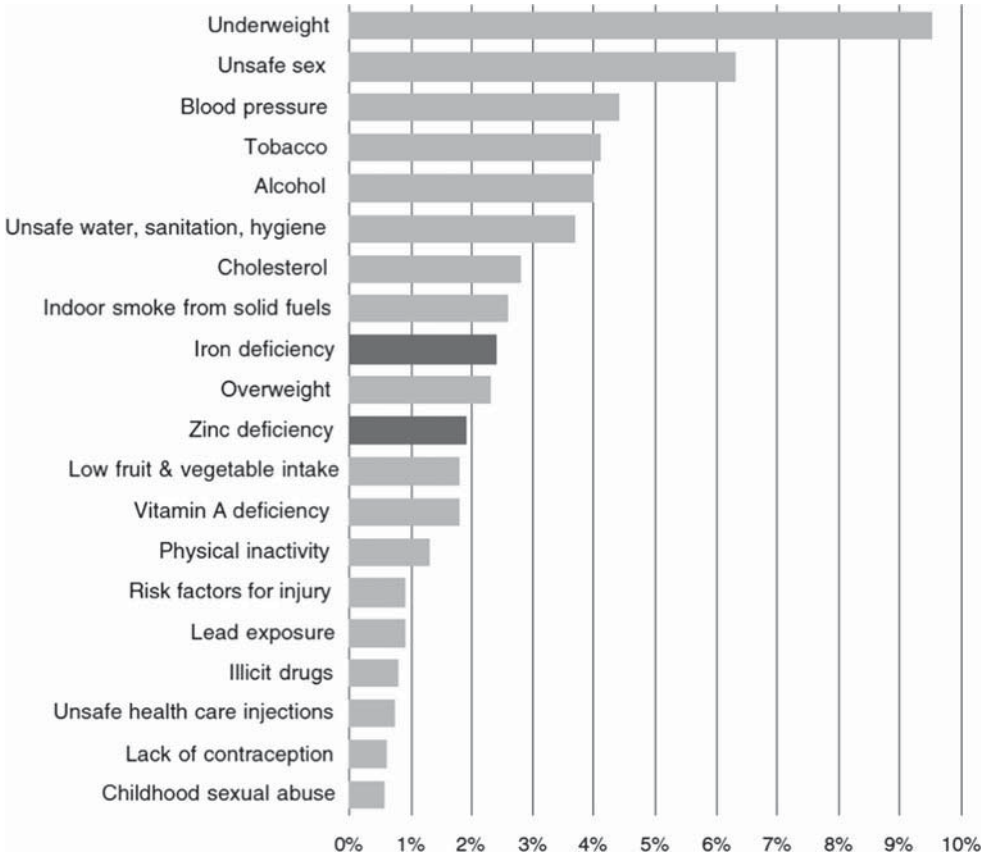

Fig. 4 The top 20 major disease burdens in the developing world. The x-axis represents the attributable DALYs (% of global DALY). *Source*: Stein (2010)

reduced by 1.5 % for every 1 % loss of hemoglobin (Dickinson et al. 2009).

The cost of unsustainable solutions (10-year survey)

The most effective solution to food insecurity is a varied diet including fresh fruits, vegetables, fish and meat. This is impractical in many countries resulting in persistent malnutrition at the population level (Gómez-Galera et al. 2010). To deal with the widespread iron, zinc and vitamin A deficiency in developing countries, nutritional planners have developed three solutions: short-term supplementation, mid-term fortification and long-term dietary modification. The short-term solution involves the provision of high-dose micronutrient capsules (e.g. 200,000 IU of vitamin A to all young children at 6-month intervals) and is the most widely implemented but also the least sustainable intervention (Greiner 2012). The mid-term fortification of staples or condiments and the long-term modification of diets for vulnerable groups are more sustainable but also more difficult to establish in developing countries.

The total costs of short-term micronutrient intervention have been estimated at US$ 1.5 billion per year, followed by an additional US$ 2.9 billion over 10 years to implement behavioral interventions and a further US$ 1.0 billion to establish more complex and targeted programs so that nutrition can be improved on a sustainable basis. Another US$ 100 million would be needed to monitor and evaluate these large-scale programs, conduct follow-up research and provide technical support (Horton et al. 2010). The initial US$ 1.5 billion annual investment would provide therapeutic supplements of iron, zinc and vitamin A, as well as universal salt iodization and iron-folate fortification during pregnancy, but even this intervention is not yet included in the WHO recommendations (Table 3) (Horton et al. 2010). These costs are estimated for the 36 countries identified in the 2008 Lancet series on maternal and child malnutrition, which are home to 90 % of moderately or severely stunted children worldwide (Horton et al. 2010). Additional costs for scaling up these interventions to include 32 smaller countries where 20 % or more of all children under the age of five are stunted or underweight (mainly in Sub-Saharan Africa) were estimated, showing that this expansion of coverage would increase the target population by 6 % and increase overall costs by a comparable amount (Horton et al. 2010) (Table 4).

There are at least four potential sources of funding for such interventions: private-sector households, private-sector corporations, public/government funding and innovative financing models such as the High Level Taskforce on Innovative Financing for Health Systems (Horton et al.

Table 3 Estimated investment costs and annual operational costs for micronutrient interventions (Horton et al. 2010)

Intervention	Additional investment (per year)	Additional annual cost
Vitamin A supplementation	US$ 1.20 per children 6–59 months of age	US$ 130 million
Therapeutic zinc supplementation	US$ 1 per children 6–59 months of age	US$ 360 million
Iron-folic acid supplementation	US$ 2.00 per pregnancy	US$ 85 million
Iron fortification of staple and other foods	US$ 0.20 per person	US$ 599 million
Multiple micronutrient powder	US$ 3.60 per children 6–23 months of age	US$ 216 million
Universal salt iodization	US$ 0.05 per person	US$ 80 million

All costs are shown in US dollars

2010). Households are accustomed to bearing most of the recurrent costs of market-based strategies, such as salt iodization (estimated at US$ 400 million) and fortification (Horton et al. 2010). Developing country governments already contribute substantial amounts to nutritional programs, e.g. India allocated nearly US$ 1.3 billion for its Integrated Children Development Service Program in 2007–2008 (Horton et al. 2010).

Private charitable foundations and philanthropists such as the Bill and Melinda Gates Foundation and the Children's Investment Fund are also emerging as a significant financing source for nutritional programs (Horton et al. 2010). Unlike the US donor agencies, which must demonstrate the worth and impact of their work to taxpayers, European donor agencies tend to work quietly, knowing they have the support of most of the public, although this makes the process less transparent (Greiner 2012).

Cost savings with nutritionally-enhanced crops

Vitamin A deficiency poses a significant public health challenge in 118 countries, especially in Africa and Southeast Asia (Van Wijk 2002). Genetic engineering is an emerging strategy for the control of VAD in the developing world, typically the development of β-carotene enriched crops, such as canola (*Brassica napus*) (Shewmaker et al. 1999) and mustard (*Brassica juncea*) (Agricultural Biotechnology Support Project 2003). Golden Rice (Ye et al. 2000; Paine et al. 2005), Multivitamin Corn (Naqvi et al. 2009), and high lutein/zeaxanthin corn (Naqvi et al. 2011) are further examples of crops that have been engineered to increase their content of β-carotene and other nutritionally important carotenoids.

Several recent studies have examined the potential economic impact of Golden Rice and Golden Mustard on VAD by calculating the avertable health burden in terms of DALYs in the Philippines and India (Zimmerman and Qaim 2004; Stein et al. 2006; Chow et al. 2010). The widely-used DALY metric allows comparisons of alternative health strategies using a single index that combines information about mortality and morbidity (Zimmerman and Qaim 2004; Stein et al. 2006; Chow et al. 2010).

Golden Rice

There are no conventional rice varieties with enough β-carotene in the grain to use in conventional breeding strategies to increase β-carotene levels and combat VAD. Golden Rice has been engineered to produce β-carotene (pro-vitamin A) in the grain endosperm, so that polished rice grains can be used to prevent VAD in the developing world. Golden Rice grains contain up to 23-fold more total carotenoids than conventional rice (37 μg/g dry weight) including β-carotene levels of up to 31 μg/g dry weight (Paine et al. 2005). An ex ante analysis of Golden Rice in India was carried out considering the entire sequence of events between cultivation and consumption to calculate its overall impact on health (Stein et al. 2006). In a high-impact scenario, India's annual burden of VAD (2.3 million DALYs lost) could be reduced by up to 59.4 % by the consumption of Golden Rice, saving 1.4 million healthy life years. In a low-impact scenario, where Golden Rice is consumed less frequently and delivers less β-carotene, the burden of VAD would be reduced by 8.8 %. However, in both scenarios, the cost per DALY saved by using Golden Rice (US$ 3.06–19.40) is lower than the cost of supplementation, and Golden Rice outperforms international cost-effectiveness thresholds. The total annual cost of vitamin A supplementation is approximately $US 21 million, and the total annual cost of a flour fortification program has been estimated at $US 4–6 million (Fiedler et al. 2000). Golden Rice should therefore be introduced immediately as a complementary intervention to fight VAD in rice-eating populations (Stein et al. 2006).

Another ex ante analysis of Golden Rice was carried out in the Philippines, suggesting a gain of 15,000–85,000 DALYs per annum and a reduction in the health burden of 5.7–31.5 %, with the lower figures representing a pessimistic scenario and the higher figures representing an optimistic one (Zimmerman and Qaim 2004). The greatest

Table 4 The 36 countries with 90 % of the global burden of stunting and an additional 32 high-burden countries with underweight or stunting rates greater than 20 % (Horton et al. 2010)

36 countries identified in the lancet that carry 90 % of the stunting burden for which financing needs are estimated	32 smaller countries with rates of child stunting and/or underweight > 20 % that could be added to these estimates at an additional cost of 6 %
Afghanistan	Albania
Angola	Bhutan
Bangladesh	Bolivia
Burkina Faso	Botswana
Burundi	Central African Republic
Cambodia	Comoros
Cameroon	Congo, Republic of
Congo, Democratic Republic of	Ecuador
Côte d'Ivoire	Equatorial Guinea
Egypt, Arab Republic of	Eritrea
Ethiopia	Djibouti
Ghana	Gambia, The
Guatemala	Guinea
India	Guinea-Bissau
Indonesia	Haiti
Iraq	Honduras
Kenya	Lesotho
Madagascar	Liberia
Malawi	Maldives
Mali	Mauritania
Mozambique	Mongolia
Myanmar	Namibia
Nepal	Rwanda
Niger	São Tomé and Príncipe
Nigeria	Sierra Leone
Pakistan	Somalia
Peru	Sri Lanka
Philippines	Swaziland
South Africa	Tajikistan
Sudan	Timor-Leste
Tanzania	Togo
Turkey	Zimbabwe
Uganda	
Vietnam	
Yemen, Republic of	
Zambia	

overall benefits were predicted in children, reflecting the lower initial prevalence of corneal xerophthalmia among children in the Philippines. Golden Rice was predicted to avert 798 child deaths per year in the optimistic scenario (Zimmerman and Qaim 2004).

Bioavailability studies demonstrated that the total amount of β-carotene in Golden Rice (35 μg/g dry weight) was the same before and after cooking, i.e. boiling for 30 min (Tang et al. 2009). Therefore, eating 348 g of Golden Rice per day would achieve the dietary reference intake for vitamin A.

Golden Mustard

Golden Mustard has been engineered to accumulate up to 600 μg/g β-carotene (Agricultural Biotechnology Support Project 2003) and is particularly suitable for deployment in India, which consumes large amounts of mustard oil (Chow et al. 2010). India has the greatest number of clinical VAD cases in the world (more than 35 million) and the greatest percentage of subclinical VAD in children under six (31–57 % of the population) (West 2002). A cost analysis of Golden Mustard was carried out based on a conservative efficacy rate of 4 % and an optimistic efficacy rate of 23 % averted mortality. The number of DALYs averted over a 20-year time frame was estimated at 18–34 million, and the number of lives saved was 113,000–654,000, with the lower figures representing the conservative scenario and the higher figures representing the optimistic one (Chow et al. 2010). Golden Mustard was also estimated to avert 5–6 million more DALYs and 8,000–46,000 more deaths than supplementation, mainly because it would benefit the entire population and not only children and women (Chow et al. 2010).

The amount of β-carotene in mustard oil derived from Golden Mustard containing 600 μg/g β-carotene has been estimated at 185 μg/g (Chow et al. 2010), although only 71 % remained after baking, seasoning, deep-frying and shallow frying (Manorama and Rukmini 1991). The mustard variety used to produce Golden Mustard accounts for 70–80 % of the mustard grown in India, therefore only 75 % of the mustard seed pressed into oil would be the fortified variety. Based on these assumptions, the effective concentration of β-carotene would be 49.3 μg/g of consumed oil and the fixed costs would amount to approximately US$ 0.01 per person. In conclusion, only a few drops of the fortified oil would satisfy the dietary reference intake for vitamin A.

The current cost of technology

GE crops offer a number of potential solutions to tackle food insecurity in developing countries but the adoption rate for such crops is low at present (Qaim 2009; Ramasamy et al. 2007). This is often because developing countries have a limited capacity to carry out research and

development (R&D), coupled with the high regulatory burden of GE technology, market barriers and the inadequate protection of intellectual property (Cohen 2004; Pray et al. 2005; Ramessar et al. 2009; Paarlberg 2001). In this context, it is important to establish a cost-effective approach for the development of nutritionally-enhanced GE crops. Currently, GE technology requires substantial upfront R&D investment plus additional funding to overcome the immense regulatory burden (Ramessar et al. 2010; Twyman et al. 2009; Ramessar et al. 2007). Even when a GE crop has been authorized for cultivation, a breeding program is required to commercialize the novel trait into locally adapted varieties. Additional marketing costs are necessary to promote the public acceptance of GE crops.

Following the lead of Golden Rice (Ye et al. 2000), several other nutritionally-enhanced crops have been developed, including Multivitamin Corn which accumulates β–carotene, ascorbate and folate (Naqvi et al. 2009). However, none of these enhanced varieties have been commercialized. In the case of Golden Rice, the R&D costs (involving international projects) reached US$ 3 million (Zimmerman and Qaim 2004) but this may increase to $7.5 million for other crops depending on the circumstances (Stein et al. 2006).

GE crops must go through a risk assessment procedure where they are evaluated in laboratory tests and field trials, and must undergo safety analysis (Gómez-Galera et al. 2012; Arjó et al. 2012). The costs of regulatory compliance include the direct costs of testing (to provide information for the regulators) and also the costs of the administrative structure to ensure compliance (Pray et al. 2005). The necessary tests include molecular characterization, compositional assessment and 90-day rat toxicity assays which can cost $US 4.2–7.7 million (Kalaitzandonakes et al. 2007). Agronomic, phenotypic, environmental and allergenicity testing may also be required (EFSA 2010). After field trials, regional breeding programs must be carried out to introduce the desirable characteristics into high-yielding varieties and/or hybrids grown in those areas, resulting in additional costs. For example, the cost of the first breeding program for Golden Rice in India was approximately $US 1 million (Stein et al. 2006).

The costs associated with regulatory compliance represent a significant portion of the total costs of bringing a GE product to market, erecting a significant barrier to adoption particularly in developing countries (Jaffe 2006; Kalaitzandonakes et al. 2007; Pray et al. 2005). However, assuming that GE regulatory mechanisms for licensing such products are already in place and the R&D program is complete, one-time fixed costs for the adoption of GE crops in India have been estimated at $US 5.6 million for Golden Mustard (Chow et al. 2010) and at least $US 2 million for Golden Rice (Stein et al. 2006).

Once GE crops are approved for cultivation, promotion campaigns and social marketing is necessary to ensure consumer acceptance. In this context, marketing costs for Golden Rice could exceed $US 15 million in India (Stein et al. 2006) although these costs may be lower for second-generation, quality-enhanced crops combining agronomic and quality traits (Qaim 2009). The new phenotype of GE crops (such as the different color of rice or corn seeds containing high levels of β-carotene) can also affect public acceptance. However, a study in Mozambique looking at the consumer acceptance of an orange corn variety with high levels of β-carotene showed that existing preferences for white corn do not prevent the acceptance of orange biofortified corn, and that the colored kernels may act as a self-targeting nutritional intervention (Stevens and Winter-Nelson 2008). Taking previous data from Golden Rice together with the acceptance of orange corn, it is expected that promotional campaigns and social marketing for Multivitamin Corn will cost less than $US 15 million. Altogether, these data suggest that the total technology costs for GE crops include $US 3–7.7 million (32 %) for R&D, $US 1 million (4 %) for breeding, $US 2–5.6 million (23 %) for regulatory compliance and up to $US 15 million (41 %) for marketing, making a total of US$ 20–29 million (Fig. 5).

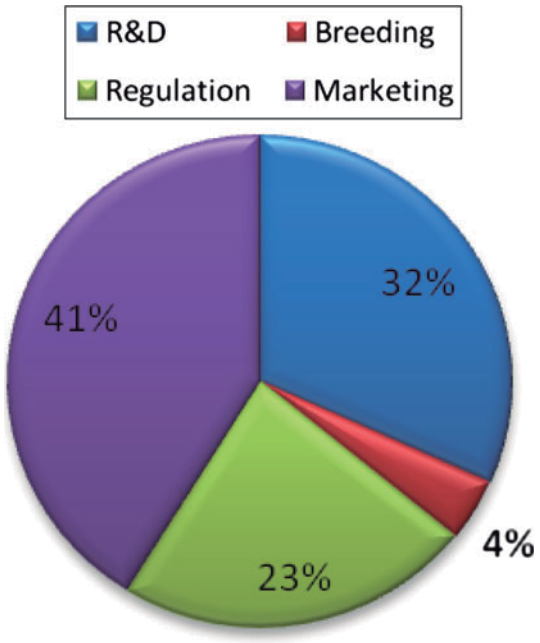

Fig. 5 The proportion of R&D, breeding, regulation and marketing costs required for the development of a genetically engineered crop (adapted from Stein et al. 2006)

In addition to technology costs, the adoption and implementation of a regulatory framework for GE crops in developing countries may have a direct impact on the economy, particularly on current and future agricultural exports to countries with stringent regulations, such as those in the EU (Zarrilli 2005; Sabalza et al. 2011). For example, the approval of Bt eggplant (Chong 2005) led to the exclusion of India from international markets and blocked financial support for biotechnology research from EU countries (Ramessar et al. 2008, 2009). The Indian Government therefore banned cultivation of this eggplant variety due to political pressure rather than scientific evidence.

Intellectual property (IP) issues also need to be taken into consideration. Even in the case of the more recent biotechnology patents, IP is built at least in part on earlier innovations which have been protected through patents, including vectors, selectable markers, transcription regulatory elements, sub-cellular targeting systems, etc. (Krattiger and Mahoney 2007). Consequently, IP issues influence decision making in transgenic crop development (Sechley and Schroeder 2002). Humanitarian applications such as Golden Rice (GR), was constrained by up to 70 patents. Thus the creation of GR required the use of many technologies that were patented by different companies, including Bayer AG, Monsanto, Novartis AG, Orynova BV and Zeneca Mogen BV (Krattiger and Potrykus 2007). Following discussions and negotiations with IP holders, the developers of GR were able to secure free access to all constraining IP gratis for defined humanitarian research purposes only, allowing the use of GR in developing countries by resource-poor farmers (Krattiger and Potrykus 2007). Although GR demonstrated that IP and Freedom to Operate (FTO) issues can be resolved, biotechnology companies are generally uneasy about such humanitarian ventures because most developing countries lack enforceable IP policies that would ensure that their IP rights are protected adequately (Wendt and Izquierdo 2001). In order to establish and maintain international technology transfer agreements, industrialized and developing countries need to cooperate in the development of a manageable system for IP protection. This can be achieved in a number of ways, e.g. local ownership and the involvement of scientists from developing countries or by the implementation of appropriate IP policies and effective enforcement procedures in developing countries (Kowalski 2002). Several organizations have been set up to promote biotechnology in developing countries, particularly in the area of subsistence agriculture, focusing on IP access and management They offer advice on a complete set of biotechnology technologies under IP by third parties and a road map for FTO. Some examples are CAMBIA (Center for the Application of Molecular Biology to International Agriculture), PIPRA (Public Intellectual Property Resource for Agriculture), AATF (African Agricultural Technology Foundation) and SIPPI (Science and Intellectual Property in the Public Interest).

Conclusions and recommendations

Numerous studies have shown the social and economic benefits of GE technology, including its ability to address global food insecurity. GE crops can tackle food insecurity in a number of ways. First-generation crops, with modified input traits, can address food insecurity by increasing the yields of food crops grown by subsistence farmers to avoid hunger, and can increase the profit margins of smallholder farmers growing cash crops e.g. by reducing labor and pesticides, thereby reducing poverty and empowering a greater proportion of the population (Qaim 2010; Sanahuja et al. 2011). For example, the major benefits of the herbicide tolerant Roundup Ready soybean in Bolivia include a 30 % increase in yield, a 22 % savings in labor and other variable costs (ISAAA 2012). Herbicide tolerant soybean adopters in general cultivate larger areas, are more educated and are more likely to own their farm and farm machinery (Smale et al. 2012). Second-generation crops, with modified output traits, can address food insecurity directly by increasing the nutritional value of food, e.g. the examples of Golden Rice, Golden Mustard and Multivitamin Corn, all of which have higher levels of key vitamins compared to conventional varieties (Naqvi et al. 2009).

A number of studies have considered the relative costs and benefits of GE technology in terms of the overall development costs compared to conventional intervention strategies and the benefits on the ground to farmers. These studies have clearly demonstrated the benefits of first-generation crops to farmers in many developing countries and the consequential positive effects on national GDP and GNP values thus increasing the ability of governments to invest in infrastructure and improve the health and well-being of their populations. For example, the adoption of Bt cotton in India has generated a profit of US$ 51 billion during the period 2002–2008 (Devasahayam et al. 2011) and herbicide tolerant soybean in Bolivia provided a net return of US$196 per hectare, resulting in US$175 million benefits at the national level (ISAAA 2012).

Furthermore, we have reviewed several cost–benefit studies relating to nutritionally enhanced GE crops. For example, the expected cost of developing Golden Rice, including R&D costs, was estimated to be $US 10.7 million, with a continuing cost of $US 0.5 million per year (Zimmerman and Qaim 2004). Balanced against this, Stein et al. (2006) showed that introducing Golden Rice 2 which accumulates substantially more β-carotene in the polished

grain than the original variety, could recover between 204,000 and 1.4 million DALYs per annum in India at a cost of only $US 21.4–27.9 million over 30 years, which is an average annual cost of only $US 713,000–931,000. Providing vitamin A supplements was an inexpensive intervention, at $US 23–50 per DALY and US$ 1000–6000 per death averted, although this would target the most vulnerable groups such as children and pregnant women (Chow et al. 2010). GE crops could avert 5–6 million more DALYs and 8,000–46,000 more deaths by covering the entire population rather than just the most vulnerable groups. Although the cost of GE crops was up to five times higher than their non-engineered counterparts, this predominantly reflected the one-off cost of regulatory compliance and approval ($US 5.6 million) which could be reduced if the regulatory burden on GE technology was lowered (Chow et al. 2010). The deployment of Golden Rice and other nutritionally-enhanced crops should also be considered in a geographical context – for example, rice is a staple in Asia but not in Sub-Saharan Africa, where nutritionally-enhanced corn varieties would be preferable (Zhu et al. 2008; Naqvi et al. 2009).

The global costs of food security need to be considered when developing efficient strategies to address hunger and malnutrition. It would cost $US 130 million to provide supplements for the 17.3 million acutely-affected children (6–59 months of age) in the 36 countries with the highest burden of malnutrition (Horton et al. 2010) but it is important to consider that these are recurrent costs, necessary to address the symptoms but not the causes of malnutrition. In contrast, the costs of developing GE crops have to be borne upfront in order to overcome technological and regulatory barriers as well as sociopolitical factors. However, once crops have been cultivated they are largely sustainable without further investment and the running costs are mainly associated with distribution and formulation to ensure adequate doses. Rice is a staple in South Asia, East Asia and the Pacific so nearly 2 billion people could benefit from Golden Rice, whereas corn is a staple in sub-Saharan Africa, South America and the Caribbean, where an additional 700 million people could benefit from Multivitamin Corn. It is also clear that GE crops enhanced with multiple vitamins and minerals are desperately needed because this would allow a single crop to treat multiple deficiency diseases. Stacking nutritionally-enhanced GE crops with agronomic traits will be the next logical step in addressing food insecurity in developing countries in a more meaningful way. This will assure that subsistent farmers will maximize the benefits of the new products and technologies in the most optimal way.

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Engineering metabolic pathways in plants by multigene transformation

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ABSTRACT Metabolic engineering in plants can be used to increase the abundance of specific valuable metabolites, but single-point interventions generally do not improve the yields of target metabolites unless that product is immediately downstream of the intervention point and there is a plentiful supply of precursors. In many cases, an intervention is necessary at an early bottleneck, sometimes the first committed step in the pathway, but is often only successful in shifting the bottleneck downstream, sometimes also causing the accumulation of an undesirable metabolic intermediate. Occasionally it has been possible to induce multiple genes in a pathway by controlling the expression of a key regulator, such as a transcription factor, but this strategy is only possible if such master regulators exist and can be identified. A more robust approach is the simultaneous expression of multiple genes in the pathway, preferably representing every critical enzymatic step, therefore removing all bottlenecks and ensuring completely unrestricted metabolic flux. This approach requires the transfer of multiple enzyme-encoding genes to the recipient plant, which is achieved most efficiently if all genes are transferred at the same time. Here we review the state of the art in multigene transformation as applied to metabolic engineering in plants, highlighting some of the most significant recent advances in the field.

KEY WORDS: *direct DNA transfer, multigene transformation, metabolic pathway, genetic engineering*

Introduction

Most agronomic traits in plants are controlled by multiple genes, as is also the case for the synthesis of complex organic compounds from primary and secondary metabolisms, which often represent the outputs of long and convoluted metabolic pathways. Therefore, genetic engineering has seen a progressive change from single-gene intervention to multigene transformation to tackle increasingly ambitious objectives (Halpin, 2005).

In the early years of plant biotechnology, gene transfer experiments typically involved two transgenes: one selectable marker under the control of a constitutive promoter to facilitate the selective propagation of transformed cells, and a 'primary transgene' or 'gene of interest' which could be under the control of any promoter and was intended to alter the phenotype of the plant in a specific manner (Peremarti *et al.*, 2010). This principle was adopted in the first examples of metabolic engineering, which involves the modulation of endogenous metabolic pathways to increase flux towards particular desirable molecules or even new molecules (Capell and Christou, 2004). Multigene transformation (MGT) is being gradually accepted as an approach to generate plants with more ambitious phenotypes, including more complex examples of metabolic engineering (Naqvi *et al.*, 2009). To this end, methods had to be developed for the coordinated expression of larger groups

Abbreviations used in this paper: BAC, bacterial artificial chromosome; CaMV35S, cauliflower mosaic virus 35S; ORF, open reading frame; MGT, multigene transformation; PHB, polyhydroxybutyrate; PUFA, polyunsaturated fatty acid.

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of genes (Capell and Christou, 2004) to attain objectives, such as: (a) enhance the activity of enzymes at multiple rate-limiting steps in target pathways, e.g. by expression of enzymes that are released from feedback inhibition; (b) increase the availability of upstream precursors to increase flux through the target pathway; (c) modulate pathway branch points to prevent the loss of flux; and (d) promote the development of sink compartments to store target compounds (Fig. 1) (Zhu *et al.,* 2013).

Examples of metabolic engineering in plants include primary metabolic pathways (carbohydrates, amino acids, and lipids) and secondary metabolic pathways (e.g. alkaloids, terpenoids, flavonoids, lignins, quinones, and other benzoic acid derivatives; Gomez Galera *et al*., 2007). These pathways generate a large number of compounds that are useful to humans, including energy-rich foods, vitamins and many different pharmaceuticals. In this review, we focus on the metabolic engineering of vitamins, polyunsaturated fatty acids, and secondary metabolites, because they provide illustrative examples of applied MGT.

The scope of the challenge

The simultaneous transfer of multiple genes into plants (co-transformation) can be achieved using two main approaches, one involving linked genes (multiple genes on the same plasmid) and the other involving unlinked genes (different genes on different plasmids). The two methods can be used with both major strategies for gene transfer to plants, i.e. transformation with *Agrobacterium tumefaciens* and direct DNA transfer (Naqvi *et al.,* 2009).

Multiple linked genes can be transferred by *Agrobacterium*-mediated transformation using standard binary vectors containing multiple genes within a single T-DNA or multiple T-DNAs each containing a single gene, whereas for direct DNA transfer methods the genes can be linked on conventional vectors (Naqvi *et al.,* 2009). The transgenes tend to integrate at a single locus, although the precise arrangement of multiple T-DNAs depends on the bacterial strain (Twyman *et al.,* 2002). This strategy is robust for a small number of input genes, but as the number increases, the vectors become increasingly cumbersome and unstable; the effective upper capacity using standard vectors is approximately 50 kb due to dwindling efficiency (Naqvi *et al.,* 2009). High-capacity binary vectors (BIBAC, BIBAC2, and TAC) that allow the transfer of up to 200 kb of insert DNA are discussed below.

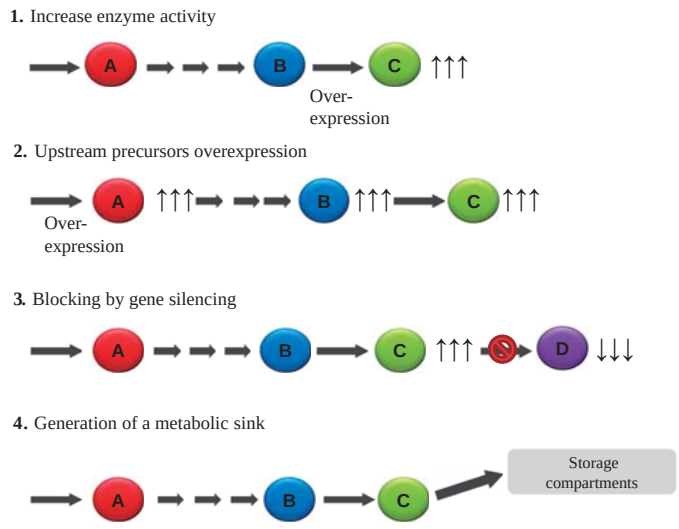

Fig. 1. Strategies to modulate organic compound levels in plants. *A and B are the precursors of C; C is the target product; D is the result of the target product conversion.* **(1)** *Modification of the activity of enzymes implicated in rate-limiting steps in the target pathway by modulation of one or two key enzymes, or multiple enzymes.* **(2)** *Upstream precursors enhancement by increasing flux through the pathway by overexpressing the enzyme(s) that catalyze(s) the first committed step of the pathway.* **(3)** *Blocked pathway branch points by RNA interference or antisense.* **(4)** *Enhanced accumulation of target metabolite by increasing sink compartments.* (Zhu et al., 2013).

Multiple unlinked genes can be introduced by *Agrobacterium*-mediated transformation if the bacteria contain multiple compatible plasmids carrying separate T-DNAs or if the inoculum comprises a mixture of bacterial strains carrying different vectors; however, the ratio of different input genes is difficult to control and multiple T-DNAs tend to integrate inefficiently (Naqvi *et al.,* 2009). Currently, only direct DNA transfer can introduce routinely and reliably multiple unlinked genes into plants, allowing plants carrying up to 15 different transgenes to be produced in one generation (Naqvi *et al.,* 2009).

Direct DNA transfer with separate vectors usually results in transgene integration at a single random locus in the form of a multigene array, regardless of how many different transformation cassettes have been used (Altpeter *et al.,* 2005; Kohli *et al.,* 2006). The integrated array may contain any number of transgenes from 1 to *n* (where *n* is the maximum input gene number) with the distribution within the transgenic population tending to describe a skewed normal curve as would be expected from random sampling with selection against zero integration events and for larger numbers of integrated transgenes (Kohli *et al.,* 2003). Input transgenes once integrated remain linked and do not segregate in subsequent generations (Wu *et al.,* 2002; Altpeter *et al.,* 2005). This feature is important when large numbers of genes are considered, because a much larger transgenic population would be required if each integration event were independent (Altpeter *et al.,* 2005).

Chen *et al.,* (1998) successfully transformed rice (*Oryza sativa*) plants by particle bombardment with 13 separate plasmids containing different marker genes, and regenerated plants carrying and expressing all the input genes at one locus. Subsequently, Wu *et al.,* (2002) transformed rice with nine transgenes also by particle bombardment and found that nonselected transgenes were present along with the selectable marker in approximately 70% of the plants and that 56% carried seven or more genes. This percentage was much higher than expected given the independent integration frequencies, in accordance with a model suggesting that the integration of one transgene promotes the cointegration of more input DNA at the same locus (Kohli *et al.,* 1998). All nine transgenes were expressed, and the expression of each gene was independent of the others (Wu *et al.,* 2002) (Table 1).

The position of transgene integration also influences the level and stability of expression in both transformation methods. For example, the transgenes can be integrated at a silencing locus (position-dependent silencing) or influenced by nearby regulatory sequences, such as enhancers (Topping *et al.,* 1991). The integration mechanism does not appear to be sequence dependent. Contrary to the prevailing view that the repetitious use of the same promoter may lead to the likelihood of transcriptional silencing,

a number of transgenic plants have been generated containing five or more transgenes controlled by the same promoter with no untoward effects (Naqvi *et al.,* 2009). Particle bombardment often generates large, high-copy-number transgenic loci, which are believed to be prone to instability and silencing, but there are many instances where this is not the case. For example, Golden Rice provides a clear example in which higher transgene copy numbers correspond to higher expression levels, ultimately leading to more β-carotene production in the endosperm (Datta *et al.,* 2003). *Bacillus thuringiensis* (*Bt*)-resistant rice containing multiple transgene copies also performed well against a number of insect pests in the greenhouse (Maqbool and Christou, 1999; Maqbool *et al.,* 2001) and under field conditions (Tu *et al.,* 2000; Ye *et al.,* 2001), indicating that the transgenes were expressed efficiently.

Development of multigene transformation methods

Stacking and retransformation

Prior to the development of simultaneous transfer methods suitable for many genes, multiple transgenes could be stacked in plants through successive rounds of crosses between different transgenic lines (Ma *et al.,* 1995; Datta *et al.,* 2002) or by the retransformation of transformed plants with additional transgenes (Jobling *et al.,* 2002). However, both methods are time-consuming and labor-intensive because of the need of multiple breeding generations to complete the stacking process and the important segregation risk, unless all the genes can be stacked together in a homozygous plant. Both the time necessary for stacking and the segregation risk increase with the number of transgenes. In the case of sequential transformation, multiple selectable markers (or marker excision and reuse) are also required.

Standard T-DNA and bombardment vectors

Both *Agrobacterium*-mediated transformation and direct DNA transfer involve the use of vectors that are optimized to replicate efficiently in *Escherichia coli* and to facilitate subcloning, which benefit from the vector remaining small. Vectors become increasingly unstable and prone to eject DNA when too much is inserted. The shear forces during particle bombardment can lead to fragmentation as well. It also becomes increasingly difficult to find restriction enzymes that cut at a unique site as more transgenes are introduced into the vector. Therefore, as the number of input genes increases, standard vectors are largely restricted to use with unlinked transgenes. As discussed above, *Agrobacterium*-mediated transformation turns out to be progressively less efficient as the number of separate T-DNAs increases, so for the highest numbers of transgenes only direct DNA transfer can be carried out with standard vectors. This problem has been solved to a certain extent by transforming plants with two bacterial strains, each carrying T-DNAs containing two or more transgenes; however, direct DNA transfer remains efficient with up to 15 unlinked transgenes and no upper limit has yet been determined. In the context of metabolic engineering, standard expression vectors have allowed the stable expression of several transgenes in maize (*Zea mays*) to recreate partial metabolic pathways (Zhu *et al.,* 2008; Naqvi *et al.,* 2010).

High-capacity T-DNA vectors

The limitations of MGT using *A. tumefaciens* have been addressed in part by the development of systems based on high-capacity artificial chromosome vectors with the ability to integrate large DNA fragments. These systems use the capacity of bacterial artificial chromosome (BAC) vectors and combine them with the components of standard binary vectors, resulting in chimeric binary vectors, such as BIBAC and TAC. Initially, these vectors still suffered from the cumbersome cloning procedure due to the lack of unique restriction sites, but this issue has been taken care of by combining the vectors with Gateway site-specific recombination technology (Vega *et al.,* 2008). Multisite and MultiRound Gateway systems have been used to integrate up to seven genes into the plant genome (Buntru *et al.,* 2013).

Split reading frames

The use of linker peptides can also facilitate MGT by letting several polypeptides to be encoded in a single open reading frame (ORF) controlled by a single promoter. For example, the *Foot-and-mouth disease virus* 2A polyprotein system allows the coexpression of up to four polypeptides in tobacco (*Nicotiana tabacum*) plants (Møldrup *et al.,* 2011; Lee *et al.,* 2012; Sun *et*

TABLE 1

EXAMPLES OF UNLINKED GENES FOR CO-TRANSFORMATION USING MULTIGENE TRANSFORMATION IN PLANTS

Number of input transgenes	Plant	Results	References
Three	Potato	17% of plants contained all input transgenes.	Romano *et al.* (2003)
Three	Rice	60% of all transgenic lines carried all three transgenes.	Sivamani *et al.* (1999); Maqbool *et al.* (2001)
Four	Maize	Introduced *psy1* and *crtI* (carotenoid pathway), *Dhar* (ascorbate pathway) and *folE* (folate pathway) using an unlinked direct DNA transfer co-transformation strategy to increase levels of β-carotene, folate and ascorbate in the endosperm. Achieved significant increases in all three nutrients providing the first example towards 'super-nutritious' cereals.	Naqvi *et al.* (2009)
Four	Rice	50% of transgenic plants contained all four input transgenes.	Wu *et al.* (2002); Altpeter *et al.* (2005)
Four	Rice	More than 20% of the plants contained and expressed all four input transgenes (fully assembled secretory antibody).	Nicholson *et al.* (2005)
Five	Rice	All transgenic plants contained at least two transgenes (mostly marker genes) and 16% contained all input transgenes (five).	Agrawal *et al.* (2005)
Up to five	Maize	Introduced *psy1, crtI, lycb, bch* and *crtW* genes using an unlinked direct DNA transfer co-transformation strategy aiming to generate a range of genotypes and phenotypes to dissect the carotenoid pathway. Recovered maize plants with a range of phenotypes reflecting different carotenoid profiles.	Zhu *et al.* (2008)
Nine	Rice	Non-selected transgenes were present along with the selectable marker: 70% of the plants; 56% carried seven or more transgenes.	Wu *et al.* (2002)
Thirteen	Rice	85% of the plants contained more than two, and 17% more than nine of the introduced transgenes.	Chen *et al.* (1998)

Adapted from (Naqvi *et al.,* 2010).

al., 2012). The 2A linker is less than 20 amino acids in length and has the ability to cleave its own C-terminus, thus releasing downstream polypeptides after synthesis (Halpin, 2005). In the context of metabolic engineering, the *Paracoccus crtW* and *crtZ* genes were simultaneously expressed as a polyprotein with an intervening 2A linker in transgenic tobacco and tomato (*Solanum lycopersicum*) plants to generate novel ketocarotenoids (Ralley *et al.,* 2004). More recently, the genes for phytoene synthase and carotene desaturase have been expressed in soybean (*Glycine max*) seeds with an intervening 2A linker, using either the β-conglycinin or the cauliflower mosaic virus 35S (CaMV35S) promoter (Kim *et al.,* 2012). Only the β-conglycinin promoter produced seeds with orange endosperm, indicating the accumulation of β-carotene, and this corresponded to high mRNA levels in the transgenic seeds. In contrast, the CaMV35S construct generated high mRNA levels in the leaves of transgenic plants (Kim *et al.,* 2012). Attributes and limitations of the key MGT methods are illustrated in Fig. 2.

Controlling the expression of multiple transgenes

As discussed above, a number of studies have shown that the same promoter can be used to drive multiple transgenes without negative effects, such as the strong endosperm-specific expression of three transgenes in maize achieved using the barley (*Hordeum vulgare*) D-hordein promoter (Naqvi *et al.,* 2009). Other studies have indicated that repetitive use of the same promoter can encourage (although probably not directly trigger) transgene silencing (Mourrain *et al.,* 2007). This observation may reflect several underlying factors, such as the presence of potential secondary structures that could interact in *trans* to promote *de novo* methylation, or the intrinsic activity of the promoters generating enough mRNA to saturate the polyadenylation machinery of the cell, allowing the formation of hairpin RNAs. These effects are also context dependent, based on the integration site and the juxtaposition of transgene copies, some of which may integrate 'head-to-head', thus encouraging the formation of double-stranded RNA at the junction of two opposing promoters (Kohli *et al.,* 2006).

Therefore, although it is by no means certain that using the same promoter for different transgenes will have a negative impact on transgene expression, various strategies have been devised to avoid the possibility. Examples include the use of natural diverse promoters with the same or similar activity (for instance, five different endosperm-specific promoters were used in maize to achieve

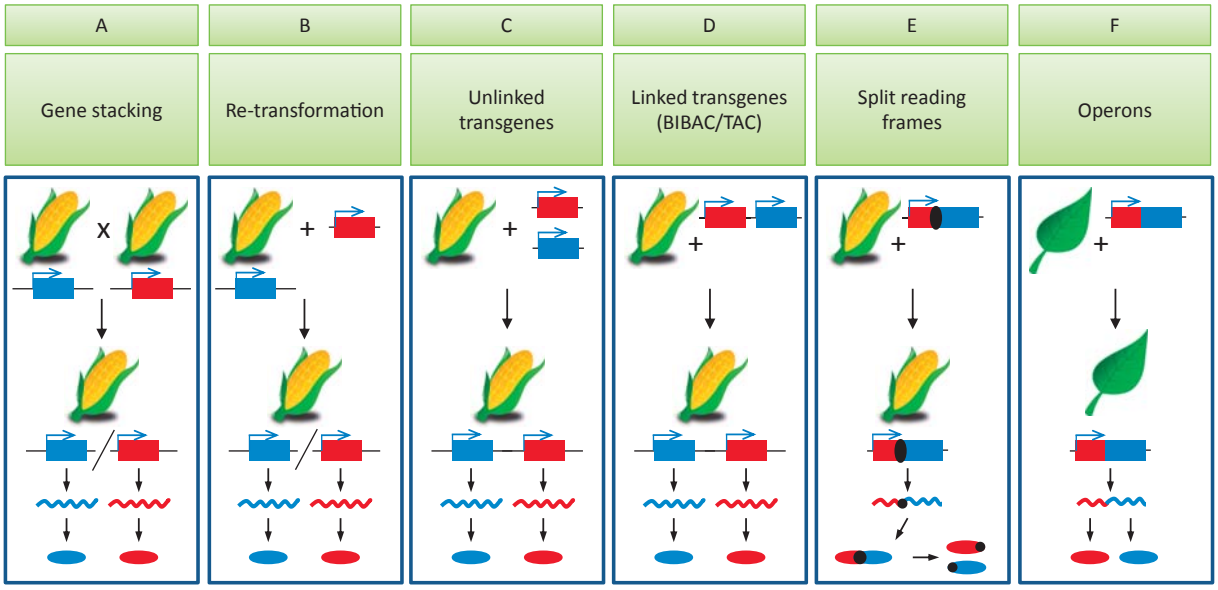

Fig. 2. Multigene transformation (MGT) methods for metabolic engineering. *Schematic summary of the principles of different methods for multigene transfer. Each panel shows a different method and charts the origin, fate, and activity of two different transgenes (red and blue blocks, with promoters shown as sideways arrows). The corresponding products at the level of mRNA (undulating lines) and polypeptides (discs) are shown in matching colors.* **(A)** *In the gene stacking approach, plants already carrying transgenes 1 and 2 are crossed to bring both genes into the same line. The genes are integrated and expressed independently (diagonal slash) and may therefore segregate in later generations. Therefore, a backcross program is needed to bring the two transgenes to homozygocity.* **(B)** *In the retransformation approach, plants already carrying transgene 1 are transformed with transgene 2, to bring both genes into the same line. The genes are integrated and expressed independently (diagonal slash) and may therefore segregate in later generations. A backcross program is also needed in this case to bring the two transgenes to homozygocity.* **(C)** *In the unlinked transformation approach, transgenes 1 and 2 are introduced into wild-type plants using separate vectors. All genes tend to integrate at the same locus, which is random, and may integrate in tandem (shown here) or in head-to-head or tail-to-tail conformations, occasionally with intervening genomic DNA sequences. Although panels (A-C) show individual transgenes as blue and red blocks, the same principles of integration and segregation also apply to groups of linked transgenes.* **(D)** *In the linked transformation approach, the transgenes are arranged in tandem on a single vector. The entire construct tends to integrate so the integrated transgenes are arranged in the same order as on the vector. This approach becomes increasingly difficult with more transgenes, unless high-capacity BIBAC/TAC vectors are employed.* **(E)** *In the split reading frame approach, two genes are expressed as a fusion protein linked by the 2A peptide from the Food-and-mouth disease virus, resulting in the expression of polycistronic mRNA and a polyprotein, which is self-cleaved into proteins 1 and 2, although each retains part of the 2A peptide (black circles).* **(F)** *In the operon approach, two or more genes are expressed as an operon yielding a polycistronic mRNA, but the proteins are translated independently via internal ribosome entry sites. This approach is only feasible for genes expressed in plastids and is therefore suitable for plants, such as tobacco and a small number of other species that are amenable to plastid transformation (as shown), but not currently for cereal crops, such as maize (shown in the other panels).*

the high-level expression of five carotenogenic genes (Zhu *et al.*, 2008), and the use of synthetic or modified promoters to reduce the amount of sequence identity (Naqvi *et al.*, 2010; Peremarti *et al.*, 2010).

Most promoters used in plant biotechnology are unidirectional, but bidirectional promoters are becoming increasingly useful for MGT because they allow the simultaneous expression of two gene products. For example, the human β-casein gene and a bacterial marker gene encoding luciferase have been expressed using the auxin-inducible, bidirectional mannopine synthase (*mas*) promoter in transgenic potato (*Solanum tuberosum* cv. Bintje) plants to increase their nutritional value (Chong *et al.*, 1997).

Promoter activity depends on the availability and activity of the transcription factors, so that the expression of such transcription factors can activate several target genes. For example, ectopic expression of the maize *C1* and *R* chimeric transcription factors in soybean upregulated a suite of endogenous isoflavonoid biosynthetic genes encoding phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase, chalcone isomerase, chalcone reductase, flavanone 3-hydroxylase, dihydroflavonol reductase, and flavonol synthase, doubling the isoflavonoid levels in the seeds (Yu *et al.*, 2003).

The number of promoters can also be reduced by using the split ORF method based on the 2A linker peptide discussed above, or operon-based methods in which the genes are arranged in tandem to yield a polycistronic mRNA, of which the ORFs are translated independently. The latter method is only suitable for plastid transformation, because the plastid genome is arranged into operons reflecting its prokaryotic origin. Plastid transformation

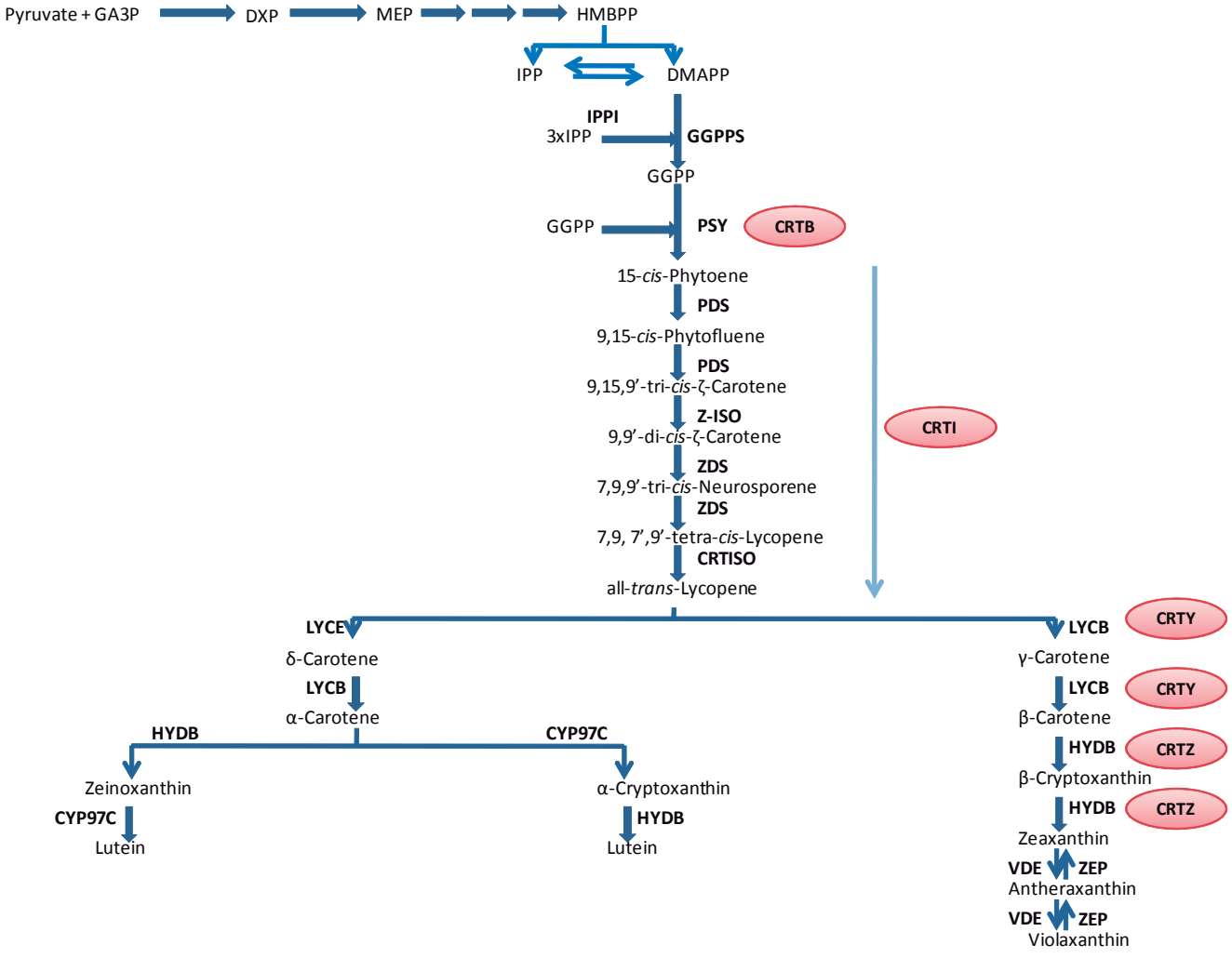

Fig. 3. Carotenoid biosynthetic pathway in plants and equivalent steps in bacteria. *Enzymes in the red ovals are from bacteria. Abbreviations: CRTB, bacterial phytoene synthase; CRTI, bacterial phytoene desaturase, which catalyze all desaturation and isomerization reaction from phytoene to lycopene; CRTISO, carotenoid isomerase; CRTY, bacterial lycopene β-cyclase; CRTZ, bacterial β-carotene hydroxylase; CYP97C, heme-containing cytochrome P450 carotene ε-ring hydroxylase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; GA3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; HDR, HMBPP reductase; HMBPP, hydroxymethylbutenyl 4-diphosphate; HYDB, β-carotene hydroxylase [non-heme di-iron β-carotene hydroxylase (BCH) and heme-containing cytochrome P450 β-ring hydroxyalses (CYP97A and CYP97B)]; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; MEP, methylerythritol 4-phosphate; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin deepoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, ζ-carotene isomerase. See Farré* et al., *2010, 2011.*

with operon-like multigene constructs has been used to produce astaxanthin in tobacco by expressing β-carotene ketolase and β-carotene hydroxylase (Hasunuma *et al.,* 2008). Similarly, the production of polyhydroxybutyric acid (PHB) in plastids has been achieved by expressing the *phbC-phbB-phbA* genes of *Ralstonia eutropha* using the T7g10 promoter (Lössl *et al.,* 2005).

Combinatorial transformation

One of the key challenges in metabolic engineering is that any targeted pathway must be understood in detail before interventions are made, to avoid wasting resources on the development of futile transgenic lines. In other words, for the longer and more complex pathways, large numbers of transgenic lines must be developed and tested independently before the most suitable intervention points are identified. Combinatorial transformation, a concept developed by Zhu *et al.,* (2008), elegantly solves this challenge and simultaneously turns the irritating random nature of transgene integration during gene transfer to plants into an advantage. The approach is based on the creation of metabolic libraries comprising plants transformed with random selections of particular transgenes. For example, the targeted analysis of five transgenes would require the generation of five transgenic lines carrying individual transgenes, plus other lines carrying combinations (perhaps created by stacking), each of which would then be subject to metabolic profiling to determine the impact on the target pathway. In combinatorial transformation, this idea is reversed by taking advantage of the scattergun nature of transgene integration: instead of selecting specific transgenic lines containing particular combinations of transgenes, the aim is to look at all the transgenic lines and with as much diversity as possible. Combinatorial transformation with five transgenes would therefore generate many different lines, some containing single transgenes, others two or three or four, and some with all five. These lines constitute a diverse library of metabolic potential, produced in a single generation. Hence, subsequent metabolic profiling helps to identify bottlenecks in the pathway and the best intervention points, even if effective intervention can only be achieved by multiple transgenes. The combinatorial approach is analogous to the use of factorial designs to test different parameters rather than focusing on the variation of one parameter at a time.

In the context of metabolic engineering, the carotenoid biosynthesis pathway in maize has been investigated by combinatorial transformation, allowing the identification and complementation of rate-limiting steps that affect the accumulation of β-carotene and other nutritionally important carotenoids, such as lutein, zeaxanthin, and lycopene. This approach has also allowed the pathway to be extended beyond its natural end-point to produce compounds, such as astaxanthin, revealing competition between β-carotene

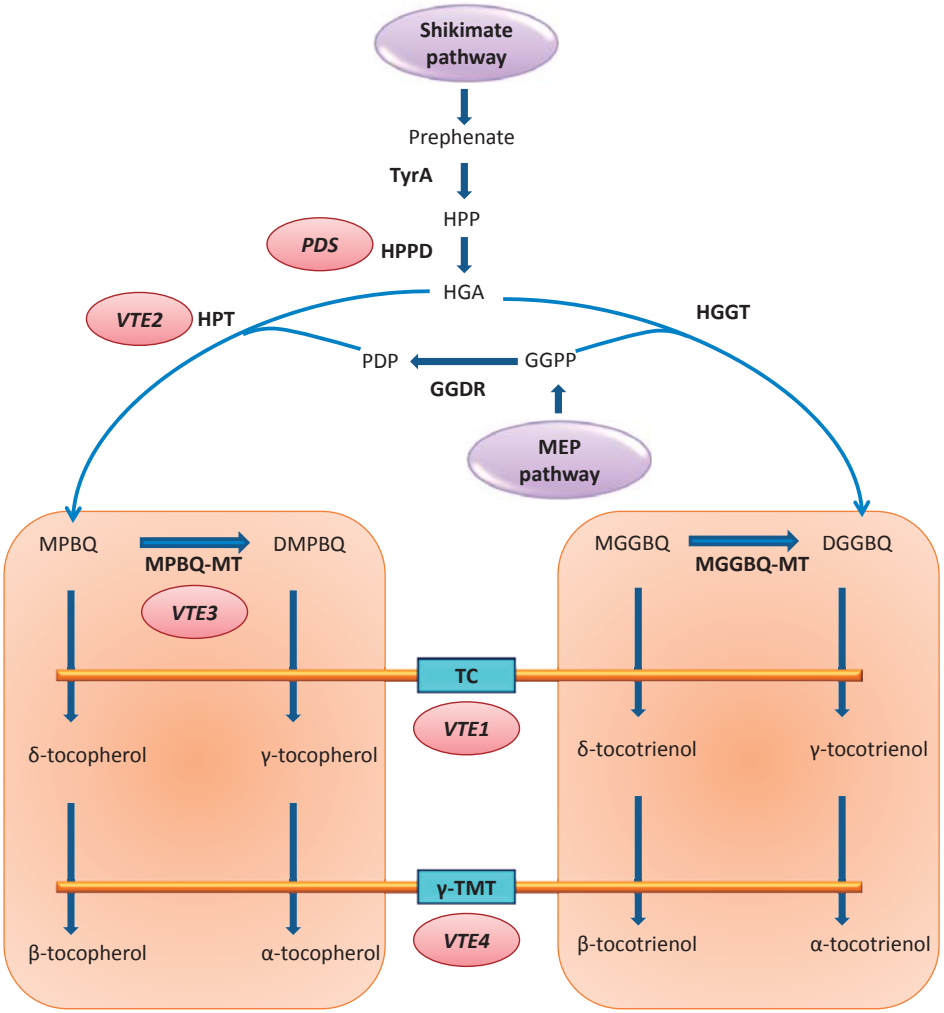

Fig. 4. Vitamin E biosynthesis in plants *(Farré* et al., *2012). Tocochromanols are synthesized on the inner chloroplast membrane from precursors derived from the shikimate and methylerythritol 4-phosphate (MEP) pathways. The shikimate pathway contributes the head-group precursor homogentisic acid (HGA), whereas the MEP pathway gives rise to the side-chain precursors phytyldiphosphate (PDP) and geranylgeranyldiphosphate (GGDP). The first committed step in the reaction is the cytosolic conversion of p-hydroxyphenylpyruvic acid (HPP) to HGA by p-hydroxyphenylpyruvic acid dioxygenase (HPPD). HGA is then prenylated with either PDP or GGDP to produce the intermediates 2-methyl-6-phytyl benzoquinone (MPBQ) and 2-methyl-6-geranylgeranylplastoquinol (MGGBQ). A second methyl group is added by MPBQ methyltransferase (MPBQ-MT) in the tocopherol branch and MGGBQ methyltransferase (MGGBQ-MT) in the tocotrienol branch, producing the intermediates 3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) and 2-dimethyl-6-geranylgeranylbenzoquinol (DMGGBQ). All four of these intermediates are substrates for tocopherol cyclase (TC), which produces δ and γ tocopherols and tocotrienols. Finally, γ-tocopherol methyltransferase (γ-TMT) catalyses a second ring methylation to yield α and β tocopherols and tocotrienols. Other abbreviations: GGDR, geranylgeranyl diphosphate reductase; HGGT, homogentisate geranylgeranyl transferase; HPT, homogentisate phytyltransferase. PDS, VTE1, VTE2, VTE3 and VTE4 correspond to genes cloned from* Arabidopsis Thaliana *that are homologous to HPPD, HPT, MPBQ-MT, TC, and γ-TMT genes, respectively.*

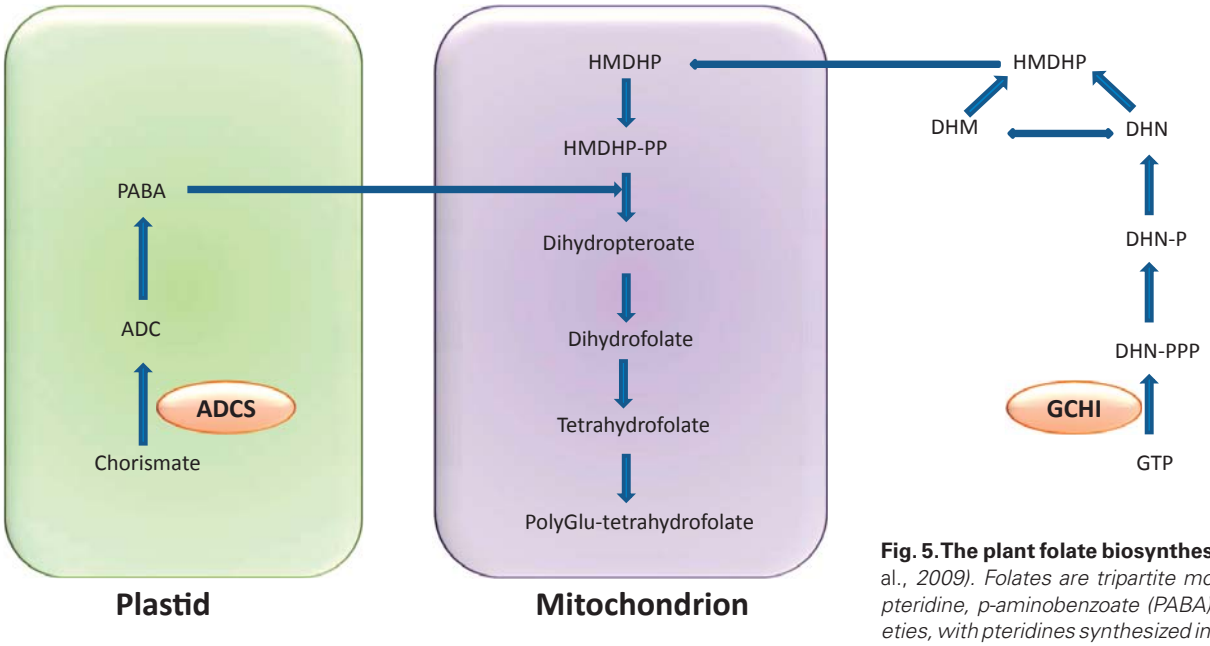

Fig. 5. The plant folate biosynthesis pathway *(Naqvi* et al., *2009). Folates are tripartite molecules consisting of pteridine, p-aminobenzoate (PABA), and glutamate moieties, with pteridines synthesized in the cytosol and PABA in the plastids. These moieties are transported to the mitochondria, where they condense to form dihydropteroate and are conjugated to glutamate. DHN, dihydroneopterin; -P/-PP/-PPP, mono/di/triphosphate; DHM, dihydromonapterin; HMDHP, hydroxymethyldihydropterin.*

hydroxylase and bacterial β-carotene ketolase for substrates (Zhu *et al.,* 2008).

Combinatorial transformation has also been used to combine genes from several different metabolic pathways to identify combinations that allow the simultaneous accumulation of different compounds. For example, maize plants had been generated that coincidently accumulated high levels of vitamins A, C and B_9 (folate) (Naqvi *et al.,* 2009).

Synthetic biology as the next step for multigene metabolic engineering

Synthetic biology describes the *de novo* assembly of genetic systems using prevalidated components (Haseloff and Ajioka, 2009). In the context of metabolic engineering in plants, a synthetic biology approach would utilize specific promoters, genes, and other regulatory elements to create ideal genetic circuits that facilitate the accumulation of particular metabolites. The concept of synthetic biology creates engineering and mathematical modeling to predict and test the behavior of the resulting system, which can be considered as the next step in multigene metabolic engineering because it removes any dependence on naturally occurring sequences and allows the design of ideal functional genetic circuits from first principles. Thus far, most work on synthetic biology has been accomplished with microorganisms, in spite of still some limiting factors, such as the ability of current methods to assemble complex DNA molecules encoding multiple genetic components in predefined arrangements (Weber *et al.,* 2011). Simple synthetic biology approaches have been described in plants, mostly in the context of signaling pathways and development, but also in the development of phytodetectors (Zurbriggen *et al.,* 2012) and biofortified crops (Naqvi *et al.,* 2009).

The use of synthetic biology in development as well as metabolism is important because it not only controls the metabolic capacity of a cell, but also steps one level up in terms of organization and use of particular promoters and genes that control developmental processes to generate novel tissues, in which the cells have specialized biosynthetic or storage functions to accumulate target products in particular organs. This approach will facilitate the achievement of goals that are unattainable by conventional genetic engineering, such as the development of novel organisms with medical functions, the production of biofuels, and the removal of hazardous waste (Purnick and Weiss, 2009).

Applications of MGT for pathway engineering

Metabolic pathways leading to complex organic molecules, such as vitamins (Figs 3, 4 and 5), polyunsaturated fatty acids (Fig. 6), and secondary metabolites often comprise a large number of genes, enzymes, and feedback mechanisms, limiting our ability to modulate these pathways by single-gene transformation. The introduction of multiple genes is necessary to understand the bottlenecks and identify and complement the rate-limiting steps (Zhu *et al.,* 2008).

The metabolic engineering of vitamin synthesis is necessary because many staple crops lack adequate amounts of these vital compounds. For example, vitamin A is required (as retinal) for blindness prevention and (as retinoic acid) for development and maintenance of a healthy immune system; vitamin E is an important antioxidant defense compound that quenches free radicals and protects against lipid peroxidation (Zhu *et al.,* 2013); and folate plays a central metabolic role, including DNA synthesis. None of these compounds are present at high levels in cereal grains, and more than one half of the world's population suffers from deficiency diseases because they rely on a cereal-based diet (Fitzpatrick *et al.,* 2012). Consequently, multigene metabolic engineering in plants has focused on carotenoid biosynthesis (vitamin A), tocochromanol synthesis (vitamin E), and folate synthesis (Naqvi *et al.,* 2009, 2011).

Polyunsaturated fatty acids (PUFAs) are lipids that are needed

not only as energy molecules, but for more specific activities, such as maintenance of the nervous system, the immune system, and prevention of atherosclerosis (Benatti *et al.,* 2004). PUFAs with dual roles as energy providers and essential nutrients include the omega-3 group, e.g. α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), and the omega-6 group, e.g. linoleic acid (LA) and arachidonic acid (AA) (Fig. 6). Humans cannot synthetize PUFAs because they lack methyl-end desaturases, and such modules must be accessed through the diet, particularly in fish and other seafood (Benatti *et al.,* 2004). Because there is little access to seafood across large parts of the world, metabolic engineering has been used to increase the abundance of essential PUFAs in transgenic plants.

Finally, secondary metabolites are complex molecules that are not required for housekeeping functions, but, nevertheless, provide advantages to plants, e.g. by attracting pollinators and repelling pests and pathogens. The three major types of secondary metabolite are the alkaloids, terpenoids/isoprenoids, and phenolics. Because plants have evolved to produce such molecules to control the behavior of animals and microbes, many secondary metabolites have pharmacological properties in humans or can be used as flavors, fragrances, and crop protection products (Miralpeix *et al.,* 2013).

Vitamins

The first use of multigene engineering to modulate the vitamin content of plants was the development of Golden Rice (Ye *et al.,* 2000). This is a transgenic rice line engineered to produce high levels of β-carotene through the expression of *Pantoea ananatis* phytoene desaturase (*PaCrtI*), daffodil (*Narcissus* spp.) phytoene synthase (*psy1*), and daffodil lycopene β-cyclase (*lycb*) (Fig. 3). The original Golden Rice line produced 1.6 μg/g dry weight (DW) of β-carotene, but the replacement of daffodil *psy1* with the more active maize enzyme in Golden Rice 2 boosted the β-carotene content to 31 μg/g DW (Paine *et al.,* 2005).

General strategies to increase carotenoid levels in plants include increasing the availability of carotenoid precursors, expressing enzymes in the common (linear) part of the pathway, and shifting the flux from the α- to the β-branch (Fig. 3). In canola (*Brassica napus*), *Agrobacterium*-mediated MGT was used to introduce seven different transgenes in order to reconstruct the entire carotenoid pathway, including an extension which allowed the production of ketocarotenoids (Fujisawa *et al.,* 2009). The input genes were isopentenyl pyrophosphate isomerase (*idi*), geranylgeranyldiphosphate (GGPP) synthase (*CrtE*), bacterial phytoene synthase (*CrtB*), *CrtI*, lycopene β-cyclase (*CrtY*), and the genes for two additional enzymes (*CrtZ* and *CrtW*) that catalyze downstream steps converting β-carotene into ketocarotenoids. This strategy achieved a 30-fold increase in total carotenoid content (657 μg/g fresh weight [FW]) and a 1070-fold increase in β-carotene (214 μg/g FW). In maize, MGT with maize *psy1*, *PaCrtI, lycb* of *Gentiana lutea* (great yellow gentian) (*Gllycb*), and *Paracoccus* sp. *CrtW* produced 35.64 μg/g DW of β-carotene (Zhu *et al.,* 2008). Carotenoid multigene engineering has also been applied in tomato, potato, and wheat (*Triticum aestivum*) (Dharmapuri *et al.,* 2002; Diretto *et al.,* 2007; Cong *et al.,* 2009).

The folate biosynthesis pathway (Fig. 4) involves the integration of two independent branches (pterin and p-aminobenzoate). The total folate content can be increased by modulating individual enzymes in either branch, but the best results are achieved by the simultaneous modulation of both branches by multigene engineering. In the most successful report, a 100-fold increase of total folate (38.3 nmol/g FW) was achieved in rice by expression of the *Arabidopsis thaliana* GTP cyclohydrolase 1 (GCH1) and aminodeoxychorismate synthase (ADCS) that enhances the the cytosolic (pterin) branch and the plastidic p-aminobenzoate branch of the pathway, respectively (Storozhenko *et al.,* 2007).

The synthesis of tocochromanols (vitamin E) involves a complex pathway (Fig. 5). Vitamin E levels can be elevated by increasing the total tocopherol content or enhancing the production of specific tocochromanols with the most potent vitamin E activity (α-tocopherol). The constitutive expression of two *Arabidopsis* cDNA

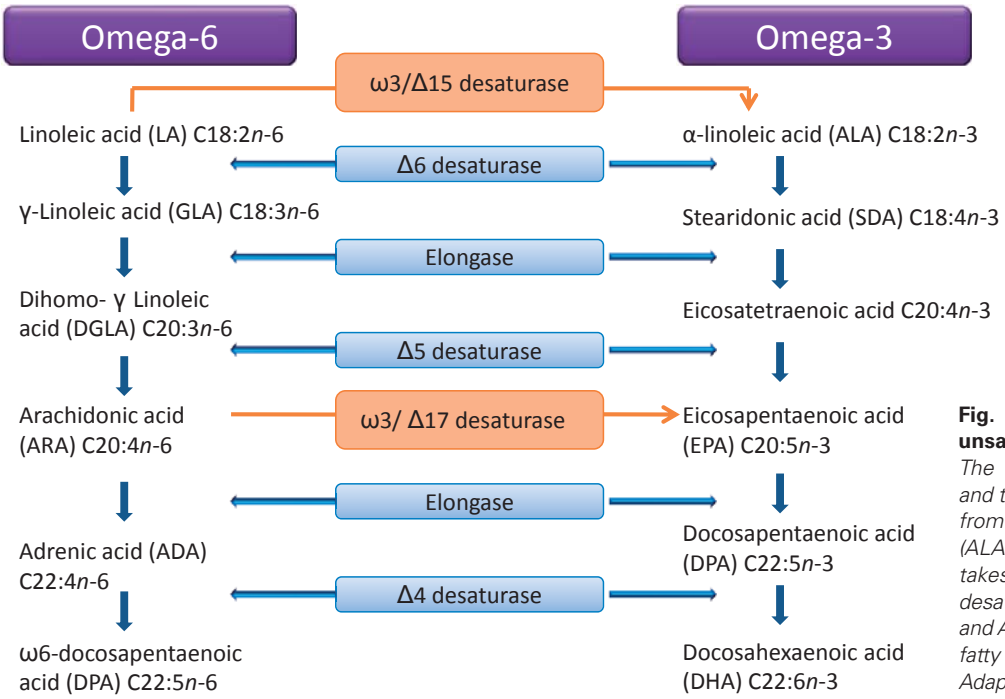

Fig. 6. The biosynthetic pathway of polyunsaturated fatty acids (PUFAs) in plants. *The conventional Δ6-desaturase/Δ6-elongase and the alternative Δ9-elongase pathways start from linolenic (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3), respectively. PUFAs synthesis takes place in plastids. Since mammals lack the desaturases which are responsible to produce LA and ALA, both of them are considered essential fatty acids and must be obtained from the diet. Adapted from Vrinten* et al., *(2007).*

clones encoding p-hydroxyphenylpyruvate dioxygenase (HPPD) and 2-methyl-6-phytylplastoquinol methyltransferase (MPBQ MT) increased the tocopherol content 3-fold in transgenic maize (Naqvi *et al.,* 2011). In soybean, the expression of homogentisate phytyltransferase (HPT1), HPPD, *TyrA* (responsible for the synthesis of HPP from prephenate), and geranylgeranyldiphosphate reductase (GGDR) increased the tocochromanol content by 15-fold (4806 μg/g DW) (Karunanandaa *et al.,* 2005).

The current state-of-the-art in vitamin engineering is the simultaneous modulation of multiple vitamin pathways in the same plant, as reported by Naqvi *et al.* (2009) through the expression of maize *psy1* and *PaCrtI*, representing the carotenoid biosynthesis pathway, rice *dehydroascorbate reductase (dhar)* to increase vitamin C (ascorbate) levels, and *E. coli FolE* to enhance folate accumulation (Figs 3, 4 and 5). The transgenic kernels contained 169-fold the normal amount of β-carotene, 6-fold the normal amount of ascorbate, and 2-fold the normal amount of folate.

Long-chain polyunsaturated fatty acids

Several different oil-seed crops have been transformed with multiple genes representing the PUFA biosynthesis pathway (Sayanova and Napier, 2004). For example, to obtain very long chain PUFAs from ALA and LA, it is necessary to introduce at least three transgenes encoding the desaturases and elongases required for sequential enzymatic reactions (Beaudoin *et al.,* 2000; Hong, 2002) (Fig. 6). Soybean seeds with higher levels of EPA have been produced by expressing *Mortierella alpina* Δ6-desaturase, Δ5-desaturase and Δ6-elongase transgenes plus omega-3Δ17-desaturase from *Saprolegnia diclina*, and omega-3Δ15-desaturase from *Arabidopsis* (Kinney *et al.,* 2011) (Fig. 6). This strategy was chosen to maximize the accumulation of omega-3 very long chain PUFAs by converting omega-6 PUFAs into their omega-3 counterparts.

Secondary metabolites

Although metabolic engineering can be used to enhance the production of secondary metabolites, it is challenging because of the complexity of the pathways and the shuffling of precursors and intermediates between compartments (Miralpeix *et al.,* 2013). The availability of precursors can be augmented by modulating the accessibility of basic nitrogen, carbon, and sulfur compounds, including the synthesis of amino acids, such as phenylalanine, tryptophan and tyrosine, and enhance both primary and secondary metabolism simultaneously (Pichersky and Gang, 2000).

Artemisinin is used as a drug against malaria caused by *Plasmodium falciparum* and has been produced in transgenic tobacco by multigene engineering (Farhi *et al.,* 2011). A mega-vector was constructed, containing the *Artemisia annua* (sweet wormwood) genes for cytochrome P450 reductase (*CPR*), amorpha-4,11-diene synthase (*ADS*), amorpha-4,11-diene monooxygenase (*CYP71AV1*), and artemisinic aldehyde Δ-11(13) reductase (*DBR2*), and the yeast 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*), each under the control of a different promoter. In a separate vector, the *ADS* sequence was fused to a COX4 signal peptide for import into the mitochondria, to boost the production of terpenoids. The vectors were introduced into tobacco plants by *Agrobacterium*-mediated transformation and the resulting transgenic plants produced amorpha-4,11-diene at levels of 26–72 ng/g FW (normal ADS) and 137–827 ng/g fresh weight (mitochondrial ADS).

MGT has also been used to boost the production of the natural polyester PHB in sugarcane (*Saccharum* spp.), but required the introduction of the enzymes β-ketothiolase (PHAA), acetoacetyl-reductase (PHAB), and PHB synthase (PHAC) from *Ralstonia eutropha*, and was achieved by particle bombardment with separate vectors (Petrasovits *et al.,* 2007). The resulting plants accumulated PHB to 1.88% DW in their leaves.

Opium poppy (*Papaver somniferum*) is one of the most important medicinal plants because it is the source of the cancer drug noscapine, the muscle relaxant papaverine, and analgesic and narcotic drugs, such as morphine and codeine. Morphine-type alkaloids have been produced in plant cell cultures since the 1970s (Rischer *et al.,* 2013). MGT has been used in this case to inhibit several genes in a pathway by virus-induced gene silencing to gain insight into the final six steps of morphine biosynthesis (Wijekoon and Facchini, 2012). The inhibition of SalSyn, SalR, T6ODM and CODM protein levels correlated with lower morphine yields and a substantial increase in the accumulation of reticuline, salutaridine, thebaine, and codeine, respectively. In contrast, the inhibition of SalAT and COR resulted in higher levels of salutaridine and reticuline.

Looking to the future

MGT is becoming essential as a strategy for metabolic engineering because it is clear that single-point interventions are inadequate to achieve ambitious metabolic goals even when dealing with a single pathway, and are unsuitable for the simultaneous engineering of different pathways, as illustrated by multivitamin corn.

Although it is now straightforward to introduce and express 5-10 transgenes in the same transgenic line, this is not the ceiling of the technique but rather the current status quo. Theoretically, there is no maximum number of transgenes that can be introduced at once, as demonstrated in microbes in which large, low-copy number vectors, such as BACs, P1-derived artificial chromosomes in bacteria, and yeast artificial chromosomes, are suitable for the introduction of hundreds of genes. This trend is emerging in plants, with large-capacity T-DNA-based vectors, but unlinked genes and direct DNA transfer allow the use of smaller vectors and achieve the same goals, because integration occurs at a single locus, therefore providing a suitable platform for strategies based on synthetic biology. In order to improve the potential of multigene transfer, a combination of linked and unlinked strategies could be developed to engineer more complex novel high-flux pathways and even combine these with the strategies shown in Fig. 1.

Multigene transfer must still overcome certain practical barriers that occur after gene integration, e.g. silencing, transgene rearrangement, and interactions between transgenes. As discussed above, repeated use of the same promoter does not necessarily encourage silencing, but may be a factor when another trigger is present; therefore, strategies have been developed based on promoter diversity or use of artificial or chimeric promoters to reduce the risk of unproductive interactions (Peremarti *et al.,* 2010). Novel strategies to assess the risk of transgene rearrangement and interactions with surrounding loci include site-specific recombination, targeted integration, and the use of engineered restriction enzymes, especially those based on zinc fingers and transcription activator-like effectors (Li *et al.,* 2012).

Metabolic pathways display a high degree of connectivity in larger networks, especially when metabolites are involved in two or more pathways; hence, the introduction of a large number of input genes has the potential to generate unintended and unpredicted effects. However, an interesting study showed that transfer of the entire pathway for dhurrin biosynthesis (a tyrosine-derived cyanogenic glucoside) into *Arabidopsis* had no significant impact on the wider transcriptome and metabolome, whereas the transfer of an incomplete pathway induced significant changes in morphology, transcriptome, and metabolome, probably through metabolic crosstalk or detoxification reactions (Kristensen *et al.,* 2005). Monitoring changes at the gene, transcript, protein, and metabolite levels is a challenge. In the future, it will be necessary to integrate these data in the context of systems biology, in which modeling is becoming a standard analytical tool for understanding whole biological systems and predicting gene behavior (Purnick and Weiss, 2009). Systems biology is also a necessary component of synthetic biology, because it is critical to foresee the behavior of synthetic genetic circuits in the context of the wider organism. Advances in systems biology and synthetic biology offer enormous potential in terms of development of novel materials and energy sources, improvement of agronomic traits, human health applications, and a better understanding of natural gene regulation (Naqvi *et al.,* 2009; Zurbriggen *et al.,* 2012). For example, the expression of three genes required for the conversion of acetyl-CoA to PHB in plastids allows the production of bioplastics in plants (Bohmert-Tatarev *et al.,* 2011), and the introduction of five genes of the *E. coli* glycolate catabolic pathway into *Arabidopsis thaliana* plastids reduces the loss of fixed carbon and nitrogen during photorespiration, increasing plant biomass (Kebeish *et al.,* 2007).

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Review article

Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies

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Summary

Antioxidants are protective molecules that neutralize reactive oxygen species and prevent oxidative damage to cellular components such as membranes, proteins and nucleic acids, therefore reducing the rate of cell death and hence the effects of ageing and ageing-related diseases. The fortification of food with antioxidants represents an overlap between two diverse environments, namely fortification of staple foods with essential nutrients that happen to have antioxidant properties (e.g. vitamins C and E) and the fortification of luxury foods with health-promoting but non-essential antioxidants such as flavonoids as part of the nutraceuticals/functional foods industry. Although processed foods can be artificially fortified with vitamins, minerals and nutraceuticals, a more sustainable approach is to introduce the traits for such health-promoting compounds at source, an approach known as biofortification. Regardless of the target compound, the same challenges arise when considering the biofortification of plants with antioxidants, that is the need to modulate endogenous metabolic pathways to increase the production of specific antioxidants without affecting plant growth and development and without collateral effects on other metabolic pathways. These challenges become even more intricate as we move from the engineering of individual pathways to several pathways simultaneously. In this review, we consider the state of the art in antioxidant biofortification and discuss the challenges that remain to be overcome in the development of nutritionally complete and health-promoting functional foods.

Introduction

Fortification is the process of adding essential micronutrients and other health-promoting compounds to foods. The fortification of processed foods such as flour, bread, packaged cereals, dairy products and salt is a public health policy in the industrialized world, aiming to reduce the number of people suffering from malnutrition and to increase general health and wellbeing in the population. In developing countries, fortification programmes are often unsustainable due to poor governance, inefficient food-distribution networks and the prevalence of subsistence agriculture in rural populations, which means that most agricultural products are not processed centrally before distribution and consumption. Approximately 50% of the global population is thought to be malnourished but the vast majority of malnourished people are the rural poor in developing countries, subsisting on a diet of milled cereal grains lacking many essential nutrients and other health-promoting compounds (Farré *et al.*, 2011a,b; Yuan *et al.*, 2011). In these settings, biofortification is a more sustainable strategy because this involves the fortification of crops at source either through the application of nutrient-rich fertilizers or the breeding or engineering of crops to synthesize and/or accumulate nutritionally important compounds, therefore avoiding the need to fortify processed food products (Gómez-Galera *et al.*, 2010).

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 (Zhu *et al.*, 2007; Gómez-Galera *et al.*, 2010; Table S1). The major deficiency diseases in developing countries correspond to essential nutrients that tend to be present at low levels in milled cereal grains, for example vitamin A, iron, iodine, zinc, vitamin C and folic acid. As well as their requirement for particular metabolic processes, certain 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. Many non-essential molecules consumed in the diet are also antioxidants with health-promoting effects, and hence there is an overlap between essential nutrients and non-essential compounds (sometimes described as nutraceuticals) that act as antioxidants.

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 carotenoids 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 a

co-regulator of gene expression (retinoic acid); β-carotene is also an antioxidant, as are many other (non-essential) carotenoids. Similarly vitamin C (ascorbate) 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. Many non-essential compounds are known to have health-promoting antioxidant activities, including anthocyanins, resveratrol and flavonoids. 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. In the industrialized world, the fortification of processed foods with essential nutrients is taken for granted and additional fortification with non-essential but health promoting antioxidant compounds forms a large part of the luxury functional foods market (Espín *et al.*, 2007).

Several recent reviews have focused on the fortification of staple foods with essential nutrients, predominantly as a strategy to alleviate micronutrient deficiency in developing country settings (Bai *et al.*, 2011; Gómez-Galera *et al.*, 2010; Yuan *et al.*, 2011; Zhu *et al.*, 2007). Here we discuss the development of strategies for the biofortification of plants with antioxidants or molecules that promote antioxidant activity, including essential nutrients and nutraceuticals.

Antioxidants confer health-promoting effects through a variety of mechanisms

Antioxidants inhibit the oxidation of other molecules and thereby prevent them from causing oxidative damage, which is a major contributory factor to diseases associated with ageing and with ageing itself. Antioxidant molecules are generally either lipophilic or hydrophilic, but both types employ common molecular mechanisms, including hydrogen atom transfer (HAT), single-electron transfer followed by proton transfer (SET-PT), sequential proton loss electron transfer (SPLET) and the formation of radical adducts (Ghanta and Chattopadhyay, 2011; Gülçin, 2012). These mechanisms are summarized in Figure 1. Some antioxidants have a single active mechanism, for example ascorbate/vitamin C uses HAT alone, whereas others employ multiple mechanisms, for example flavonoids can use HAT, SET-PT and SPLET depending on their molecular structure (Gülçin, 2012).

The three major lipophilic antioxidant classes in mammals are carotenoids, tocochromanols and coenzyme Q_{10}, all of which are derived from terpenoids. Carotenoids and tocochromanols are obtained from the diet whereas coenzyme Q_{10} is synthesized *de novo* in a multistep pathway starting with acetyl-CoA. All three classes act primarily by scavenging lipid peroxyl radicals ($ROO^{\cdot}$) and by disrupting free radical chain reactions in membranes. Carotenoids are tetraterpenoids (C40 isoprenoids) produced mainly by photosynthetic organisms and some fungi, bacteria, algae and archaea, and they can be classed either as carotenes or xanthophylls, the latter distinguished by the presence of oxygen atom(s) (Fraser and Bramley, 2004). The best known carotenoids are those with pro-vitamin A activity (particularly β-carotene) because these represent a major dietary source of vitamin A, but others with important antioxidant effects include lycopene, lutein and zeaxanthin. Lycopene is the red pigment present at high levels in tomatoes and has been shown to reduce the risk of cardiovascular disease and some types of cancer (Fraser and Bramley, 2004). Lutein is very abundant in green tissue, but zeaxanthin is restricted to a few sources like pepper and a number of corn varieties. The latter are not particularly abundant in western diets. They are particularly important in the macula and lens of the human eye, where they filter high-energy blue light and counteract oxidative damage to protect against age-related macular degeneration (Fraser and Bramley, 2004). The tocochromanols are a group of eight structurally related compounds collectively known as vitamin E obtained primarily from green leaves and oilseeds. The most potent form in humans is α-tocopherol, not because it is intrinsically any more active than the others but because it is absorbed most efficiently (Brigelius-Flohe and Traber, 1999). Coenzyme Q_{10} is present in the mitochondria where it functions in the electron transport chain and helps to prevent oxidative damage to membranes. Higher levels of coenzyme Q_{10} may help to prevent cardiovascular and neurological disorders as well as

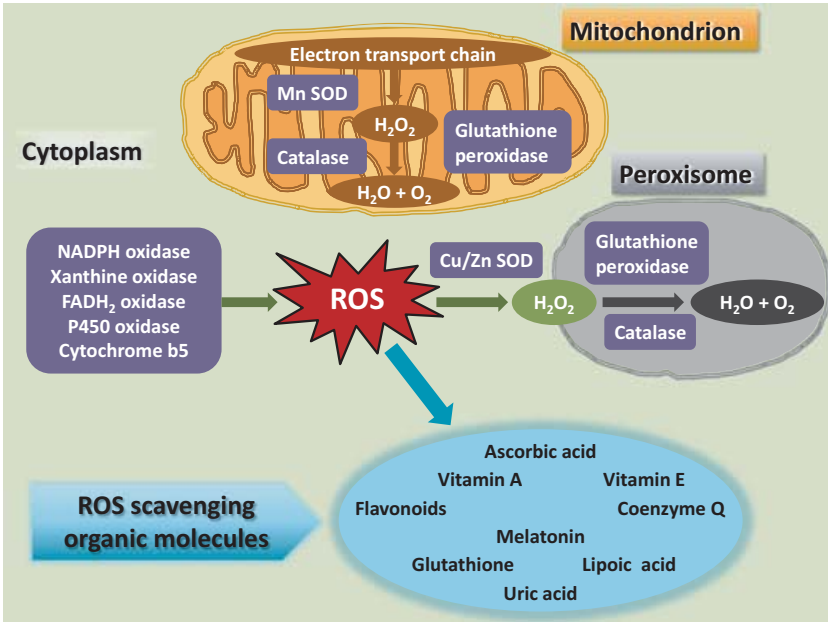

Figure 1 Mechanism of ROS generation and detoxification in humans (Mates *et al.*, 1999).

certain cancers and is used as an orphan drug for the treatment of congestive heart failure in children (Dhanasekaran and Ren, 2005).

There are many water-soluble antioxidants in mammalian diets, ranging from simple compounds such as ascorbate to complex molecules such as the flavonoids. The tripeptide glutathione has an antioxidant activity which is conferred by the sulfhydryl group of cysteine (Rennenberg, 1980). Lipoic acid uses a thiol functional group to neutralize free radicals (Goraca *et al.*, 2011). As well as scavenging free radicals, hydrophilic antioxidants may also chelate free metal ions that promote oxidation reactions. In plants, flavonoids, phenolic acids and glutathione are responsible for this activity, whereas uric acid and lipoic acid perform the same function in animals (Goraca *et al.*, 2011; Gülçin, 2012). However, the consumption of antioxidant phytochemicals in the diet increases the ability of mammals to counteract oxidative stress, reducing the frequency of cell death promoted by oxidative damage and therefore providing benefits such as a healthy immune system, a lower risk of disease and increased longevity (Ghanta and Chattopadhyay, 2011).

Biofortification increases the availability of essential nutrients and health-promoting compounds at source

Three staple crops—rice, maize and wheat—provide 60% of the calories consumed by humans (FAO, 2010). Milled cereals are deficient in many nutrients, including essential amino acids (particularly lysine), essential fatty acids, vitamins and minerals. Human populations that subsist entirely or predominantly on milled cereals are therefore the most at risk of deficiency diseases, and these tend to be the rural poor in developing countries, who are also the least likely to benefit from organized programmes to distribute fortified processed foods or vitamin and mineral supplements (Gómez-Galera *et al.*, 2010; Underwood and Smitasiri, 1999).

The limited impact of conventional interventions in developing country settings has promoted the use of biofortification as a sustainable approach that is equally beneficial to subsistence farmers and consumers of processed foods (Zhu *et al.*, 2007). 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. Mineral biofortification can be achieved in limited cases by using mineral-rich fertilizers although the impact of this approach depends on the mobility of mineral ions in the soil and the ability of plants to concentrate them in edible tissues such as cereal grains. There are two general strategies that are suitable for biofortification with any organic or inorganic nutrient, that is conventional breeding and genetic engineering. These are similar in aim, albeit different in scope. Both attempt to create plant lines carrying genes that favour the most efficient biosynthesis and/or accumulation of essential micronutrients and other health-promoting compounds. Conventional breeding achieves this by crossing the best performing plants and selecting those with favourable traits over many generations, sometimes in combination with biotechnology tools such as mutagenesis or marker assisted selection, whereas genetic engineering introduces the traits as recombinant DNA and allows the best-performing plants to be selected in a single generation (Harjes *et al.*, 2008; White and Broadley, 2005). Conventional breeding is limited to genes that can be sourced from sexually compatible plants and requires lengthy breeding programmes to introduce traits into locally adapted elite varieties, whereas genetic engineering has no such limitations and novel genes can be introduced directly into local cultivars. First-generation engineered crops with modified input traits have already shown their potential to enhance agricultural productivity and reduce poverty in developing countries (Christou and Twyman, 2004; Farré *et al.*, 2010a, 2011a), and now second-generation crops that address nutritional requirements through the direct modification of output traits are under development (Pérez-Massot *et al.*, 2012; Yuan *et al.*, 2011). Genetic engineering also allows nutritional traits to be targeted to specific organs (e.g. cereal seeds) and multiple traits can be combined in the same plants without complex breeding programmes (Naqvi *et al.*, 2009, 2010).

Organic antioxidants, such as carotenoids, tocochromanols, ascorbic acid and flavonoids, are synthesized *de novo* by plants, so engineering strategies aiming to enhance the availability of such compounds involve the modification of endogenous plant metabolism (Capell and Christou, 2004). This can be achieved using a variety of strategies as outlined in Figure 2. The most popular 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).

Different approaches must be used to achieve the accumulation of minerals because these cannot be synthesized *de novo* and must instead be taken up from the environment (Gómez-Galera *et al.*, 2010; Zhu *et al.*, 2007). Strategies to enhance mineral accumulation include the introduction of genes that improve (i) the efficiency of mineral uptake from the soil, (ii) the efficiency of transport form the roots to storage organs, (iii) the storage capacity and (iv) the bioavailability of the stored mineral (Gómez-Galera *et al.*, 2010). These strategies are summarized in Figure 3.

It is important to emphasize the difference between bioaccumulation (the amount of a particular nutrient that can be stored in plant tissues) and bioavailability (the amount that can be absorbed when the plant tissue is consumed as food). Whereas most studies have focused on bioaccumulation, the bioavailability of nutrients in engineered crops is a more important indicator of its nutritional quality (Hirschi, 2008). 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

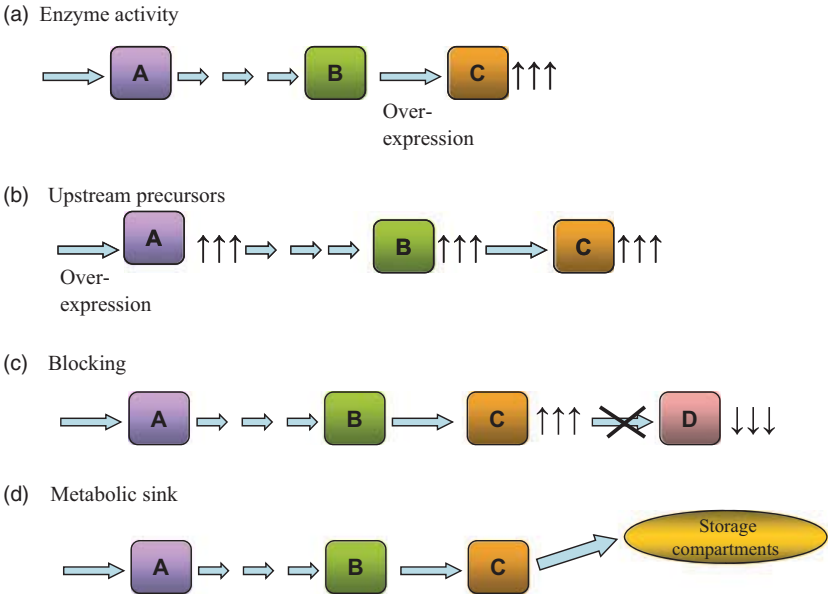

Figure 2 Strategies to modulate organic compound levels in plants. These strategies comprise the modification of: (a) activity of enzymes implicated in rate limiting steps in target pathways by modulation of one or two key enzymes, or multiple enzymes; (b) upstream precursors to increase flux through the pathway by overexpressing the enzyme that catalyses 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.

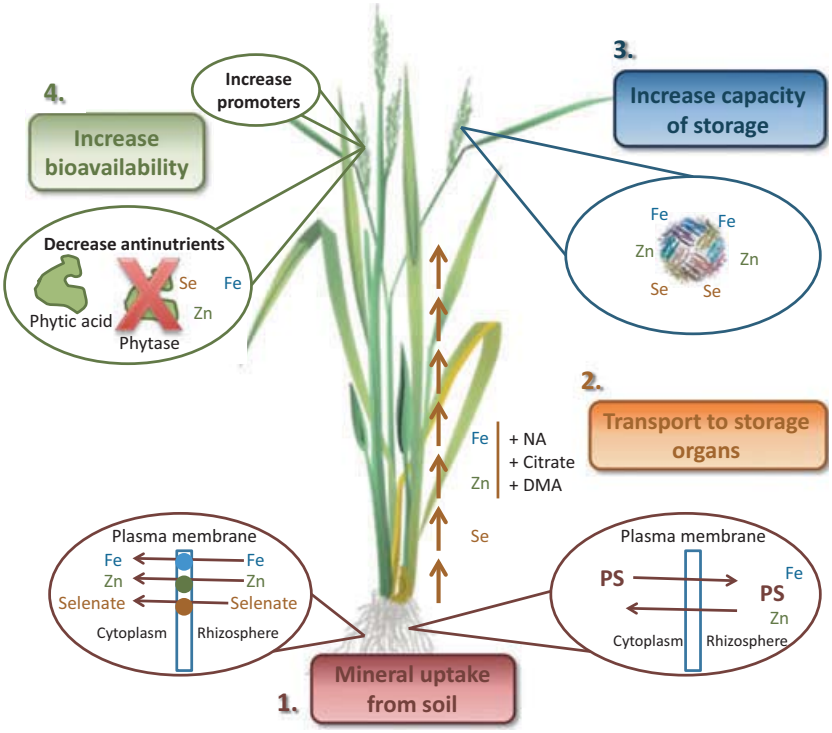

Figure 3 Strategies for inorganic compounds: 1. Mineral uptake from the soil by specific transporters which allow direct uptake from the rhizosphere to the cytoplasm (Connolly *et al.*, 2002) and/or excretion of phytosiderophores (PS), which bind Fe and Zn resulting in transport into the cytoplasm (Johnson *et al.*, 2011). 2. Efficient transport of minerals from the roots to storage organs-nicotianamine (NA), mineral-citrate or mineral-deoxymugineic acid (DMA) complexes (Ishimaru *et al.*, 2010). 3. Storage of minerals in the edible part of the plant in a form that is available in the diet and non-toxic to the plant; for example, ferritin specifically binds Fe and Zn (Drakakaki *et al.*, 2005) and selenocysteine is a sink for Se (Ellis *et al.*, 2004). 4. Increase the amount of bioavailable minerals accumulated in plants by degradation of phytic acid through phytase and then make Fe, Zn and Se available for absorption in the human gut (Drakakaki *et al.*, 2005) and/or use of compounds known to promote mineral absorption such as inulin, β-carotene or ascorbic acid (White and Broadley, 2005).

promote solubility (Jeanes *et al.*, 2004), and the bioavailability of ascorbate is enhanced by copresentation with proteins in the food matrix (Vinson and Bose, 1988). In the case of minerals, the presence of antinutritional compounds such as phytate and oxalate in vegetables can inhibit mineral absorption because they act as chelating agents (Gómez-Galera *et al.*, 2010), whereas nutritional enhancers such as inulin can promote mineral absorption by slowing down the movement of food through the gut (Gómez-Galera *et al.*, 2010). Reducing the quantities of antinutritional compounds and/or increasing the quantities of nutritional enhancers can therefore increase the bioavailability of nutrients. Bioavailability may also depend on the chemical form in which a nutrient is presented, for example selenium is absorbed more efficiently when presented in an organic form such as selenomethionine rather than as inorganic metal ions (Combs, 2001), and iron presented as a complex with ferritin is less susceptible to the effects of antinutritional compounds than non-heme iron (Lönnerdal, 2009).

Transgenic flavonoid-enriched tomato intake reduced C-reactive protein in human C-reactive protein transgenic mice expressing markers of cardiovascular risk more than in wild-type tomato (Rein *et al.*, 2006). Moreover, the life span of cancer-susceptible mice fed on a diet supplemented with high-anthocyanin tomatoes was increased substantially (Butelli *et al.*, 2008). 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). Toxicity assessment in mice fed with multivitamin maize showed no adverse health effects and did not induce any clinical sign of toxicity (Arjó *et al.*, 2012).

Genetic engineering for biofortification with lipophilic antioxidants

Carotenoids

The carotenoid biosynthesis pathway in plants (and equivalent bacterial enzymes) is shown in Figure 4a. The key aspects of the pathway in the context of metabolic engineering are that the first committed step is catalysed by the enzyme phytoene synthase (PSY/CRTB) and that the next four desaturation and isomerization steps which produce all-trans lycopene are carried out by four enzymes in plants but by a single bacterial enzyme known as CRTI (phytoene desaturase), which is therefore preferred for genetic engineering to reduce the number of transgenes required. Lycopene represents a branch point in the pathway, where the competing activities of beta and epsilon cyclases (LYCB/CRTY and LYCE) result in the production of β-carotene (pro-vitamin A) and α-carotene. These products are then converted into lutein and zeaxanthin, respectively, by the activity of carotene hydroxylases.

As discussed above, there are multiple strategies available to enhance carotenoid production and one popular approach has been the overexpression of PSY/CRTB to alleviate the metabolic bottleneck at the first committed step in the pathway. One successful example of this approach in canola resulted in a 50-fold increase in carotenoid levels in the seeds (to 1617 μg/g fresh weight, FW), predominantly represented by α-carotene and β-carotene (394 and 949 μg/g FW, respectively), and was achieved by expressing the *CRTB* gene under the control of a seed-specific promoter (Shewmaker *et al.*, 1999). The engineered canola was the pioneering work for the successful enhancement of carotenoid production in crop plants. The success of this approach relied on the fact that enzymes acting downstream of PSY were not limiting in canola seeds and the phytoene was successfully converted into downstream carotenoids.

However, the expression of daffodil PSY in rice endosperm only resulted in the accumulation of phytoene because the subsequent desaturation steps were also limiting (Burkhardt *et al.*, 1997; Schaub *et al.*, 2005). This is in contrast to canola which contains a native carotenogenic pathway. Therefore, to boost carotenoid levels in rice grains, it was necessary to express multiple enzymes from the pathway. Initially, daffodil PSY was combined with the multifunctional bacterial enzyme CRTI resulting in the accumulation of β-carotene and xanthophylls in 'Golden Rice' (Ye *et al.*, 2000). These results indicated that the endogenous levels of lycopene cyclases and carotene hydroxylases were sufficient to convert the lycopene generated by CRTI into downstream products (Schaub *et al.*, 2005). Later, the amount of β-carotene was increased to 31 μg/g dry weight (DW), a 17-fold improvement, by replacing the daffodil PSY with its more efficient corn ortholog, resulting in the higher performance Golden Rice II (Paine *et al.*, 2005). Tissue-specific transgene expression is critical for successful carotenoid modulation. For example, the first report of tomato engineered to expressed tomato PSY1 constitutively resulted in a dwarft phenotype due to the depletion of the endogenous precursor pool of geranylgeranyl diphosphate (GGPP) leading to a shortage of gibberellins (Fray *et al.*, 1995). This was overcome subsequently by fruit-specific expression of bacterial CRTB (Fraser *et al.*, 2002). Flux through the carotenoid pathway can also be increased by making more precursors available by engineering upstream pathways [e.g. by overexpressing 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS) to provide more DXP in the methylerythritol phosphate (MEP) pathway; Enfissi *et al.*, 2005] but this also increases flux through unrelated pathways that utilize the same precursors, so is less efficient and targeted.

Whereas the results described above appear to reflect the additive effects of transgenic and endogenous metabolic capabilities, more complex interactions have been observed when feedback effects occur between the superimposed pathways. The expression of CRTI in tomato was envisaged to enhance the lycopene content but actually resulted in a threefold increase in β-carotene levels while reducing the overall level of carotenoids including lycopene (Römer *et al.*, 2000). The elevated β-carotene content was unexpected because of the low LYCB levels normally found in tomato fruits, but further investigation revealed that the endogenous *LYCB* gene had been induced in the transgenic plants (Römer *et al.*, 2000). The outcome of the above strategy therefore depends on the relative activities of endogenous LYCB and LYCE which vary in different plants and even in different cultivars, so more predictable results can be achieved by deliberately expressing one or other of the enzymes to increase flux in the appropriate direction beyond the branch point. As an example, the overexpression of LYCB in tomato fruits increased the accumulation of β-carotene and zeaxanthin at the expense of α-carotene and lutein (D'Ambrosio *et al.*, 2004; Rosati *et al.*, 2000). Similarly, transgenic canola seeds expressing CRTB, CRTI and CRTY accumulated more carotenoids than wild-type seeds and the β:α-carotene ratio increased from 2:1 to 3:1, showing that the additional lycopene β-cyclase (CRTY) activity skewed the competition for the common precursor lycopene and increased flux specifically towards β-carotene (Ravanello *et al.*, 2003). In corn, the outcome of such experiments depends on competition and complementarity between the transgenic and endogenous pathways. For example, in the white inbred M37W which lacks significant amounts of carotenoids in the endosperm, the expression of PSY, CRTI and LYCB increased the β:α–carotene ratio from 1.21 to 3.51 (Zhu *et al.*, 2008) and zeaxanthin levels were 29.64 μg/g DW, but also enhanced flux through the competing branch, resulting in nearly 25-fold the normal levels of lutein (up to 10.76 μg/g DW) showing that LYCE activity is not a limiting step in corn endosperm. In contrast, the introgression of the same pathway into two yellow-endosperm varieties with opposing β:α–carotene ratios (0.61 for EP42 and 1.90 for A632) generated one hybrid accumulating lutein (23.41 μg/g DW) with a β:α–carotene ratio of 2.05 and the other accumulating high levels of zeaxanthin (56.9 μg/g DW) with a β:α–carotene ratio of 6.8 (Naqvi *et al.*, 2011a).

Carotenoid profiles can also be modulated by inhibiting endogenous carotenogenic enzymes, for example by a tuber-specific antisense approach against LYCE in potato which increased β-carotene levels by up to 14-fold and total carotenoid levels by up to 2.5-fold. However, there was no corresponding reduction in lutein levels again suggesting that LYCE is not a rate-limiting step (Diretto *et al.*, 2006). Similar results were observed in canola seeds by RNA interference against LYCE (Yu *et al.*, 2008). The inhibition of β-carotene hydroxylase (BCH) prevents β-carotene from being converted into zeaxanthin, and this strategy has achieved 38-fold increase in β-carotene levels in

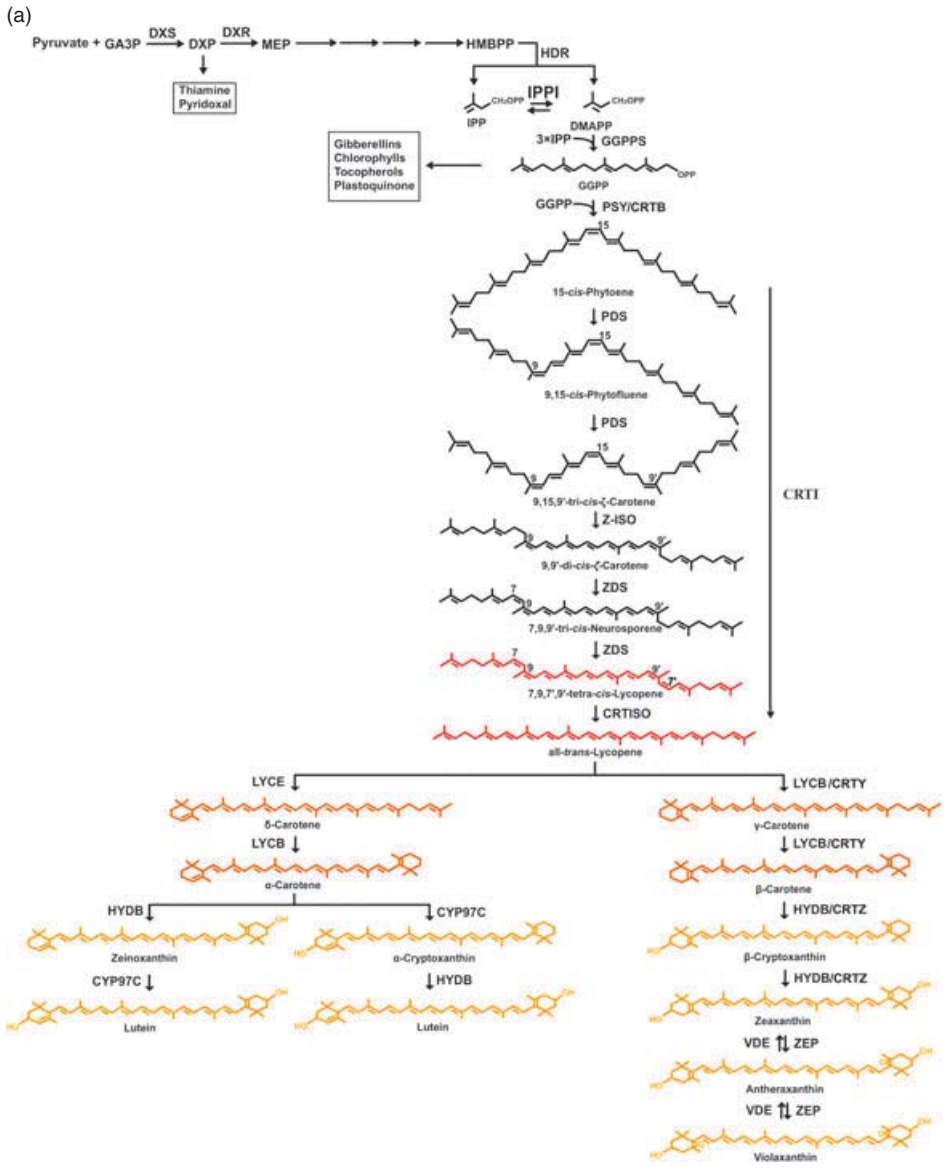

Figure 4 (a) Carotenoid biosynthetic pathway in plants and equivalent steps in bacteria (Farré *et al.*, 2010b, 2011b). Abbreviations: CRTB, bacterial phytoene synthase; CRTI, bacterial phytoene desaturase; CRTISO, carotenoid isomerase; CRTY, bacterial lycopene β-cyclase; CRTZ, bacterial β-carotene hydroxylase; CYP97C, heme-containing cytochrome P450 carotene ε-ring hydroxylase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; GA3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; HDR, HMBPP reductase; HMBPP, hydroxymethylbutenyl 4-diphosphate; HYDB, β-carotene hydroxylase [non-heme di-iron β-carotene hydroxylase (BCH) and heme-containing cytochrome P450 β-ring hydroxyalses (CYP97A and CYP97B)]; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; MEP, methylerythritol 4-phosphate; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, ζ-carotene isomerase. (b) Vitamin E biosynthesis in plants (Farré *et al.*, 2012a). Tocochromanols are synthesized on the inner chloroplast membrane from precursors derived from the shikimate and MEP pathways. The shikimate pathway contributes the head-group precursor homogentisic acid (HGA), whereas the MEP pathway gives rise to the side-chain precursors phytyldiphosphate (PDP) and geranylgeranyldiphosphate (GGDP). The first committed step in the reaction is the cytosolic conversion of ρ-hydroxyphenylpyruvic acid (HPP) to HGA by ρ–hydroxyphenylpyruvic acid dioxygenase (HPPD). HGA is then prenylated with either PDP or GGDP to produce the intermediates 2-methyl-6-phytyl benzoquinone (MPBQ) and 2-methyl-6-geranylgeranylplastoquinol (MGGBQ). A second methyl group is added by MPBQ methyltransferase (MPBQ-MT) in the tocopherol branch and MGGBQ methyltransferase (MGGBQ-MT) in the tocotrienol branch, producing the intermediates 3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) and 2-dimethyl-6-geranylgeranylbenzoquinol (DMGGBQ). All four of these intermediates are substrates for tocopherol cyclase (TC), which produces δ and γ tocopherols and tocotrienols. Finally, γ-tocopherol methyltransferase (γ-TMT) catalyses a second ring methylation to yield α and β tocopherols and tocotrienols. Other abbreviations: GGDR, geranylgeranyl diphosphate reductase; HGGT; homogentisate geranylgeranyl transferase; HPT, homogentisate phytyltransferase. (c) Postulated ascorbate biosynthesis and recycling pathways (Ishikawa and Shigeoka, 2008; Ishikawa *et al.*, 2006). Abbreviations: AO, ascorbate oxidase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GalLDH, L-galactono-1,4-lactone dehydrogenase; GalUR, D-galacturonate reductase; GDH, L-galactose dehydrogenase; GGP, GDP-L-galactose phosphorylase; GlOase, L-gulonolactone oxidase; GME, GDP-mannose-3′,5′ epimerase; GMP, GDP-mannose pyrophosphorylase; GPP, L-galactose 1-phosphate phosphatase; MDHAR, monodehydroascorbate reductase; Miox, *myo*-inositol oxidase.

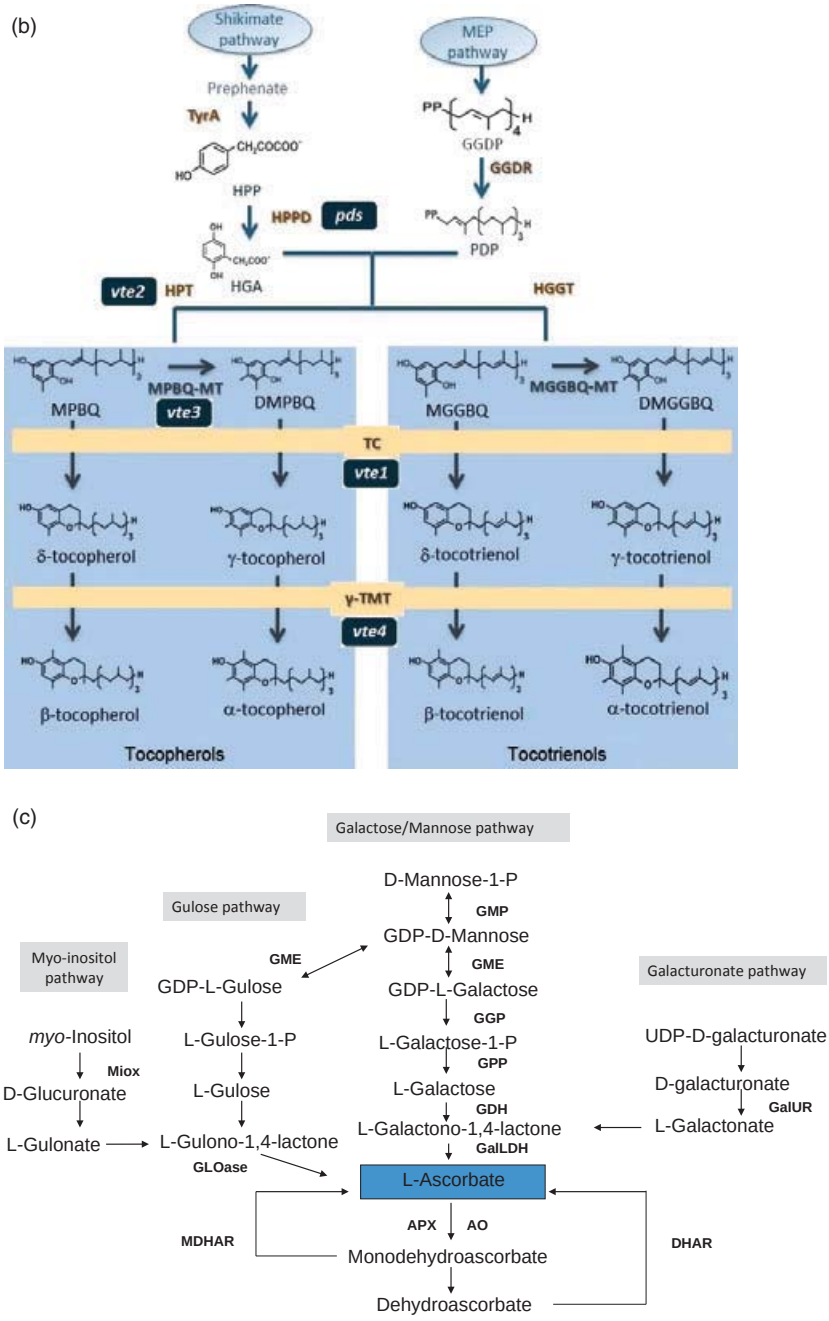

Figure 4 (Continued)

potatoes, in concert with a 3.7-fold increase in lutein levels and a 0.5 fold reduction in zeaxanthin levels (Diretto *et al.*, 2007). Targeting the next step in the pathway (zeaxanthin epoxidase, ZEP) prevents zeaxanthin from being converted into downstream products, and this strategy increased total carotenoid levels 5.7-fold, β-carotene levels 3.4-fold, lutein levels 1.9-fold and zeaxanthin levels 133-fold (Römer *et al.*, 2002). Vitamin E (α–tocopherol) was increased two to threefold in the transgenic potatoes. Fine-tuning these alterations has the potential to significantly enhance the nutritional value of potatoes. These studies show that carotenoid levels in plants can be increased both by alleviating early bottlenecks and introducing bottlenecks at later steps or in competitive branches.

A final strategy that has been used to enhance β-carotene and total carotenoid levels in plants is to increase the number of storage compartments (Lopez *et al.*, 2008; Lu *et al.*, 2006). Carotenoids are stored in adapted plastids, so increasing the number of these organelles or encouraging their differentiation can act as a metabolic sink, shifting the metabolic equilibrium towards carotenoid synthesis. The cauliflower *Or* gene was identified through the discovery of a dominant allele that causes the curd to become orange, and the overexpression of this allele in potato under the control of the granule-bound starch synthase (GBSS) promoter produced potato tubers with bright orange flesh and carotenoid levels of up to 31 μg/g DW, including 3.75 μg/g DW β-carotene (Lopez *et al.*, 2008).

In some of the examples discussed above, the introduction of heterologous enzymes has enhanced the production of carotenoids that are already produced in moderate to large amounts or has modified the carotenoid profile of edible plant tissues, whereas in other cases the endogenous tissues do not produce carotenoids and the entire pathway must be introduced *de novo* (e.g. Golden Rice). Similarly, most plants do not produce ketocarotenoids such as astaxanthin and canthaxanthin, which are the pink/red pigments most frequently found in seafood and have potent, health-promoting antioxidant activities (Zhu *et al.*, 2009). Ketocarotenoids are currently produced in bacteria and are added artificially to fish feed pellets to increase the pigmentation of for example farmed salmon and trout. The expression of bacterial β-carotene ketolases in corn endosperm has resulted in the production of astaxanthin corn lines that could be valuable sources of ketocarotenoids in the aquaculture, nutraceutical and cosmetic industries (Zhu *et al.*, 2008).

Tocochromanols

The tocochromanol biosynthesis pathway in plants is shown in Figure 4b. The key aspects of this pathway in the context of metabolic engineering are that it begins with the prenylation of homogentisic acid (HGA), derived from the shikimate pathway, with phytyldiphosphate (PDP), derived from the MEP pathway, and that prenylation may be carried out by two different enzymes —homogentisate phytyltransferase (HPT) or homogentisate geranylgeranyl transferase (HGGT)—to generate alternative intermediates that give rise to the tocopherol and tocotrienol branches of the pathway, respectively. These intermediates are substrates for the same three enzymes, yielding eight different products, so genetic engineering can be used not only to increase the total tocochromanol content, but also to alter the relative levels of the different forms. This provides a strategy to boost levels of the most potent form, α-tocopherol (Farré *et al.*, 2012a).

As with carotenoid engineering, total tocochromanol levels in plants can be increased by expressing single or multiple rate-limiting enzymes. HGA is the key target because it is the last common precursor before the pathway splits into multiple branches, and HGA levels can be boosted by expressing enzymes in the shikimate pathway such as TyrA and/or HPPD which increase total tocochromanol levels without significantly affecting the balance between different forms (Karunananda *et al.*, 2005). In contrast, the expression of downstream enzymes such as MPBQ-MT and γ-tocopherol methyltransferase (γ-TMT) has the effect of interconverting different forms of vitamin E without a significant impact on total tocochromanol levels. For example, the expression of MPBQ-MT (VTE3) in Arabidopsis seeds increased the total tocopherol content marginally but caused the preferential accumulation of γ-tocopherol (75%–85% of total tocopherols; Van Eenennaam *et al.*, 2003). Similarly, the expression of γ-TMT had no impact on total tocochromanol levels but resulted in 85% –95% of the total tocopherol pool being converted into α–tocopherol, representing an 80-fold increase over wild-type seeds and a ninefold increase in total vitamin E activity indicating that flux was diverted into the α-branch of the pathway (Shintani and DellaPenna, 1998). Transgenic soybeans expressing MPBQ-MT and γ-TMT accumulated >95% α-tocopherol, resulting in a greater than eightfold increase of α-tocopherol and an up to fivefold increase in seed vitamin E activity (Van Eenennaam *et al.*, 2003). The result was a fivefold increase in vitamin E activity.

Similar increases in vitamin E activity were achieved following the overexpression of γ-TMT in lettuce (Cho *et al.*, 2005), but the most promising results were achieved by crossing transgenic lettuce lines expressing HPT (thus a higher flux through the entire pathway) with those expressing γ-TMT (skewing the pathway in favour of α-tocopherol). This resulted in an increase of both the total tocopherol content and the α/γ tocopherol ratio (Cho *et al.*, 2005). More recently, lettuce plants simultaneously transformed with the Arabidopsis genes encoding HPT and γ-TMT also showed both quantitative and qualitative increases in vitamin E activity, a sixfold increase in the total tocopherol content and also a sixfold increase in the α/γ ratio (Li *et al.*, 2011).

The overexpression of HGGT should favour the tocotrienol branch of the pathway, but corn seeds overexpressing HGGT accumulated six times the normal levels of both tocotrienols and tocopherols (Cahoon *et al.*, 2003). Recently, rice seeds expressing Arabidopsis *HPPD* resulted in a small increase in absolute tocotrienol synthesis (but no change in the relative abundance of the γ and α isoforms). In contrast, there was no change in the absolute tocopherol level, but a significant shift from the α to the γ isoform. These data confirm that HPPD is not rate limiting, and that increasing flux through the early pathway reveals downstream bottlenecks that act as metabolic tipping points (Farré *et al.*, 2012b). The combined expression of HPPD and MPBQ-MT resulted in a threefold increase in γ-tocopherol levels without changing the total tocopherol content (Naqvi *et al.*, 2011b). These experiments again showed that flux was directed into the α-branch but in this case it was blocked by low γ–TMT activity, forcing the accumulation of γ–tocopherol (Naqvi *et al.*, 2011b).

Coenzyme Q_{10}

The metabolic engineering of fat-soluble antioxidants has understandably focused on carotenoids and tocochromanols because of their status as essential nutrients. However, the beneficial properties of coenzyme Q_{10} have prompted investigations into good dietary sources of this compound and the development of strategies to increase its abundance by genetic engineering, potentially to provide additional sources for the nutraceutical industry. The most significant studies involved the expression of the *Gluconobacter suboxydans* decaprenyl diphosphate synthase gene in rice, with the corresponding enzyme targeted for import into the mitochondria which is the normal site of coenzyme Q_{10} activity in humans and the site of coenzyme Q_9 synthesis in wild-type rice (Takahashi *et al.*, 2006, 2009, 2010). This approach was chosen because it allowed the production of coenzyme Q_{10} through the extension of an existing metabolic pathway using a single enzyme, and because coenzyme Q_{10} is preferentially localized in the bran and germ suggesting that the ingestion of coenzyme Q_{10} could be increased by the consumption of unmilled brown rice as a health food (Takahashi *et al.*, 2010).

Genetic engineering for fortification with hydrophilic antioxidants

Ascorbate

Unlike carotenoids and tocochromanols, which are synthesized *de novo* in plants through a unique pathway, a key aspect of ascorbate biosynthesis in the context of metabolic engineering is that it may be synthesized *de novo* via several pathways or recycled from oxidation products, offering multiple intervention points for nutritional enhancement (Ishikawa *et al.*, 2006). An overview of ascorbate synthesis and recycling is shown in Figure 4c. The major *de novo* synthesis pathway involves the intermediate L–galactose (Wheeler *et al.*, 1998) but alternative

routes via L-gulose (Wolucka and Van Montagu, 2003), D–galacturonic acid (Agius *et al.*, 2003) and *myo*-inositol (Lorence *et al.*, 2004) have been identified in particular species and tissues.

As discussed earlier, one of the key intervention methods is to overexpress a rate-limiting enzyme, and in the L-galactose pathway this has been achieved by targeting GDP-L-galactose phosphorylase (GGP), resulting in increases of between two and sixfold in crops such as tomato, potato and strawberry (Bulley *et al.*, 2012). The first committed step in the L–galactose pathway is catalysed by GDP-mannose-3′,5′-epimerase, and transgenic tomato plants expressing two isoforms of this enzyme showed a modest 1.6-fold increase in ascorbate levels in ripe tomato fruits (Zhang *et al.*, 2011). Success has also been achieved by targeting the alternative biosynthesis pathways, for example the rate-limiting L-gulonolactone oxidase in the *myo*-inositol pathway which resulted in a sevenfold increase in ascorbate in lettuce, although only a 1.4-fold increase in potato (Hemavathi *et al.*, 2010; Jain and Nessler, 2000).

The ascorbate recycling pathway has been utilized for metabolic engineering in corn (Chen *et al.*, 2003; Naqvi *et al.*, 2009), potato (Goo *et al.*, 2008) and tomato (Haroldsen *et al.*, 2011) by overexpressing the key enzyme dehydroascorbate reductase (DHAR). Ascorbate levels in corn endosperm increased twofold following the constitutive overexpression of wheat DHAR (Chen *et al.*, 2003) but sixfold following endosperm-specific expression of rice DHAR (Naqvi *et al.*, 2009). A 1.6-fold increase in ascorbate was achieved in ripe tomato fruits constitutively expressing tomato DHAR (Haroldsen *et al.*, 2011). Potato tubers constitutively expressing a sesame DHAR enzyme showed a 1.6-fold increase in ascorbate, while the use of a tuber-specific promoter resulted in a marginally lower 1.3-fold increase (Goo *et al.*, 2008). It therefore appears that ascorbate metabolic engineering is exquisitely sensitive to the source of the heterologous enzyme and its spatiotemporal expression profile in the transgenic plants, which is likely to reflect the complex regulatory systems to control ascorbate accumulation.

Flavonoids

Flavonoids, a large structurally diverse class of more than 9000 polyphenolic compounds, are synthesized via the phenylpropanoid pathway by plants and microbes. They include chalcones, flavones, flavonols, flavanones, anthocyanins and isoflavonoids (Wang *et al.*, 2011). Although the phenylpropanoid pathway is complex and incompletely understood, key rate-limiting enzymes have been identified allowing the use of metabolic engineering to increase the overall flavonoid content, and the levels of particular subclasses (Wang *et al.*, 2011).

Flavonoid metabolic engineering has highlighted two strategies for the production of antioxidant compounds. The first is the introduction of different structural genes from diverse plants to create a recombinant biosynthesis pathway. For example, genes encoding stilbene synthase, chalcone synthase, chalcone reductase, chalcone isomerase and flavone synthase from different sources were combined in transgenic tomatoes, resulting in the accumulation of stilbenes (resveratrol and piceid), deoxychalcones (butein and isoliquiritigenin), flavones (luteolin-7-glucoside and luteolin aglycon) and flavonols (quercetin glycosides and kaempferol glycosides) in the fruit peel (Schijlen *et al.*, 2006). The total antioxidant capacity of the transgenic fruit peel was more than three times higher than wild-type fruits. These experiments show that genetic engineering can not only increase the levels of potentially health-promoting antioxidant compounds in fruits, but can also provide insight into the underlying metabolic pathways and allow the selection of particular flavonoid compounds.

The second approach involves the combinatorial use of different regulatory factors to increase flavonoid levels. For example, tomato fruits produce only low amounts of flavonols such as kaempferol and quercetin, which are concentrated in the fruit peel. However, the expression of corn regulatory genes *Lc* and *C1* induced the production of kaempferol in the fruit flesh, increasing overall levels by 60% (de Vos *et al.*, 2000). The additional expression of chalcone isomerase increased the flavonol concentration in the fruit peel by 78-fold, mainly due to the accumulation of rutin (Muir *et al.*, 2001). Corn regulatory genes (this time *C1* and *R*) have also been expressed in soybean, resulting in a twofold increase in isoflavonoids levels. The expression of these genes in conjunction with cosuppression of flavanone 3 hydroxylase (F3H) to block the anthocyanin and flavonol pathways resulted in a fourfold increase in the isoflavone content (Yu *et al.*, 2003). Finally, tomato plants expressing two snapdragon-derived transcription factors accumulated anthocyanins at levels comparable to those found in blackberries and blueberries, which are among the best sources of dietary anthocyanins although they are more expensive and are consumed in smaller quantities than tomatoes (Butelli *et al.*, 2008). The hydrophilic antioxidant capacity of the tomato fruits was threefold higher than wild type fruits, and the peel and flesh were both intense purple in coloration.

Melatonin

Melatonin is known as a terminal antioxidant because it forms stable oxidation products that cannot be recycled. It is synthesized *de novo* in humans and has a major role in the regulation of circadian rhythms, but it is also produced by plants where it fulfils an analogous role in the regulation of photoperiod responses as well as acting as an antioxidant. Melatonin is used as a drug to treat certain circadian disorders such as delayed sleep phase syndrome and seasonal affective disorder, but its antioxidant properties are also relevant as it has been indicated for protection against radiation and can also reduce the risk of certain forms of cancer. Melatonin is also sold as a dietary supplement in the USA.

The biosynthesis of melatonin in plants is incompletely understood, but several enzymes in the pathway have been expressed in transgenic plants including arylalkylamine *N*-acetyltransferase (AANAT), *N*-acetylserotonin *O*-methyltransferase (ASMT) and 2,3-dioxygenase (IDO) (Okazaki *et al.*, 2009, 2010). For example, an algal AANAT enzyme has been expressed in Micro-Tom tomato (Okazaki *et al.*, 2009) and human AANAT has been expressed in rice (Kang *et al.*, 2010), in both cases leading to elevated melatonin levels in the transgenic plants. In contrast, melatonin levels decreased in transgenic rice plants expressing IDO, potentially reflecting the induction of a feedback mechanism (Okazaki *et al.*, 2010). Because melatonin is a natural bioregulator in plants, its synthesis can also be induced by the application of exogenous chemicals such as benzothiadiazole (BTH) and chitosan (CHT) (Vitalini *et al.*, 2011). Interestingly, it has been shown that plants with higher levels of melatonin have a normal level of β-carotenoids, but double the normal amount of vitamin E, and significantly higher quantities of vitamin C and glutathione, suggesting that part of the defense pathway induced by melatonin includes the induction of additional antioxidants (Wang *et al.*, 2012).

Antioxidant activity can depend on the abundance of metal ions

A number of metals are required by humans in small amounts because they act as cofactors for enzymes, transcription factors and signalling proteins. This includes several enzymes whose function is to detoxify reactive oxygen species, which means metal ions are required as cofactors for antioxidant enzymes, including different forms of superoxide dismutase (requiring Cu, Mn and/or Zn; Johnson and Giulivi, 2005), catalase (requiring Fe) and glutathione peroxidase (requiring Se) (Mates *et al.*, 1999). This presents an interesting quandary because metal ions are also responsible for oxidation reactions and the body has numerous mechanisms to sequester and/or compartmentalize metals to ensure they do not cause oxidative damage. A key example is iron, which generates hydroxyl radicals through the Fenton reaction, a process which is facilitated by metal ion reduction mediated by ascorbate. Iron is therefore both necessary and potentially toxic, which means it must be stored and transported in a bioavailable but inactive form using proteins such as transferrin and ferritin (Crichton and Charloteaux-Wauters, 1987; Theil, 1987).

The biofortification of crops with metals is nutritionally relevant because iron and zinc deficiencies are prevalent in developing countries, but selenium is also an important target for biofortification particularly in regions with selenium-depleted soils. The strategies used for mineral biofortification depend on the mobility of the metal in the soil and its metabolism by the plant. For example, iron tends to be immobilized by interactions with soil particles (particularly in alkaline soils) so biofortification strategies must increase its mobility as well as its transport and storage (Lee *et al.*, 2012), whereas zinc is highly mobile in the soil and biofortification strategies focus on transport and storage alone (Palmgren *et al.*, 2008; Stomph *et al.*, 2009). Selenium is unusual because it can be incorporated into organic compounds in place of sulphur, such compounds being essential in humans but metabolic byproducts in plants.

Plants take up minerals from the soil in two different ways, one involving the direct absorption of mobile ions through specific transporters in the root plasma membrane, and the other involving the synthesis and secretion of chelating agents known as phytosiderophores which mobilize metals that have adsorbed onto soil particles, particularly iron. Genetic engineering can be used to enhance both mechanisms for example by overexpressing transporters to increase the uptake capacity of roots (Connolly *et al.*, 2002; Grotz *et al.*, 1998; Terry *et al.*, 2000) and by expressing enzymes in the phytosiderophore biosynthesis pathway such as nitotianamine synthase (NAS; Johnson *et al.*, 2011) or nicotianamine aminotransferase (NAAT; Takahashi *et al.*, 2001) to increase the mobilization of metal ions in the soil. However, because such proteins are often promiscuous, these strategies often lead to the coaccumulation of iron and zinc. For example, the overexpression of rice NAS in transgenic rice plants under the control of a seed-specific promoter resulted in the accumulation of up to 19 μg/g DW of Fe and 52–76 μg/g DW of Zn in rice grains (Johnson *et al.*, 2011). In cassava, the expression of the algal iron-transport protein FEA1 increased the level of Fe fourfold to 40 μg/g DW in transgenic tubers whereas the expression of Arabidopsis zinc transporters *AtZAT1* and *AtZIP1* increased the zinc content by up to fourfold and 10-fold, respectively (Sayre *et al.*, 2011).

After absorption by the roots, metals are transported to sink tissues such as fruits or seeds through the phloem, so the overexpression of phloem plasma membrane transporters (YSL family transporters) can play an important role in biofortification (Ishimaru *et al.*, 2010). Once they reach the sink tissues, metals must be stored in a bioavailable form if they are to be useful for nutritional purposes. This can be achieved by expressing metal storage proteins such as ferritin, which store both Fe and Zn (Drakakaki *et al.*, 2005; Wirth *et al.*, 2009) or enzymes such as selenocysteine methyltransferase (Ellis *et al.*, 2004), which converts inorganic Se into an organic form. The overexpression of phytase, a fungal enzyme which degrades phytic acid, is a useful strategy to increase mineral bioavailability in rice (Wirth *et al.*, 2009) and corn (Drakakaki *et al.*, 2005) because this prevents phytic acid in the gut chelating metal ions and preventing absorption.

Conclusions and perspectives

Antioxidants are interesting targets for biofortification because they encompass essential nutrients for vulnerable populations in many developing countries as well as non-essential nutraceutical compounds which are presented as food additives or functional foods for the luxury markets in the West. Notwithstanding these differences, many of the same technical issues must be addressed in both environments, that is the need to modulate endogenous plant metabolic pathways to ensure that flux is directed to the appropriate compounds, the need to ensure such compounds accumulate in the most appropriate tissues and the focus on bioavailability rather than bioaccumulation. The examples discussed above provide a snapshot of the diverse approaches to antioxidant biofortification which are driven by the nature of the endogenous metabolic pathways. Where there is a single, well-characterized pathway (e.g. carotenoids and tocochromanols) it is often appropriate to target individual rate-limiting steps or key branch points, often through a combination of interventions to relieve early bottlenecks and reduce metabolic loss through downstream catabolism. In these cases, it is possible to affect the pathway either quantitatively (by targeting early, common steps) or qualitatively (by targeting branch points) to generate novel profiles of compounds. Where multiple pathways are present (e.g. ascorbate), there is also a tendency towards complex regulation that favours homeostasis. In these cases, it is often more productive to focus metabolic interventions in a localized manner (e.g. in seeds) to avoid feedback that can occur when transgenes are expressed constitutively. In contrast, the highly complex and multiple branched flavonoid pathway provides an example where targeting the regulatory genes is more beneficial.

In the future, it is likely that genetic engineering will be used to modify different pathways simultaneously, as recently demonstrated by the production of multivitamin corn with elevated levels of three vitamins (β-carotene, ascorbate and folic acid) but also other carotenoids, providing higher levels of several key antioxidants (Naqvi *et al.*, 2009). As such ventures become more ambitious, it is likely that the complex interactions between pathways will become more apparent, meaning that interventions will need to become more refined and focused to avoid conflicts such as competition for substrates, precursors and intermediates (Zhu *et al.*, 2007). It is therefore clear that as well as looking at the structural genes that represent each metabolic pathway, we also need to consider the regulatory factors, the

interplay between metabolic flux and enzyme activity, the compartmentalization of different enzymes and the shuttling of intermediates, the interplay between endogenous and heterologous pathways in transgenic plants and the impact of metabolic interventions at the level of the metabolome.

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Supporting information

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

Table S1 Water and fat soluble antioxidants.
Table S2 Transgenic plants with enhanced levels of provitamin A and vitamin E content and coenzyme Q10.
Table S3 Transgenic plants with enhanced levels of vitamin C content.
Table S4 Transgenic crops with enhanced mineral content.

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Review

Feature Review

Paradoxical EU agricultural policies on genetically engineered crops

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European Union (EU) agricultural policy has been developed in the pursuit of laudable goals such as a competitive economy and regulatory harmony across the union. However, what has emerged is a fragmented, contradictory, and unworkable legislative framework that threatens economic disaster. In this review, we present case studies highlighting differences in the regulations applied to foods grown in EU countries and identical imported products, which show that the EU is undermining its own competitiveness in the agricultural sector, damaging both the EU and its humanitarian activities in the developing world. We recommend the adoption of rational, science-based principles for the harmonization of agricultural policies to prevent economic decline and lower standards of living across the continent.

Importance of agriculture in the EU

Agriculture is one of the most important pillars of social and economic development in the EU, and Europe remains one of the world's largest traders in agricultural products (Figure 1) (http://ec.europa.eu/agriculture/publi/map/01_12_en.pdf). However, a substantial genetic gain in yield potential and stress resistance is required to ensure that sustainable agricultural practices can be developed to meet the demands of a growing population in Europe and in the many agriculture-dependent developing countries (http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf) [1].

EU agricultural policy is proposed by the European Commission, approved by agriculture ministers in EU member states, and ratified by the European Council and Parliament. The stated objectives are to support farm incomes, encourage the production of high-quality goods led by domestic and export market demands, promote environmentally sustainable practices, and increase the competitiveness of European agriculture (http://ec.europa.eu/agriculture/cap-overview/2012_en.pdf). However, the common agricultural policy (CAP) of the EU has provoked intense criticism because it reduces competitiveness, productivity, and sustainability, and ultimately invites economic and social instability (http://www.agriregionieuropa.univpm.it/materiale/2011/Erjavec_OptionsForTheCAP_16_2_2011.pdf). Although they aim to promote environmentally sustainable agricultural practices, nutritious food, and inexpensive medicines, EU policies hamper the development of key technologies to achieve those objectives.

There are three major paradoxes in current EU agricultural policy that not only affect agriculture directly but also have knock-on effects on the environment, on human health, on the wider economy, and on food security in developing countries. First, the Lisbon Strategy aims to create an EU knowledge-based bioeconomy (KBBE) and recognizes the potential of genetically engineered (GE) crops to deliver it [2], but EU policy on the cultivation of GE crops has created an environment in which the aims of the Lisbon Strategy can never be achieved. The policy sets a framework for coexistence measures ensuring sufficient segregation between GE and conventional crops, thus offering choice to farmers and consumers [3,4], but also encourages the haphazard implementation of these measures without coordination or a rational scientific basis, including plans to allow member states and their regions an unconditional opt-out. This has imposed a *de facto* moratorium on GE maize (*Zea mays*) and soybean (*Glycine max*) crops in Europe, even though these same GE products are imported because there is insufficient capacity to grow these crops using conventional agricultural practices [5].

The second paradox is the CAP, which aims to ensure a stable supply of high-quality food for the EU population at fair prices while providing farmers with a reasonable standard of living and preserving rural heritage [6]. However, most of the subsidies available under the CAP are used to benefit large producers rather than family farms (http://www.attac-netzwerk.de/fileadmin/user_upload/AGs/Agrarnetz/EU-Agrarpolitik/marita_eusubsidies.pdf), and the dumping of CAP-subsidized EU products disrupts agriculture in developing countries (http://www.cedia.eu/en/policy/2011/swiss_paper_cap_policy_2011.pdf). The third paradox is the contrast between policy aims and outcomes.

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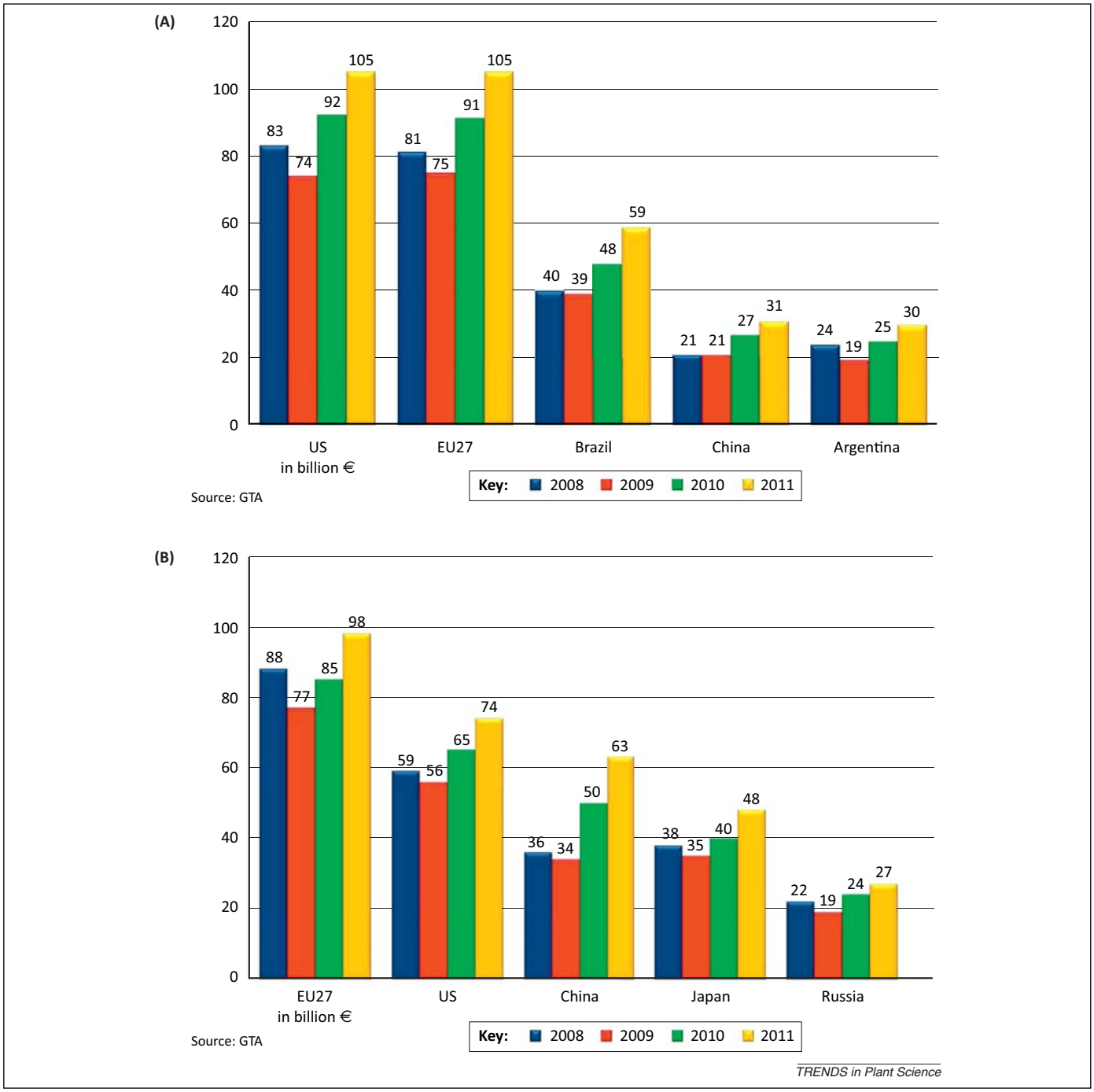


Figure 1. The world's top five **(A)** exporters and **(B)** importers of agricultural products (reproduced from http://ec.europa.eu/agriculture/trade-analysis/map/05-2012_en.pdf). (A) EU exports fell in 2009 but increased in 2010 and 2011 to record levels. (B) The EU is currently the largest importer in the world, although the USA and China have increased imports since 2009.

For example, the EU has banned many pesticides, but approves the import of food products treated with banned chemicals (http://europa.eu/legislation_summaries/food_safety/plant_health_checks/l21289_en.htm; http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:1976:340:0026:0031:EN:PDF).

This review focuses on the role of GE agriculture, how its deployment in Europe is necessary to achieve the stated goals of EU agricultural policy, and how continued resistance is placing short-term political and economic interests above the long-term goals of environmentally sustainable agriculture, food safety, and human health (Table 1). The suppression of GE agriculture in the EU is widely recognized as ideological rather than scientific, driven to a large extent by the organic food industry in an effort to protect organic food premiums at the expense of overall competitiveness [3]. This policy is actively working against the EU's own goals, driving research, development and innovation abroad, and granting commercial and economic benefits to other countries that then sell the products back to EU member states [5]. The EU is thus becoming increasingly uncompetitive and isolated in the international markets, which thrive on innovation and technological development in agriculture [7,8].

Table 1. Paradoxes among agricultural and health policies of the EU

Policy	Intention	Reality	Consequences	Solution
Knowledge-based economy				
KBBE	To promote European competitiveness based on excellent science and technology using industry as a base to deliver innovations.	The development of innovations in GE agriculture is blocked, SMEs are failing and major industry is moving overseas.	EU agriculture will not benefit from innovations and its competitiveness will be reduced.	Promote high-quality agricultural biotechnology, in both the public and private sectors by streamlining the regulatory pathway for GE crops.
Agricultural policy				
CAP	Increase agricultural productivity and ensure a good standard of living for the agricultural community, stabilize markets, and ensure fair prices for consumers.	Promotes the overproduction of noncompetitive commodities, inequality in the distribution of subsidies, and the artificial competitiveness of EU produce. Isolates the EU from world market fluctuations.	A substantial portion of the EU budget is wasted. Large producers gain subsidies at the expense of small farmers. Products are dumped on developing country markets damaging local infrastructure. The adoption of competitive technologies such as GE crops is obstructed.	Increase competitiveness by adopting agricultural innovations such as GE crops. Combine a reduction in subsidies with policies that prevent dumping. Reduce the overproduction of crops.
Trade policy				
Treaty on the Functioning of the EU Article 207 EC	Balance agricultural import and export to meet the demands of home and export markets in the most economically beneficial manner.	EU imports animal feed (mainly soybean and maize) because it cannot meet home demand. More than 80% comes from GE producers, with 39 GE crops authorized for import but only 2 (1 food crop) for cultivation. Member states do not allow their own farmers to grow GE crops even if they are identical to imported varieties. Imports must meet EU thresholds for adventitious presence.	EU farmers cannot use new technologies such as GE to increase competitiveness. Imported foods are impounded if they fail EU thresholds, creating trade barriers, economic damage to importers and exporters, and the perception of the EU as an economic risk for exporters. Decline in overall trade with the rest of the world.	Rationalize and harmonize EU policy on the cultivation and import of GE crops.
Coexistence measures				
Recommendation 2003/556/EC	Coexistence measures should be transparent, science-based, and proportionate and should embrace cross-border cooperation, equal stakeholder involvement, and coherent liability rules to provide farmers with freedom of choice.	Coexistence measures are purely an economic issue. They are neither proportionate nor science-based and discriminate against farmers seeking the choices the measures are meant to promote. They place farmers at risk of litigation and effectively ban GE agriculture in large areas of the EU.	Suppression of GE agriculture, which will damage the EU economy and global scientific standing. Increases the public's negative perception of GE crops. Reduces freedom of choice.	Harmonization of coexistence measures throughout the EU with a strict evidence-based threshold for minimum distances.
Recommendation 2010/C 200/01	Autonomy for member states to manage the cultivation of GE crops unilaterally, develop their own coexistence regulations, and overturn EFSA recommendations without presenting new evidence, ostensibly to prevent EU-wide bans caused by a minority of uncooperative member states.	The lack of EU-wide regulations means that member states can impose arbitrary minimum isolation distances that have no scientific basis and make approval decisions for political rather than scientific reasons.	The creation of GE free zones in Europe, pandering to extremist views, and further damage to the public's perception of GE agriculture.	Strict adoption of EU-wide science-based rules for the adoption of GE crops backed up by legal sanctions against countries that do not comply.

Table 1 (*Continued*)

Policy	Intention	Reality	Consequences	Solution
Environmental policy				
Regulation (EC) 1698/2005 and Regulation (EC) 1257/1999	Improve the competitiveness of the agricultural and forestry sector, improve the environment and the countryside, and improve the quality of life in rural areas by encouraging diversification of the rural economy by increasing agricultural subsidies.	Subsidies, grants, and export refunds do not provide enough to keep EU farmers competitive. Market-driven agriculture is reducing crop diversity, promoting deforestation, and reducing long-term sustainability.	Loss of competitiveness in the rural farming sector and rural economy generally.	Decentralize rural economy measures and allow farmers to use cost-saving technologies that can enhance productivity and profitability in a more sustained manner.
Regulation 396/2005/EC	Set maximum residue levels (MRLs) for pesticides used in the EU to eliminate trade barriers and increase market transparency.	Allows the import of foreign products treated with pesticides banned in the EU. Different MRLs for home-grown and imported products.	Farmers must reduce the use of pesticides but must compete with imports of crops treated with the same chemicals. Restriction to a small number of essential pesticides means that resistance becomes more likely. The MRLs set by the EU are a trade barrier for developing countries.	Harmonize MRLs based on scientific evidence and allow the cultivation of GE crops that are competitive without the need for pesticides.
Regulation 1107/2009/EC	Promote environmentally sustainable practices by banning many pesticides and encouraging the use of integrated pest management and nonchemical alternatives.	Exception for 'essential pesticides' that cannot be replaced means that farmers focus on a small number of products but overall use does not change.	Increases the risk that pests and pathogens will evolve resistant populations against the limited number of permitted chemicals. More effective chemicals cannot be used thus overall pesticide levels in the environment increase.	Allow the use of pesticides on a case-by-case basis where minimum harm is achieved. Allow the cultivation of GE crops that reduce pesticide usage.
Health and safety policy				
Regulation 165/2010/EEC	Ensure the protection of public health and, where appropriate, set maximum levels for certain contaminants.	Stringent levels for mycotoxins compared with the rest of the world.	Generates a trade barrier with countries unable to meet EU limits. Benefits the high-quality export market for which the EU pays a premium, whereas lower quality commodities are used for domestic markets in developing countries.	Harmonize levels based on scientific evidence. Allow the cultivation of Bt crops that reduce mycotoxin levels.
EU policy on nutrition	Protect consumer health while guaranteeing smooth operation of the single market by ensuring that food hygiene control standards are established and met, reducing the risk of contamination.	EU agriculture policy blocks the development of cost-effective technologies that increase the quality and quantity of food grown in Europe, and the production of more accessible pharmaceuticals for the EU and developing countries.	Budget required for the treatment of food-related diseases and to meet the costs of increased disability-adjusted life years.	Allow the cultivation of crops bred for increased nutrition and pharmaceutical production.
Directive 2001/83/EC (also Regulation EC 726/2004 and EC 1394/2007)	Directive 2001/83/EC requires individual approvals for the manufacture of health products in the EU. The other policy instruments established the EMA and set up rules to ensure the free movement of biotechnology-derived drugs within the EU.	The authorization and marketing of novel pharmaceutical products from plants is regulated jointly by the EMA and the EFSA.	The development of novel and inexpensive pharmaceutical products is hindered by regulatory complexity, meaning that both developed and developing countries need to pay more than necessary for essential drugs.	Unification of the regulations so that there is a single process for the approval of medicines derived from plants.

The CAP no longer fits

The policy instruments encompassed by the CAP were intended to provide farmers with a reasonable standard of living while preserving rural heritage and ensuring a stable supply of high-quality and fairly priced food within the EU [6]. However, the objectives have changed over the years, and now most of the subsidies go to large food-processing and trading companies along with the wealthiest farmers, protecting the larger industry players from the economic impact of competition with imports [9] (http://ec.europa.eu/agriculture/publi/app-briefs/01_en.pdf), whereas small farms are largely ignored unless they fall within the scope of rural development programs (http://www.cedia.eu/en/policy/2011/swiss_paper_cap_policy_2011.pdf) or they are linked to the organic farming industry and become automatically eligible for payments because of their perceived environmental benefit (http://www.soilassociation.org/news/newsstory/articleid/2690/soil-association-welcomes-cap-reform-announcements; http://europa.eu/rapid/press-release_IP-11-1181_en.htm?locale=en). Another major criticism is that direct payments and export refunds promote the practice of dumping (i.e., exporting at prices below the cost of production), which allows artificially competitive EU commodities to displace home-grown products in developing countries [10]. Similar criticism has been leveled at US Farm Bills [11,12].

Maintaining the CAP in its current state means that the EU will continue to waste a large proportion of its budget supporting uncompetitive producers and processers, while poorer farmers remain in poverty and agriculture in developing countries is suppressed. GE agriculture would offer a competitive advantage to food producers and processers based on the adoption of new technology rather than artificial subsidies. Some claim that reducing or abolishing subsidies will not have the predicted positive impact on prices and the welfare of small farmers [13]. Instead, they propose a combination of reduced subsidies and policies that place limits on export dumping, global commodity overproduction in key crops, and the market power of agribusiness conglomerates [14].

KBBE – support in principle, obstruction in practice

The Lisbon Strategy was launched in 2000 by the European Council to increase the productivity and competitiveness of the EU by aspiring to create '...the most dynamic and competitive knowledge-based economy in the world...' (http://www.europarl.europa.eu/summits/lis1_en.htm). Knowledge is considered a valuable resource for economic growth and social welfare, highlighting the importance of investment in research and development [2]. Biotechnology and the life sciences were identified as essential components of this strategy with the establishment of the KBBE, which accounts for €1.5–2 trillion of the EU gross domestic product (Table 2) (http://www.bio-economy.net/reports/files/KBBE_2020_BE_presidency.pdf).

A bioeconomy comprises all the industries that produce, manage, or exploit biological resources. Because crops are the major source of biomass used by humans, the EU has recognized the potential of agricultural biotechnology as a means to increase the yield and quality of economically relevant crops [15]. But despite official acknowledgement of the potential benefits, and generous funding of precompetitive research in this area, little has been done to promote translational research and the commercialization of agricultural technology so that the benefits are realized at the farm and consumer levels. Indeed, the EU's politicians and policymakers have actively obstructed the adoption of GE agriculture through the establishment of complex and inconsistent regulations that strongly discourage farmers from considering the technology.

Table 2. EU bioeconomy

Sector	Annual turnover (billions of euro)	Employment (thousands)	EU population (%)
Food	965	4400	0.880
Agriculture	381	12 000	2.400
Paper and pulp	375	1800	0.360
Forestry and wood	269	3000	0.600
Fisheries and aquaculture	32	500	0.1 00
Biobased industries			
Biochemicals and plastics	50[a]	150[a]	0.030[b]
Enzymes	0.8[a]	5[a]	0.001[b]
Biofuels	6[c]	150	0.030
Total	2078	22 005	4.400

[a]Estimate 2009.

[b]http://www.bio-economy.net/reports/files/KBBE_2020_BE_presidency.pdf.

[c]Estimate based on the production of 2.2 million tons of bioethanol and 7.7 million tons of biodiesel at average EU market price.

EU legislation for the approval of GE crops (Directive 2008/27/EC and Regulation EC 1829/2003) is the most restrictive in the world. Regulatory compliance for a new crop can cost up to €11 million and requires a dedicated legal team working for many years [16]. For example, the Amflora potato took 15 years to develop, 13 of which were required for regulatory approval. Such onerous regulation blocks the approval pathway to all but the most committed and well-funded companies, preventing the realization of innovation generated by public sector institutions and small-to-medium enterprises (SMEs) unless they agree to collaborate with major industry players [17,18].

Perhaps more importantly, the final decision for approval is political rather than scientific. As part of the regulatory process, a scientific opinion on safety must be sought from the European Food Safety Authority (EFSA), the official and expert scientific body charged with the task of safety evaluation in the EU. This opinion is based on the views of expert panels that consider the available scientific evidence. However, the opinions of the EFSA are routinely ignored by many member states and the EU has recently approved a plan to allow member states an opt-out for the cultivation of approved GE crops with no requirement for scientific justification or evidence of risk (http://www.europabio.org/sites/default/files/europabio_-_ernst_young_report___what_europe_has_to_offer_biotechnology_companies.pdf; http://www.europarl.europa.eu/news/en/headlines/content/20110627FCS22686/8/html/GMOs-Parliament-backs-national-right-to-cultivation-bans). Although the ostensible reason for the proposal is to allow member states to adopt GE agriculture on an individual basis rather than relying on voting in the European Parliament, the European

Commission, and ultimately the Council of Ministers, the opt-out may only serve to legalize the formerly illegal (although widely practiced) strategy of declaring GE-free zones within the EU, thus damaging not only the EU economy but also its global scientific standing and opposing the basis of the Common Market [5].

The lack of GE agriculture in the EU is an overall symptom of the promising but poorly executed Lisbon Strategy. By failing to uphold the rights of farmers to adopt GE crops, the EU is moving rapidly away from the KBBE model, actively discouraging innovation and investment in the sector, stifling the growth of SMEs, driving research and development abroad, and therefore handing the EU's competitive advantage to the industry in North and South America and Asia (http://www.basf.com/group/corporate/en/function/conversions:/publish/content/news-and-media-relations/news-releases/downloads/2012/P109e-PlantBiotechnology.pdf).

Farmers – choice in principle but not in practice

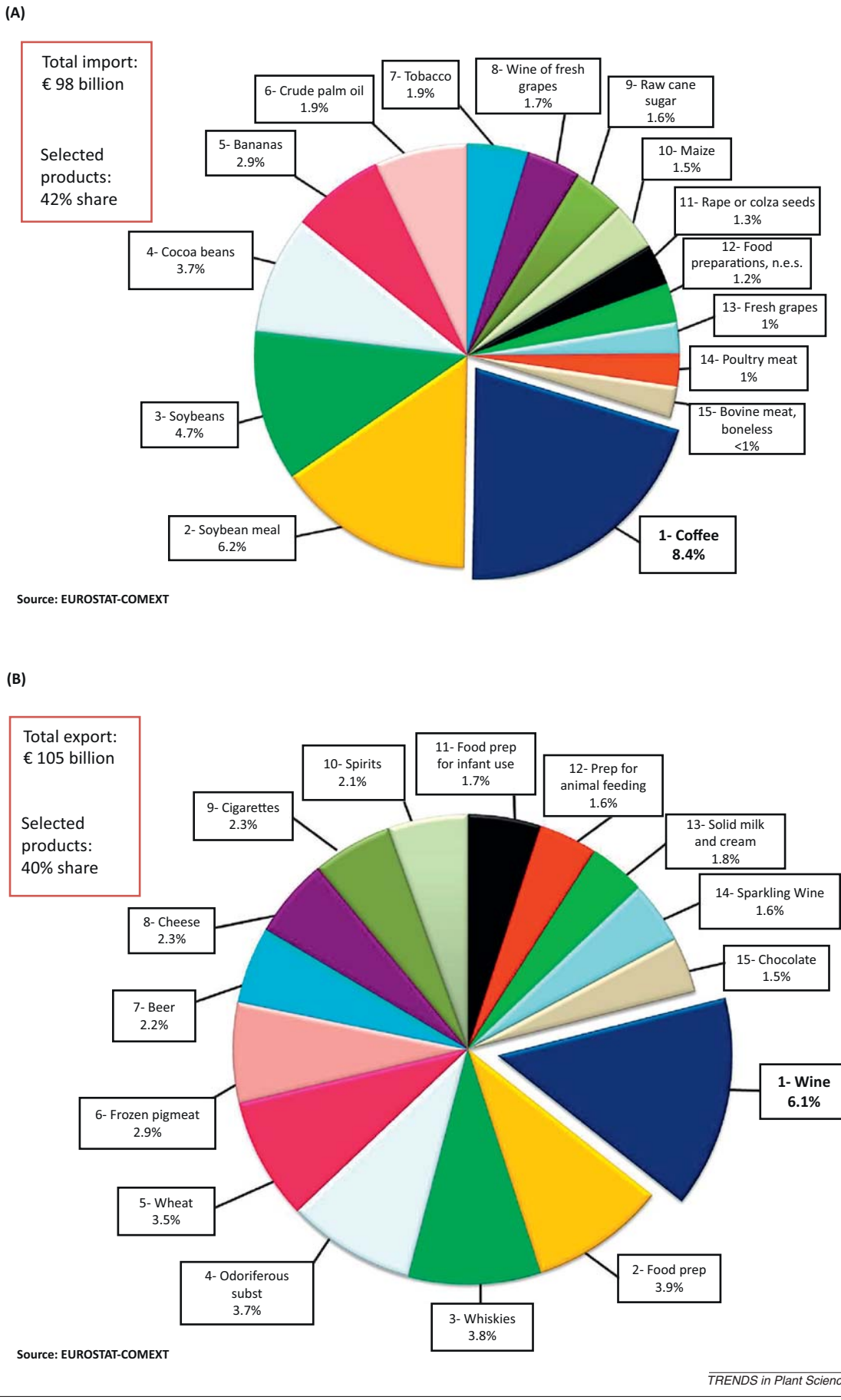
EU policy officially supports the coexistence of GE and conventional agriculture, and lays down coexistence regulations by allowing member states to establish minimum distances between fields of GE and conventional crops to prevent admixture. Coexistence refers to the ability of farmers to make a practical choice among conventional, organic and GE crops, in compliance with legal obligations for labeling and/or purity standards as defined in European Commission legislation. The European Commission has published detailed and pragmatic recommendations for the development of coexistence regulations for implementation at national or regional levels, based on a tolerance threshold for adventitious presence above which a conventional crop must be labeled as containing GE material (http://ec.europa.eu/agriculture/publi/reports/coexistence2/index_en.htm). Despite the absence of any science-based public health or environmental safety justifications, the regulations were developed in response to lobbying by self-regulating organic industry groups which claimed that adventitious presence could reduce the value of a conventional or organic crop, particularly the latter, which is often sold at a premium. This implicitly acknowledges that coexistence measures are concerned with the economic impact and not the health or environmental safety of the product, given that no GE crops can be grown without a positive safety evaluation from the EFSA [19,20] (http://www.gmcc13.org/files/proceedings_gmcc05.pdf).

The adventitious presence thresholds in the EU are the strictest in the world. There is a two-tier tolerance policy (EC 1830/2003) with a 0.9% adventitious presence limit applied to approved products and zero tolerance applied to unapproved products, replacing the temporary 0.5% second-tier limit previously approved by the EFSA. There are clear scientific principles that can be used to establish acceptable minimum distances between GE and conventional crops and other mitigation strategies to achieve these thresholds, and these principles are explained in Recommendations 2003/556/EC and 2010/C 200/01 to allow the development of national coexistence strategies and best practices. The major changes in Recommendation 2010/C 200/01 provide individual member states with greater flexibility and responsibility for their own coexistence policies, aiming to speed up pending authorizations by removing the ability of those member states to veto approvals throughout the EU. However, the practical effect of these recommendations has been to allow member states to impose arbitrarily large minimum distances between conventional and GE crops so that GE agriculture is effectively prevented unless farmers agree to surround their crops with large areas of uncultivated land or risk litigation from surrounding farms [5,21]. As a consequence, only 100 000 ha of GE crops was grown in the EU in 2012

Table 3. Paradoxes among coexistence measures of the EU[a]

Principle	Intention	Consequence
Transparency	National strategies and best practices for coexistence should be developed in a transparent manner.	Coexistence requirements are confusing and those implementing them are unaccountable [3,4].
Cross-border cooperation	Member states should ensure cross-border cooperation with neighboring countries to guarantee the effective functioning of coexistence measures in border areas.	There is no cooperation because member states act independently and national governments are responsible for coexistence policies. Each member state establishes a legislative framework on a crop-by-crop basis [3,4].
Stakeholder involvement	National strategies and best practices for coexistence should be developed in cooperation with all relevant stakeholders.	GE farmers are sidelined. Farmers who choose to grow GE crops have to invest extra money to comply with the excessive coexistence measures [21,23].
Based on scientific evidence	Management measures for coexistence should reflect the best available scientific evidence on the probability and sources of admixture between GE and non-GE crops.	The thresholds for adventitious presence are far stricter than for conventional crops and the isolation distances enforced to achieve such thresholds are arbitrary, excessive, and are politically motivated rather than reflecting scientific reality [3].
Proportionality	Measures to avoid the unintended presence of genetically modified organisms (GMOs) in other crops and vice versa should be proportionate to the intended objective (protection of the particular needs of conventional, organic, and GE farmers).	Measures are neither regionally nor economically proportionate [23]. Proportionality is still linked to economic loss even if not necessarily to the labeling thresholds [3].
Liability	The policy instruments adopted may have an impact on national liability rules in the event of economic damage resulting from admixture.	Strict liability regulations mean that GE farmers are always responsible for any admixture and risk fines or litigation from surrounding farms [3,4,21,23].

[a]http://ec.europa.eu/agriculture/publi/reports/coexistence2/index_en.htm.



(See figure legend on the bottom of the next page.)

(http://www.isaaa.org/resources/publications/briefs/43/). In Luxembourg, for example, it is necessary to leave 800 m between GE and non-GE maize plots and 3 km between GE and non-GE rapeseed (*Brassica napus*) plots. Similarly in Latvia, it is necessary to leave 4 km between GE and non-GE rapeseed and 6 km if the non-GE rapeseed is organic [4]. These national coexistence strategies do not comply with most of the key coexistence principles established by the European Commission, as summarized in Table 3.

Exaggerated coexistence measures are often justified in the name of safety even though the principal reason is to achieve established tolerance thresholds, but this nevertheless damages the perception of GE crops because most consumers only note the nature of the regulations, not the underlying justification. Coexistence measures are therefore being used as a convenient and politically expedient proxy for EU policymakers to prohibit GE agriculture, limiting the extent of GE agriculture in Europe to less than 115 000 ha in 2011 and approximately 100 000 ha in 2012 compared to 160 million ha in the rest of the world [3,4].

Most EU farmers are indirectly denied the choice of growing GE crops and must therefore continue to use unsustainable and uncompetitive farming practices, making them unwitting coconspirators to maintain the CAP. A recent study showed that coexistence measures that actually ensured the coexistence of GE and non-GE crops rather than preventing GE agriculture would alter the attitude of farmers towards the adoption of GE technology [22]. GE crops would reduce production costs by reducing pesticide use, labor, and fuel consumption, resulting in significant economic benefits (http://ec.europa.eu/food/plant/gmo/reports_studies/docs/economic_performance_report_en.pdf). However, the ability of member states to impose their own rules means that farmers choosing to adopt GE crops in nonsupportive member states lose any economic advantages the technology might bring through the costs of compliance, negotiations with surrounding farms, and insurance to cover litigation in the event of admixture [23]. The obligations placed on farmers growing conventional or organic crops are much less restrictive than those growing GE crops [23–25].

Safe to eat, but only if imported

As discussed above, food derived from approved GE crops has been deemed safe for human consumption by the EFSA. Approval must also be granted by the equivalent body in the USA, which comprises experts from the US Department of Agriculture (USDA) and the Food and Drugs Administration (FDA). Notably, even in the highly litigious USA, there have been no lawsuits, no product recalls, no reported ill effects, and no other evidence of risk from a GE product intended for human consumption since the technology was first deployed commercially in 1996.

The perceived risks of GE food and feed to human health persist in the EU despite all the contrary evidence from the regulators and the lack of harm in countries that have embraced the technology. Further evidence comes paradoxically from the population of the EU itself, which regularly consumes GE food imported from other countries because the restrictions that apply to home-grown GE foods do not apply to imports. Indeed, the EU is largely dependent on GE products from abroad [25]. Approximately 80% of animal feed consumed in the EU is imported, of which more than half is GE produce imported from countries such as Brazil, the USA, and Argentina, which are the largest exporters of GE products [25] (http://www.europabio.org/sites/default/files/position/pocket_guide_gmcrops_policy.pdf). The EU is dependent on soybean meal from South America and dried distillers' grains of maize from the USA (Figure 2) [26]. In 2009, the 12.9 million tons of maize imported into the EU included 69% (8.9 million tons) and 17% (2.2 million tons) from Brazil and the USA, respectively [25].

EU policy on GE food imports is less restrictive than the regulations covering GE agriculture in the EU owing to its dependence on imports to maintain the livestock industry. This explains the big difference between the numbers of crops approved for import and cultivation (Figure 3; http://www.europabio.org/sites/default/files/report/approvals_of_gmos_in_eu_europabio_report.pdf). A total of 39 different GE crops were approved for import in 2011/2012: 24 varieties of maize, 7 of cotton (*Gossypium* spp.), 3 of rapeseed, 3 of soybean, and 1 variety of sugar beet (*Beta vulgaris*) (http://www.gmo-compass.org/). In contrast, only two products have been approved for cultivation, the pest-resistant maize variety MON810 and the Amflora potato variety, which is for industrial starch production rather than food use (http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biotechnology%20-%20GE%20Plants%20and%20Animals_Paris_EU-27_7-23-2010.pdf). Even so, cultivation of the Amflora potato was prohibited in Germany in September 2010 due to intermixture with another GE potato variety that has not yet been approved, during its cultivation in Sweden (http://www.gmo-compass.org/eng/news/536.iamflorai_potato_intermixing_sweden_ban_germany.html). These barriers eventually persuaded the developer BASF to move production abroad because '...there is still a lack of acceptance for this technology in many parts of Europe...' (http://www.basf.com/group/corporate/en/function/conversions:/publish/content/news-and-media-relations/news-releases/downloads/2012/P109e-PlantBiotechnology.pdf).

The member state opt-out discussed above only applies to cultivated GE crops because EU member states are not legally permitted to block the marketing of approved imported GE products. This means that EU markets are flooded with imported GE products that could just as easily be grown in the EU, and that member states such as Austria, Belgium, or Luxembourg with some of the most hostile policies towards GE crops need these imported products the most. The absurdity of this position is that a ban on cultivation often means that GE soybean, maize,

Figure 2. The main agricultural **(A)** imports and **(B)** exports in the EU (reproduced from http://ec.europa.eu/agriculture/trade-analysis/map/05-2012_en.pdf) (A) Coffee is the main agricultural product imported into the EU, followed by soybean meal and soybeans, which represent 11% of EU agricultural products, then maize. After these, the next agricultural product that is imported to Europe is maize, which is tenth in the list. (B) Approximately 64% of EU agricultural exports are finished products, whereas commodities and intermediate products represent 8% and 19%, respectively (average 2009–2011). The top EU export is wine (€6.1 billion) followed by prepared foods (€4 billion) and whiskies (€3.9 billion).

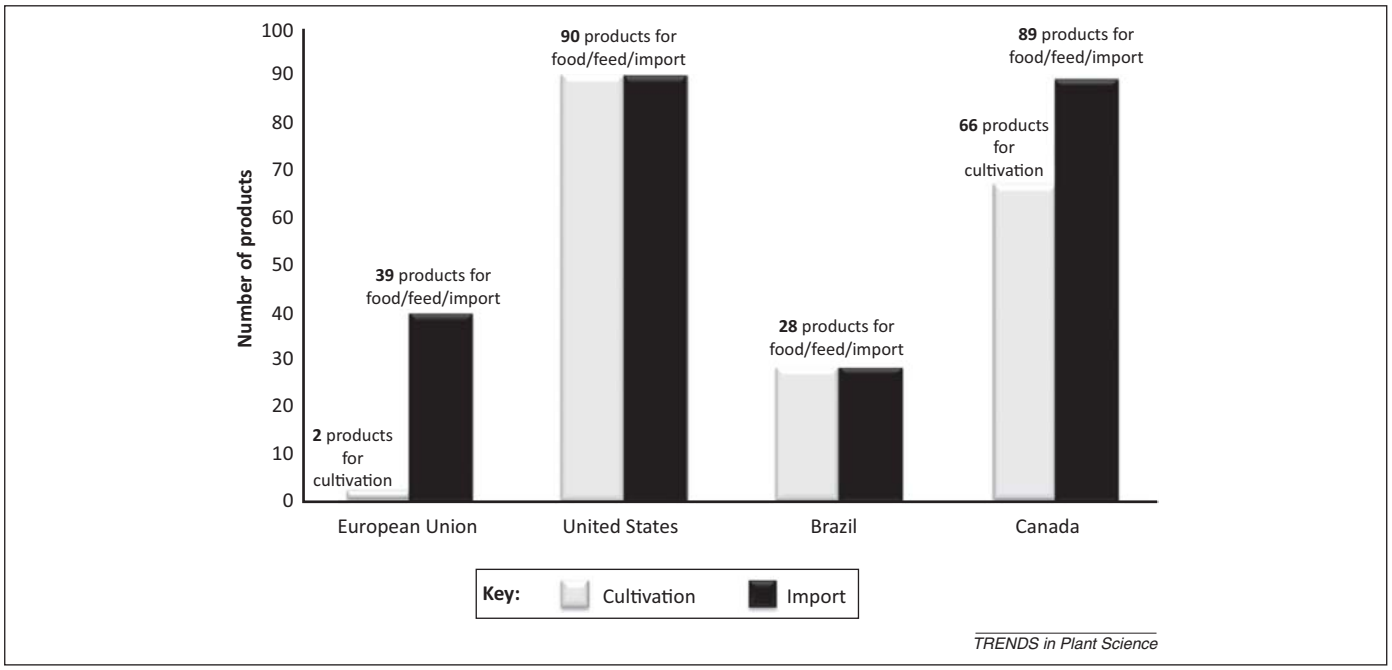


Figure 3. Number of approved genetically engineered (GE) products in the EU, USA, Brazil, and Canada (http://www.europabio.org/sites/default/files/report/approvals_of_gmos_in_eu_europabio_report.pdf). The EU has the lowest number of approved products for cultivation, MON810 and the Amflora potato. However, 39 products are approved for import, compared to the USA and Brazil, where 1 approval covers both import and cultivation.

or cotton is imported instead, which undermines claims that GE crops should not be grown because they are unsafe [5] (http://ec.europa.eu/research/biosociety/pdf/a_decade_of_eu-funded_gmo_research.pdf).

Although less stringently regulated than cultivation, the import of GE products is nevertheless still heavily controlled, causing logistic and economic problems for exporters and EU importers alike, reducing the flow of commodities and threatening the stability of the livestock industry. The import approval process differs between countries, which causes delays and asynchronous authorizations in different parts of the world [26]. The zero tolerance for the adventitious presence of GE products that have not yet been approved for import into the EU results in shipments containing traces of GE material that are approved by the exporter being rejected at the point of import, with significant economic consequences for both the EU and the supplier, particularly if the supplier is a developing country [27]. Europe is increasingly being perceived as a risky export market, resulting in preferential trading between other countries, and EU importers bearing high prices and insurance premiums to offset risks undertaken by the supplier [25] (http://ftp.jrc.es/EURdoc/report_GMOpipeline_online_preprint.pdf). The situation described above may worsen with the increasing adoption of GE agriculture as a mainstream technology outside the EU, leading to deficits initially in the demand and supply chain for soybean meal and then in the feed industry more generally, with a knock-on effect on the livestock, poultry, and dairy industries and economic decline throughout the EU [8,27].

Sustainability – promoted in principle but discouraged in practice

Agricultural sustainability is a key program within the CAP and has a strong impact on market forces and the food supply chain (http://ec.europa.eu/agriculture/publi/app-briefs/01_en.pdf). Subsidies based on land use are provided to encourage environmentally responsible cultivation methods and reduce the use of chemicals and pesticides (http://aei.pitt.edu/559/1/1_Bandarra.pdf). However, the subsidies have instead resulted in the intensification of agriculture and a dramatic increase in the use of fertilizers and pesticides, thus increasing the rate of environmental damage (http://www.wwf.org.uk/filelibrary/pdf/ag_in_the_eu.pdf). The restriction of pesticide use has encouraged farmers to rely on a small number of permitted chemicals, risking the emergence of resistant pest populations and making agricultural sustainability difficult to achieve [28].

Integrated approaches can help to achieve sustainability, and biotechnology can play a crucial role in this process as seen in other parts of the world [29]. For example, the cultivation of insect-resistant GE crops has reduced the use of pesticides in India and China, improving the environment and the health of farmers [30]. GE papaya (*Carica papaya*) that is resistant to *Papaya ring spot virus* has improved disease management in Hawaii, resulting in a sustainable supply of papaya fruits for the domestic population [31]. Furthermore, the cultivation of herbicide-tolerant crops in the Western Hemisphere has promoted reduced/zero-tillage farming to reduce soil erosion and water contamination caused by agriculture [32].

Insect-resistant maize in Europe provides efficient pest control without pesticides, also limiting the impact of agriculture on nontarget organisms while increasing yields and the net economic benefits of farming. This has been realized in countries such as Spain, where GE crops are still encouraged, whereas other parts of the EU see no benefit because of the costs of compliance with national regulations [33]. Mycotoxin levels in GE maize are much lower than those in conventional maize, therefore reducing

the risk of acute toxicity effects and long-term health issues [34]. The deployment of Bt maize has reduced pesticide use in Spain and has led to a significant reduction in mycotoxin levels, compared to conventional maize [35]. Growing Bt maize throughout the EU could achieve annual savings of up to 700 tons of pesticide active ingredient, adding to the current 443 000 tons saved globally by the cultivation of pest-resistant maize and cotton [36] (http://ec.europa.eu/food/food/biotechnology/reports_studies/docs/Europabio_contribution_II_en.pdf). Between 1996 and 2010, GE agriculture has reduced fuel use and increased carbon sequestration, thus reducing the carbon footprint of agriculture by 146 million tons [36].

Despite the economic and environmental benefits demonstrated in other regions, EU policies continue to block the adoption of approved GE crops [18,37,38]. Developing countries that formerly embraced GE agriculture as a means to improve the health and welfare of subsistence farmers are being discouraged because of the hurdles they encounter when exporting their produce to the EU. The reluctance of the EU to accept GE commodities has diminished the enthusiasm of developing countries to approve GE crops. This is the case in Egypt, where the likelihood of a ban on the import of GE potato into the EU has delayed the approval of this crop. Similarly in Thailand, the government has refused to approve the cultivation of GE crops because they fear losing export markets in Europe (http://agbioforum.org/v7n12/v7n12a12-kent.pdf).

Pesticides – banned but not forgotten

European Commission regulations EC/396/2005 and 1107/2009/EEC were introduced to harmonize the maximum residue levels (MRLs) for pesticides in food and feed and to reduce pesticide use in agriculture, particularly by prohibiting the use of certain chemicals deemed to be hazardous [39]. A special essential-use category was created to extend the use of pesticides that could not be replaced, giving manufacturers 5 years to make the product safe. However, if alternative products are still not available after that period then farmers will be able to continue using them for another 5 years (http://www.pan-europe.info/Resources/Links/Banned_in_the_EU.pdf; 1107/2009/EEC). The regulations create the impression that the EU is making efforts to reduce pesticide use in agriculture, but in reality they favor the use of a small number of chemicals thus greatly enhancing the risk that resistant pest populations will emerge. Meanwhile, imported food treated with banned products is still approved, thus EU citizens are still exposed to banned pesticide residues but through the import chain rather than those used and regulated in the EU. The EU MRLs for pesticides are exceeded in 6.9% of imported agricultural products but in only 1.5% of home-grown products (http://www.efsa.europa.eu/en/press-/news/111108.htm) because Regulation 396/2005 allows pesticide thresholds to be set at different levels for imported products, including the threshold for substances that are no longer authorized in the EU (http://europa.eu/legislation_summaries/food_safety/plant_health_checks/l21289_en.htm).

EU pesticide-use policies are intended to promote environmentally sustainable agriculture (i.e., farmers should protect their crops without using chemicals if possible or combine them with measures that reduce the amounts required). The net result is that EU farmers lose the ability to protect their crops effectively in many cases, and the lower yields need to be compensated by the import of food from outside the EU even though it has been treated with products that are banned in the EU. Because the MRLs for crops grown in EU countries are lower than those for imported crops, EU farmers are prevented from competing with farmers outside the EU because the regulations favor the market for imported foods.

Directive 2009/128/EC establishes a framework for community action to achieve the sustainable use of pesticides: 'Member States shall take all necessary measures to promote low pesticide-input pest management, giving wherever possible priority to non-chemical methods'. In practice, however, this legislation does little to promote nonchemical methods. For example, *Diabrotica virgifera virgifera* is a maize pest that was accidentally introduced into Europe from the USA in the early 1990s, and it now causes substantial yield losses in Central Europe. GE crops provide effective protection against the pest in the USA and additional pesticides are not required. Directive 2009/128/EC advocates the use of such crops because they facilitate nonchemical control methods, and an expert study commissioned by the EU states that '...the main benefits of transgenic varieties are an increased protection of the root system, a decrease of volumes of insecticides being used by farmers in soil treatments, and an easy management for the farmers...'. Even so, EU farmers in affected areas and neighboring regions are obliged to use pesticides because the only GE maize variety authorized for cultivation in the EU is MON810, which is not resistant to *D. virgifera* (http://ec.europa.eu/food/plant/organisms/emergency/final_report_Diabrotica_study.pdf). There is a similar contradiction in Regulation 1107/2009. Insect pheromones and other semiochemicals are widely used in European orchards and vineyards to control insect pests by disrupting mating. However, the regulations for marketing plant protection products apply to all substances regardless of origin and properties, which means that the same lengthy and expensive approval process must be followed for a natural and ubiquitous volatile chemical emitted by female insects to attract males as that required for a highly toxic synthetic pesticide [40]. Dozens of active natural products that are potentially useful in agriculture and that are not harmful to humans or the environment cannot be marketed in the EU due to this legislation.

The MON810 maize variety has been grown in Spain for more than 14 years. During that time there have been no reports of health issues for consumers, resistant pest populations, or of impacts on nontarget organisms [34,41]. However, there has been a significant reduction in pesticide use for borer control as well as economic benefits for farmers (http://www.europabio.org/sites/default/files/position/europabio_socioeconomics_may_2011.pdf). Many other GE varieties with favorable EFSA opinions are mired in the regulatory process, awaiting clearance for commercial cultivation, while known harmful pesticides continue to be used.

Mycotoxin safety levels – moving the goalposts

Mycotoxins are secondary metabolites produced by certain filamentous fungi that infect crops and stored food such as cereals, nuts, spices, dried fruits, apple juice, and coffee [42]. If consumed, these compounds can be acutely toxic to humans (mycotoxicosis) and cause long-term carcinogenic effects [43]. The tolerance levels set for mycotoxins are highly controversial because they imply a compromise between human health and economic factors (http://agecon.ucdavis.edu/people/faculty/roberta-cook/docs/links/LCfood-safettrade03.pdf). In 1997, the EU harmonized the acceptable level of aflatoxin contamination in groundnuts (*Arachis hypogaea*) at 10 ppb for groundnuts intended for further processing and at 4 ppb for cereals intended for direct human consumption. This caused a sudden reduction in imports into some member states and the levels were strongly criticized by many members of the World Trade Organization (WTO) because the measures would create a trade barrier for countries economically dependent on exports to the EU. In response, the thresholds were increased in 1998 to 15 ppb in groundnuts intended for further processing (8 ppb for aflatoxin B1, which is the most toxic mycotoxin) and 4 ppb for foods intended for direct consumption (2 ppb for aflatoxin B1) [44].

The policy instrument used to set the maximum levels for aflatoxins in food is Regulation 165/2010/EC, which has doubled the tolerance threshold to 8 ppb but provides an exemption in that levels are expected to be '. . .as low as can reasonably be achieved. . .' This was justified on the advice of the Scientific Panel on Contaminants in the Food Chain (CONTAM), which stated that exceeding the maximum levels occasionally would have a low overall impact on health. Therefore, standards have been lowered in developed countries that have the technology to detect and thus avoid mycotoxins in food in a cost-effective manner, but have remained stringent for developing countries without these capabilities, blocking their export markets [45]. Furthermore, attempts to meet these demands mean that developing countries export their highest quality food, leaving the poverty stricken domestic population to consume mycotoxin-contaminated food that cannot be exported [46]. The restriction of the market in this manner also results in the EU paying more for higher quality foods [47].

However, GE agriculture could provide a solution to this challenge. For example, Bt maize is resistant to insect pests that cause damage and allow penetration by mycotoxin-producing fungi [48]. MON 810 is the only GE maize variety approved in the EU, but under the proposals for a member state opt-out it could soon disappear from much of the continent. However, to meet its stringent requirements for mycotoxin-free maize, the EU imports the MON 810 GE maize variety as well as other more advanced Bt varieties from other countries [5].

Road to nutrition

One of the major global health challenges is malnutrition. Approximately half the world's population (including 5% of the EU population) suffers from malnutrition, yet the biofortification of crops could provide a method to address this problem and could potentially save lives [49–51]. EU policies on food and nutrition are described in the European Commission White Paper on Food Safety (2000) (http://ec.europa.eu/dgs/health_consumer/library/pub/pub06_en.pdf). The fortification of processed food and agronomic biofortification using nutrient-rich fertilizers has overcome the lower endogenous levels of some nutrients in the UK and Finland [52,53]. However, the biofortification of crops with essential minerals and vitamins by genetic engineering can yield nutritious foods more rapidly and more sustainably by equipping plants with the means to synthesize, absorb, and accumulate nutrients at source [54–57]. The development of Golden Rice, a variety of rice (*Oryza sativa*) enriched with β-carotene [58], multivitamin corn enriched with ascorbate, β-carotene, and folate [59], and folate-biofortified rice [60] are key examples of successful biofortification achieved through EU public sector research [50]. However, because of the regulatory burden on GE crops, no nutritionally enhanced varieties are likely to be grown for consumption in the EU [5,61] or in developing countries that are economically dependent on trade and/or aid from the EU (http://www.adelaide.edu.au/cies/publications/present/CIES_DP1012.pdf).

DNA sequencing has revealed that biotechnology is less disruptive to the genome than conventional plant breeding because the transgene insertions are localized [62]. Transgenic plants with novel traits also resemble the parental variety more closely than those generated by introgression [63]. GE biosafety research in Europe over the past 25 years has cost more than €300 million and can be summarized in one sentence: GE is no more dangerous than crop modification by any other method. This has been confirmed by a recent EU study that revealed no greater risk from the consumption of GE maize than any conventional variety [64] (ftp://ftp.cordis.europa.eu/pub/fp7/kbbe/docs/a-decade-of-eu-funded-gmo-research_en.pdf).

Even with this extensive research to support the safety of GE crops backed up by 15 years of consumer safety in the USA and elsewhere [65], GE varieties must undergo compositional, allergenicity and toxicity testing, molecular characterization, and environmental impact assessments from which conventionally bred varieties are exempt even if they are identical in every way to the GE variety [66]. Effectively, EU regulations do not focus on the product, only on the process [67].

Medical innovation – giving with one hand, taking with the other

In addition to malnutrition, several key diseases prevalent in developing countries are major global health challenges, including HIV/AIDS, tuberculosis, malaria, and rabies. Many people die from these diseases because of the lack of drugs, often reflecting poverty and limited access to medical facilities rather than the availability of those facilities *per se* [68]. The EU has invested in research projects focusing on the development of inexpensive diagnostics, drugs, and vaccines, and the platforms to produce them in developing countries (http://ec.europa.eu/research/health/infectious-diseases/antimicrobial-drug-resistance/pdf/infectious-diseases-leaflet09_en.pdf; http://www.pharma-planta.net/).

GE crops provide an alternative platform to chemical synthesis for the production of pharmaceutical molecules

because they can produce large amounts of biomass that can be scaled up and down as required to meet demand [69–72]. Diverse pharmaceutical products have been produced in plants, including vaccines, antibodies, and enzymes [69,73]. Innovative plant-derived pharmaceuticals include edible vaccines [74], microbicides to prevent the transmission of HIV [75], and recombinant versions of insulin and human growth hormone [76]. The slow adoption of pharmaceuticals produced in whole plants in the field reflects the slow development of the regulatory process, which involves not only the European Medicines Agency (EMA) but also the EFSA, which has a mandate to consider non-food plants as well as those used for food [77]. The cultivation of GE plants for pharmaceutical use needs to meet the requirements stated in Directive 2008/27/EC, which regulates 'the release' of GE plants into the field, and Regulation 1829/2003/EC, which sets out rules governing food and feed products. Finally, plant-derived pharmaceuticals must meet the specific guideline established by the EMA to regulate the production process to determine the final safety of the pharmaceutical product (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003154.pdf).

Similar to the other benefits of GE agriculture, pharmaceutical research is being hindered by the huge investment required at the precompetitive stage so that novel products can negotiate the regulatory pathway beyond the proof-of-principle stage. Drugs that can be produced inexpensively in plants are currently produced in cultivated mammalian cells at great expense [72], beyond the reach of developing countries. The luxury of onerous regulation in the EU is costing lives in the developing world.

Concluding remarks and recommendations

The EU has enacted a series of strategies whose stated aim is to develop the most competitive knowledge-based bioeconomy in the world. So far this has failed. One reason for the disappointing performance is the paradoxical nature of the agricultural policies described in this review, many of which are contradictory, anticompetitive, and actively promote the practices they claim to discourage. In many cases, this is because policies are based on political expediency and short-term economic goals rather than rational scientific evidence and long-term economic models.

To reverse this situation, the EU needs to consider rational principles as the basis for policy development, removing inconsistencies surrounding the cultivation and import of GE crops, and the acceptable levels of pesticide residues and mycotoxins. This would make the EU agricultural industry more competitive and international trade would be harmonized. Most importantly, by rationalizing its policy framework, innovative new crops, drugs, and novel and efficient production methods would come to market more rapidly and the EU would become a leading influence in the use of technology to save lives. Following the current path, the EU faces being left behind technologically, economically, and in terms of its humanitarian policies, to the detriment of the EU population and the rest of the world.

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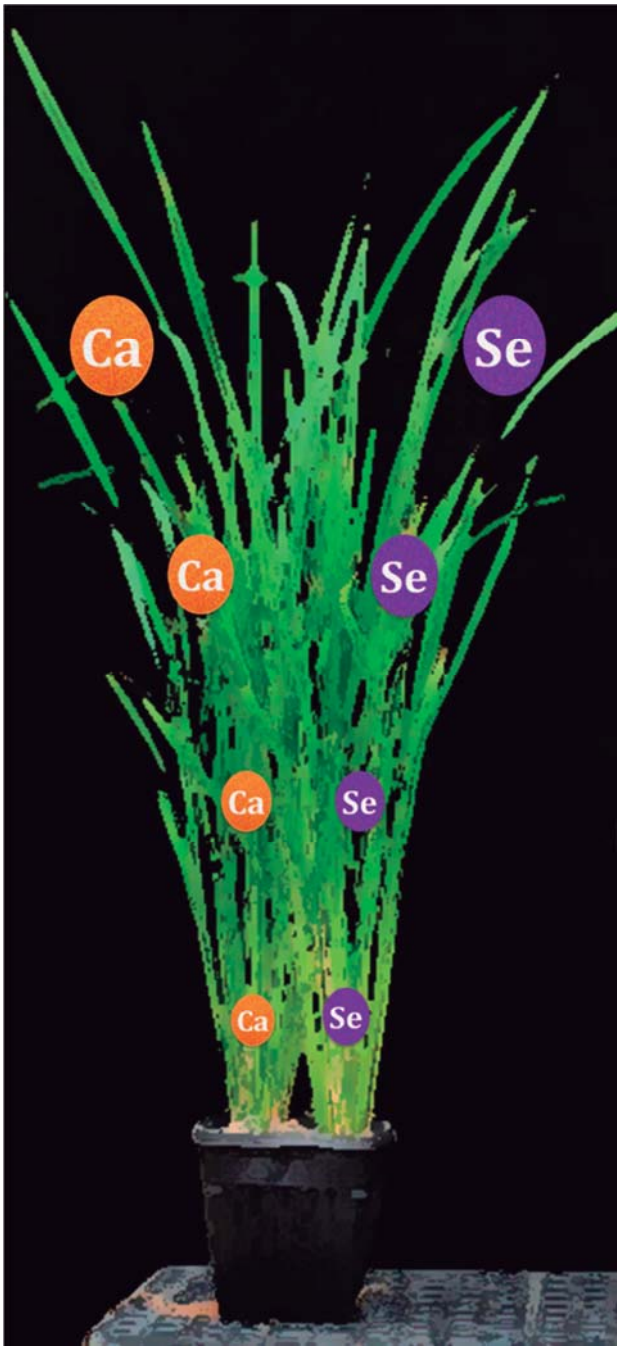

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