Towards engineering of photosynthesis in rice

Gemma Masip Vilà

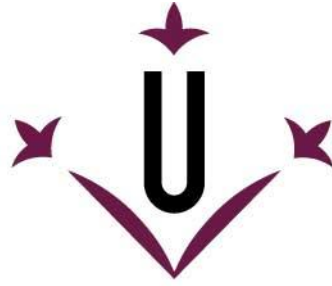**Universitat de Lleida**

Towards engineering of photosynthesis in rice

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Memòria presentada per optar al grau de Doctor per la Universitat de Lleida

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TABLE OF CONTENTS

INDEX OF FIGURES

CHAPTER 4: GENERATION AND MOLECULAR CHARACTERIZATION OF TRANSGENIC RICE PLANTS ENGINEERED WITH CARBONIC ANHYDRASES

CHAPTER 5: PHENOTYPIC ANALYSIS OF TRANSGENIC PLANTS ENGINEERED WITH CARBONIC ANHYDRASES

INDEX OF TABLES

CHAPTER 2: GENERATION AND MOLECULAR CHARACTERIZATION OF TRANSGENIC RICE PLANTS ENGINEERED WITH THE GLYCOLATE CATABOLIC BYPASS PATHWAY

CHAPTER 3: PHENOTYPIC MEASUREMENTS OF TRANSGENIC PLANTS TRANSFORMED WITH THE GLYCOLATE CATABOLIC BYPASS PATHWAY

CHAPTER 4: GENERATION AND MOLECULAR CHARACTERIZATION OF TRANSGENIC RICE PLANTS ENGINEERED WITH CARBONIC ANHYDRASES

CHAPTER 5: PHENOTYPIC ANALYSIS OF TRANSGENIC PLANTS ENGINEERED WITH CARBONIC ANHYDRASES

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SUMMARY

The yields of major crops, such as rice, have reached a plateau and are unlikely to meet the global demand for food, mostly in developing countries. Increasing rice photosynthesis has been identified as an approach to enhance rice yield. In the present study, I used two different strategies to create a population of transgenic plants expressing different combination of genes aiming to increase the capacity of rice to fix carbon dioxide during photosynthesis and thus produce rice with enhanced biomass, which is an important trait related to grain yield.

The *Escherichia coli* glycolate catabolic pathway which converts the glycolate produced during the photorespiration into glycerate has been demonstrated to be effective to improve the biomass of *Arabidopsis thaliana, Solanum tuberosum* and *Camelina sativa.* In this study I created a population of transgenic rice plants expressing the glycolate catabolic pathway to investigate if this pathway might be able to increase the biomass of rice similarly to these plants. I used direct DNA transfer to transform rice embryos with three different combinations of genes encoding the pathway, namely glycolate dehydrogenase (GDH), glyoxylate carboligase (*gcl*) and tartronic semialdehyde reductase (*tsr*). I characterized the transgenic plants molecularly at the mRNA level and I evaluated the physiological effects of the transgenes on the transgenic plants. Two lines that expressed the full glycolate catabolic bypass pathway (all five transgenes) and two lines expressing only GDH had a better overall performance compared to wild type lines in terms of plant height and grain yield. Two of the lines expressing the full glycolate catabolic pathway, one line expressing only GDH and the two lines expressing *gcl* and *tsr*, showed an inferior performance compared to wild type plants, having a smaller leaf area and a lower grain yield. However, further analyses are required to develop a better understanding of the consequences of expressing the glycolate catabolic bypass pathway in rice.

In a series of alternative experiments I introduced a carbon concentrating mechanism (CCM) into rice. I used two carbonic anhydrases (CA) from *Chlamydomonas reinhardtii* (*Cah1* and *Cah3*) in order to increase the amount of CO_2 in the vicinity of RubisCO. Transgenic plants that overexpressed one or two CA were analyzed molecularly and physiologically. I compared the maize *Ubi-1* and the duplicated CaMV *35S* promoters in terms of their ability to drive the expression of the two CA.

Preliminary data suggest that CA might have a positive effect on the performance of the regenerated transgenic rice plants in terms of total biomass.

I conclude that the introduction of the glycolate catabolic bypass pathway or carbonic anhydrases into rice might be a useful approach to increase the yield of rice plants.

RESUMEN

El rendimiento de los cultivos mayoritarios, como el arroz esta llegado a su límite y es probable que no sea suficiente para abastecer la demanda global de alimentos, sobre todo en los países en vías de desarrollo. El incremento de la capacidad fotosintética del arroz, ha sido identificado como un posible método para aumentar su rendimiento. En este estudio, he utilizado dos estrategias diferentes para crear un grupo de plantas transgénicas que expresen combinaciones diferentes de genes, con el objetivo de aumentar la capacidad de fijación del dióxido de carbono del arroz durante la fotosíntesis, para así generar un arroz con un incremento en la biomasa total, siendo el aumento en la producción de grano, uno de los caracteres más importantes.

La vía catabólica del glicolato de la bacteria *Escherichia coli*, que convierte el glicolato producido durante la fotorespiración a glicerato, ha demostrado ser efectiva en incrementar la biomasa en *Arabidopsis thaliana, Solanum tuberosum* y *Camelina sativa.* En este estudio he generado un conjunto de plantas de arroz transgénicas que expresan la ruta catabólica del glicolato, con la finalidad de investigar si esta vía catabólica puede ser capaz de aumentar la biomasa en arroz de forma parecida a las plantas mencionadas anteriormente. Para ello, he utilizado la transferencia directa de ADN para transformar embriones de arroz con tres combinaciones diferentes de genes que codifican para la indicada ruta metabólica; dichos genes son glicolato deshidrogenasa (GDH), glioxilato carboligasa (*gcl*) y tartrónico semialdehido reductasa (*tsr*). He caracterizado la plantas transgénicas a nivel molecular y de expresión (ARNm) y he evaluado el impacto de la expresión de los transgenes en la fisiología de la plantas transgénicas. De las plantas regeneradas, dos líneas que expresaban la ruta metabólica completa (los cinco transgenes) y dos líneas que expresaban solamente GDH, tuvieron un mayor rendimiento al compararlas con las plantas controles no transformadas, demostrado en parámetros de altura y rendimiento del grano. Dos de las líneas que expresaban los tres genes de la ruta catabólica del glicolato, otra línea que expresaba solo GDH y las otras dos líneas que expresan solo *gcl* y *tsr* tuvieron un comportamiento peor al compararlas con las plantas no transformadas controles, teniendo una superficie foliar menor y un rendimiento inferior. No obstante, son necesarios análisis posteriores para desarrollar una mejor comprensión de las consecuencias de la expresión de la ruta catabólica del glicolato en arroz.

En una serie de experimentos alternativos introduje las características de un mecanismo de fijación de carbono (CCM) en la planta de arroz. Utilicé para ello dos enzimas, las anhidrasas carbonicas (CA) de *Chlamydomonas reinhardtii* (*Cah1* y *Cah3*), para aumentar la cantidad de CO_2 en las áreas próximas de RubisCO. Las plantas transgénicas que sobreexpresaban una o dos de las enzimas CA fueron analizadas a nivel molecular y fisiológico. También comparé la eficiencia en la expresión de las enzimas CA al utilizar dos promotores diferentes, el promotor *Ubi-1* del maíz y del promotor duplicado del virus del mosaico de la coliflor (CaMV) *35S*. Los datos preliminares obtenidos sugieren que las enzimas CA puede tener un efecto positivo en el rendimiento de los niveles de biomasa totales de las plantas transgénicas regeneradas.

Concluyo indicando que la introducción de la ruta catabólica del glicolato o de las anhidrasas carbónicas en arroz puede ser un método útil para aumentar el rendimiento de estas plantas.

RESUM

El rendiment dels cultius majoritaris, com l'arròs està arribant al seu límit i és probable que no sigui suficient per proveir la demanda global d'aliments, sobretot en aquells països que es troben en vies de desenvolupament. Incrementar la capacitat fotosintètica de l'arròs, ha estat identificat com un possible mètode per augmentar el rendiment d'aquesta planta. En el present estudi, he utilitzat dues estratègies diferents per crear un grup de plantes transgèniques que expressin combinacions diferents de gens amb l'objectiu d'augmentar la capacitat fixadora de diòxid de carboni de l'arròs durant la fotosíntesi i així generar un arròs que sigui capaç d'augmentar la seva biomassa total, sent l'augment en la producció de gra un dels caràcters més importants.

La via catabòlica del glicolat del bacteri *Escherichia coli*, que converteix el glicolat produït durant la fotorespiració en glicerat, ha demostrat ser efectiva en augmentar la biomassa en *Arabidopsis thaliana*, *Solanum tuberosum* i *Camelina sativa*. En aquest estudi he regenerat un conjunt de plantes d'arròs transgèniques que expressen la ruta catabòlica del glicolat amb la finalitat d'investigar si aquesta via catabòlica pot ser capaç d'augmentar la biomassa en arròs, de forma semblant a les plantes esmentades anteriorment. He utilitzat la transferència directa d'ADN per transformar embrions d'arròs amb tres combinacions diferents de gens que codifiquen per a la indicada ruta metabòlica, els quals són el glicolat deshidrogenasa (GDH), el glioxilat carbolligasa (*gcl*) i el tartrónic semialdehid reductasa (*tsr*). He caracteritzat les plantes transgèniques regenerades a nivell molecular i d'expressió dels transgens (ARNm) i he avaluat l'impacte de l'expressió d'aquests en la fisiologia de la plantes transgèniques. De les plantes regenerades, dues línies que expressaven la ruta metabòlica completa (els cinc transgens) i dues línies que expressaven només GDH, van mostrar un major rendiment pel que fa a l'alçada i el nombre de grans, al comparar-les amb les plantes controls no transformades. Dues de les línies que expressaven els tres gens de la ruta catabòlica del glicolat, una línia que expressava només GDH i les altres dues línies que expressen només *gcl* i *tsr* van mostrar un comportament contrari a l'esperat al comparar-les amb les plantes no transformades controls, tenint una superfície foliar menor i un rendiment inferior. No obstant això, seria necessari realitzar anàlisis posteriors per adquirir una millor comprensió de les conseqüències de l'expressió de la ruta catabòlica del glicolat en arròs.

En una sèrie d'experiments alternatius vaig introduir les característiques d'un mecanisme de fixació de carboni (CCM) a les plantes d'arròs. Vaig utilitzar per a això dos enzims, les anhidrases carboniques (CA) de *Chlamydomonas reinhardtii* (*Cah1* i *Cah3*), per augmentar la quantitat de CO_2 en les àrees pròximes a RubisCO. Les plantes transgèniques que sobreexpresaven un o dos dels enzims CA van ser analitzades a nivell molecular i fisiològic. També vaig comparar l'eficiència en l'expressió dels enzims CA a l'utilitzar dos promotors diferents, el promotor *Ubi-1* del blat de moro i el promotor duplicat del virus del mosaic de la coliflor (CAMV) *35S*. Les dades preliminars obtingudes suggereixen que els enzims CA poden tenir un efecte positiu en el rendiment dels nivells de biomassa totals de les plantes transgèniques regenerades.

Concloc la meva tesi indicant que la introducció de la ruta catabòlica del glicolat o de les anhidrases carbòniques en l'arròs pot ser un mètode útil per augmentar el rendiment d'aquestes plantes.

LIST OF ABREVIATIONS

2,4-D: 2,4-Dichlorophenoxyacetic acid

µg: micrograms

3-PGA: 3-phosphoglycerate

*A. thaliana***:** *Arabidopsis thaliana*

ATP: Adenosine triphosphate

bp: base pair(s)

BSA: Bovine serum albumin

BSC: Bundle sheath cell

C_2-cycle: Photorespiratory cycle (2 Carbon cycle)

C_3-cycle: Calvin Benson cycle (3 Carbon cycle)

C_4-cycle: C_4-like CO_2 assimilation pathway

CA: Carbonic anhydrase

CAM: Crassulacean acid metabolism

CaMV *35S*: Cauliflower Mosaic Virus *35S*

CAT: Catalase

CCM: Carbon concentrating mechanism

cDNA: Complementary DNA

C_i: Inorganic carbon

CoA: Coenzyme A

DNA: Deoxyribonucleic acid

DW: Dry weight

EDTA: Ethylene diamine tetraacetic acid

F-6-P: Fructose-6-phosphate

FW: Fresh weigh

G-3-P: Glyceraldehyde-3-phosphate

*gcl***:** Glyoxylate carboxyligase

GDC: Glycine decarboxylase

GDC/SHMT: Glycine decarboxylase/serine hydroxymethyl transferase

GDH: Glycolate dehydrogenase

GGAT: Glutamate-glyoxylate amino transferase

GK: Glycerate kinase

*glcD***:** Coding sequence for the D subunit of glycolate oxidase in *E. coli*

glcE: Coding sequence for the E subunit of glycolate oxidase in *E. coli*

glcF: Coding sequence for the F subunit of glycolate oxidase in *E. coli*

GLYK: Glycerate kinase

GO: Glycolate oxidase

GOGAT: Glutamate-glyoxylate aminotransferase

GOPOD: Glucose oxidase/peroxidase

GOX: Glycolate oxidase

GS: Glutamine synthetase

GSC: Glycine decarboxylase

h: Hour

HCO_3^-: Bicarbonate

HEPES: N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

HPR: Peroxisomal hydroxypyruvate reductase

hpt: Hygromycin phosphotransferase

l: litre

MC: Mesophyll cell

MDH: Malate dehydrogenase

ME: Malic enzyme

mg: milligrams

min: Minutes

mL: Milliliter

MOPS: (N-morpholino) propane sulfonic acid

mRNA: Messenger RNA

MS: Murashige and Skoog

MSO: Osmoticum MS media

MSR: Regeneration MS media

MSS: Selection MS media

NAD+/NADH: Nicotinamide adenine dinucleotide (oxidized/reduced form)

NAD-ME: NAD-malic enzyme

NADPH: Nicotinamide adenine dinucleotide phosphate

OAA: Oxalo acetic acid

Os: *Oryza sativa* (rice)

PCK: Phosphoenolpyruvate carboxykinase

PEP: Phosphoenolpyruvate

PEPC: Phosphoenolpyruvate carboxylase

PG: Phosphoglycolate

PGA: Phosphoglycerate

PGP: Phosphoglycolate phosphatase

PPDK: Pyruvate-orthophosphate dikinase

PYR: Pyruvate

RNA: Ribonucleic acid

RT: Room temperature

RT-PCR: Reverse transcriptase-polymerase chain reaction

RubisCO: Ribulose-1,5-bisphosphates carboxylase/oxygenase

RuBP: Ribulose-1,5-bisphosphate

s: Second

SDS: Sodium dodecil sulfate

SGAT: Serine Glutamate aminotransferase

SHMT: Serine hydroxymethyl transferase

SS: Serine synthase

tsr: Tartronic semialdehyde reductase

U: unit

Ubi-1: *Ubiquitin-1*

WT: Wild type

CHAPTER 1

General introduction

CHAPTER 1: GENERAL INTRODUCTION

1.1 Global population growth and food security

Food security is in place "when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life" (World Food Summit 1996). However, ensuring complete food and nutrient security to all populations has become increasingly challenging (Singh et al. 2014). The required increase in agricultural productivity required by 2050 ranges between 100-110% compared with 2005 levels (Tilman et al. 2011). Increasing grain yield is crucial to meet the demand of feeding an increasing global population. However, yield increases in major crops such as rice in recent years have been very limited (Zhu et al. 2010) and it is possible that actual crop yield is approaching the ceiling of maximum yield potential (Tilman et al. 2002). One fundamental component of plant productivity to increase yield is photosynthesis. The time is opportune to employ our extensive knowledge of this fundamental process for improving crop productivity (Raines 2011).

1.2 Photosynthesis

Photosynthesis is a fundamental chemical process that plants, algae, and cyanobacteria utilize to capture energy from sunlight and transform it into biochemical energy (Liu et al. 2013). Photosynthetic organisms convert CO_2 to organic material by reducing it to carbohydrates in a complex set of reactions, which also release oxygen to the atmosphere (Edwards and Walker 1983). Photosynthesis is carried out in chloroplasts. Chloroplasts have an inner and an outer membrane containing the liquid stroma. Inside the stroma there are stacks of thylakoids, known as grana, which contain the proteins responsible for light reactions and subsequently, where photosynthesis takes place (Soll and Schleiff 2004) (**Figure 1.1**). The photosynthetic process comprises two reactions, the light and the light-independent reactions. The light reaction is carried out in the thylakoid membrane where pigments, mainly chlorophylls and carotenoids convert the energy of sunlight or other wavelengths to chemical energy. The energy harvested via the light reaction is stored in the form of ATP and NADPH molecules which will be used subsequently for the formation of carbohydrates during the light-independent

reactions. The conversion of carbon dioxide into sugars, known as carbon fixation, takes place during the light-independent (Lodish et al. 2000).
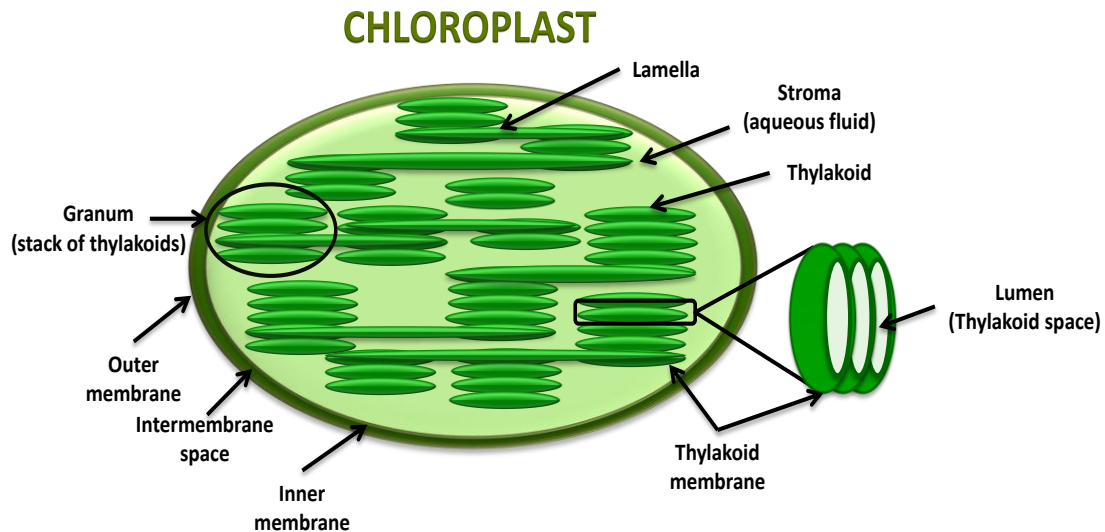
Figure 1.1-Structure of the chloroplast. Chloroplasts are surrounded by a double membrane. Inside this membrane, there is an aqueous mix of enzymes referred to as stroma. Enclosed in the stroma there is a complex network of stacks. Each stack is a granum and each of the filamented sacs which form a granum is a thylakoid. Each thylakoid is composed of a series of photosynthetic and associated proteins which carry out the photosynthetic process. Figure adapted from Lodish et al. (2000).

The starting point of the carbon fixation process involves the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). This enzyme has a dual function, being able to catalyze the fixation of both CO_2 and O_2 (Peterhänsel et al. 2010). The subsequent biochemical reaction cycle following CO_2 fixation leads to the production of glyceraldehyde-3-phosphate (G-3-P) in the Calvin-Benson cycle of C_3 photosynthesis, whereas the O_2 fixation results in the production of a toxic 2-phosphoglycolate (2-PG) molecule, which should be metabolized by photorespiration at the cost of energy and loss of CO_2 that has already been fixed (Liu et al. 2013). The probability with which RubisCO reacts with oxygen and/or CO_2 depends on the relative concentration of the two gases at the site of the reaction (Leegood et al. 2006). Although CO_2 is preferred, as the concentration is lower than that of oxygen, photorespiration occurs at significant levels (Leegood et al. 2006).

There are three biochemical pathways involved in CO_2 assimilation in higher plants: the C_3 pathway (Calvin cycle), the C_4 pathway, and the crassulacean acid metabolism (CAM) (Ehlringer and Monson 1993).

1.2.1 C_3 photosynthesis

The vast majority of terrestrial plants, including crops such as rice, wheat and oat, assimilate carbon dioxide via the C_3 photosynthetic pathway, and are known as C_3 plants (Ku et al. 1996). **Figure 1.2** shows the CO_2 assimilation pathway in C_3 plants, known as the Calvin cycle. The Calvin cycle is divided into three phases: carbon fixation, reduction, and regeneration. The first stage of the Calvin cycle, CO_2 fixation, starts when one molecule of CO_2 reacts with a five-carbon molecule, ribulose bisphosphate (RuBP), producing an unstable six-carbon intermediate that immediately breaks down into two molecules of the three-carbon compound phosphoglycerate (3-PGA), hence the name C_3 photosynthesis (Sharwood and Whitney 2014). 3-PGA formed by this reaction is used during the reduction phase to form the triose phosphates glyceraldehyde phosphate (G-3-P) and dihydroxyacetone phosphate via two reactions that consume ATP and NADHP produced during the light-reactions. The G-3-P enters the regeneration phase to produce fructose-6-phosphate (F-6-P). Different carbohydrates can be synthesized from F-6-P. In addition, F-6-P and G-3-P are used in a series of reactions to regenerate RuBP, the first compound of the Calvin cycle (Raines 2011).

The CALVIN CYCLE
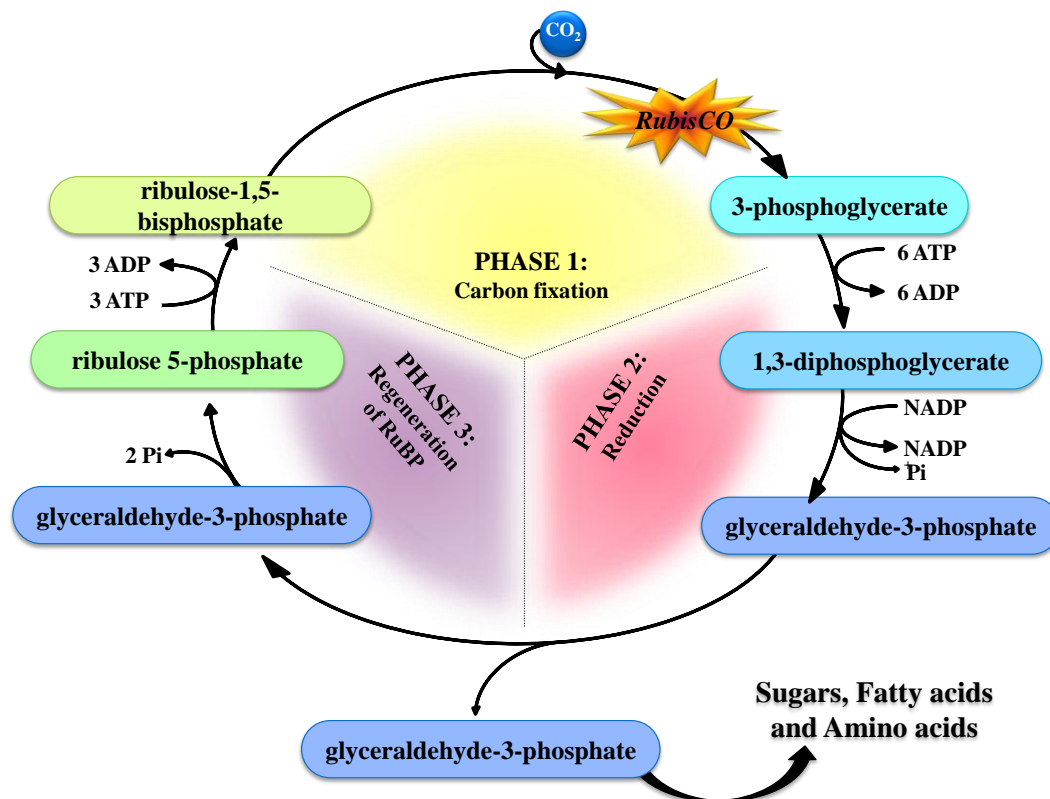
Figure 1.2-**Schematic representation of the Calvin cycle.** The decarboxylation reaction of RubisCO yields two molecules of 3-phosphoglycerate. This compound is fixed and recycled to ribulose-1,5-bisphosphate (RuBP) in a series of reactions that constitute the Calvin cycle. Fixation of six molecules of CO_2 involves twelve molecules of NADPH and eighteen molecules of ATP. In chloroplasts, CO_2 condenses with RuBP to form two molecules of 3-phosphoglycerate (3-PGA). 3-PGA is then reduced to triose phosphate consuming ATP and NADPH. The triose phosphate is then either utilized to regenerate ribulose-1,5-bisphosphate, to synthesize starch within chloroplasts or is transported into the cytosol for sucrose biosynthesis. Adapted from Lodish et al. (2000).

1.2.2 Photorespiration

Two connected pathways comprise the plant photosynthetic carbon metabolism: the reductive photosynthetic carbon metabolism, also known as the C_3 or Calvin cycle, and the oxidative photosynthetic carbon metabolism, also known as the C_2 cycle or photorespiratory pathway (Maurino and Peterhänsel 2010).

Photorespiration begins when O_2 is substituted for CO_2 in the first reaction of photosynthetic CO_2 fixation, which is catalysed by RubisCO. This substitution produces the toxic compound phosphoglycolate (2PG), which is recycled to 3-PGA.

The recycling of 2PG requires eight different enzymes in the core photorespiratory pathway. In the chloroplast, 2PG is hydrolyzed to glycolate by 2PG phosphatase (PGP). Glycolate moves out of the chloroplast through a poorly characterized glycolate-glycerate antiporter and enters the peroxisome via porin-like channels (Peterhänsel et al., 2010). Once in the peroxisome, glycolate oxidase (GOX) consumes glycolate and O_2 to produce glyoxylate and H_2O_2 in a reaction catalyzed by catalase (CAT) (Bauwe et al. 2010). Glyoxylate is then transaminated to glycine in a reaction involving glutamate by the enzyme glutamate-glyoxylate amino transferase (GGAT). The resulting glycine is transported to the mitochondria where it is decarboxylated to form the amino acid serine. The decarboxylation of glycine is mediated by the combined action of the mitochondrial glycine decarboxylase/serine hydroxymethyl transferase (GDC/SHMAT) (Oliver 1994). The reaction of GDC/SHMAT results in the release of one molecule of CO_2 and one molecule of NH_3. The NADH-dependent hydroxypyruvate reductase (HPR) reduces hydroxy-pyruvate to glycerate. Finally, glycerate is transported to the chloroplast where it is dephosphorylated to phosphoglycerate by glycerate kinase (GK) (Raghavendra et al. 1998).

The conversion of glycine to serine in the photorespiratory pathway is probably the most important process that liberates ammonia (Singh 1998). This compound rapidly diffuses to the chloroplast, where it is used by glutamine synthetase (GS) catalyzing the ATP-dependent conversion of glutamate to glutamine (Foyer 1997). The ferredoxin-dependent glutamate oxoglutarate aminotransferase (GOGAT), which is localized in the chloroplast, catalyzes the conversion of glutamine and 2-oxoglutarate to two molecules of glutamate. One molecule of glutamate is exported to the peroxisomes as an amino donor for GGAT in exchange for 2-oxoglutarate (Leegood et al. 2006). Thus the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway that re-fixes ammonia consumes both ATP and reducing power. This also can be added to the energetic cost of photorespiration. The same pathway also provides the amino groups necessary for the transamination of glyoxylate in the peroxisomes (Sharkey 2001) (**Figure 1.3).**
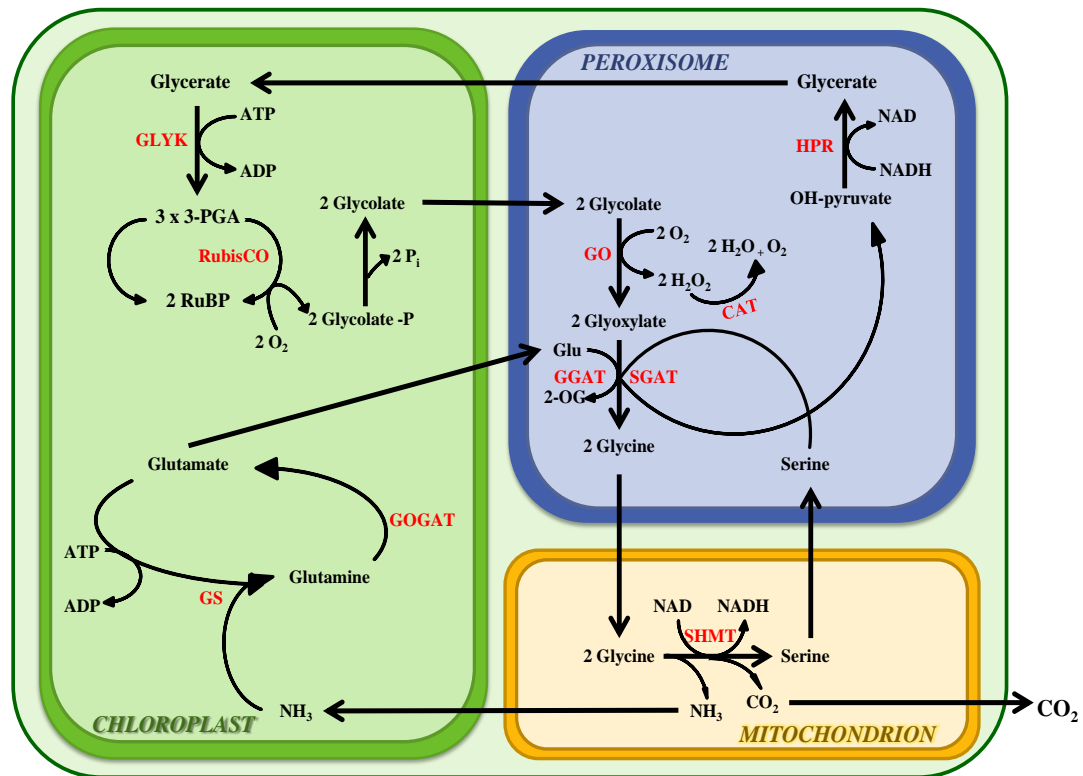
Figure 1.3-The photorespiratory pathway in C₃ plants. The photorespiratory carbon and nitrogen cycle and the enzymes distributed between chloroplasts, peroxisomes and cytosol**. CAT:** catalase; **GSC:** glycine decarboxylase; **GGAT**: glutamate:glyoxylate aminotransferase; **GLYK:** glycerate kinase; **GO:** glycolate oxidase; **GOGAT:** glutamate:oxoglutarate aminotransferase; **GS:** glutamine synthase; **HPR:** peroxisomal hydroxypyruvate reductase; **RubisCO:** ribulose-1,5-bisphosphates carboxylase/oxygenase; **RuBP:** ribulose-1,5-bisphosphate; **SGAT:** serine-glutamate aminotransferase; **SHMT:** serine hydroxymethyl transferase; **3-PGA:** 3-phosphoglycerate. Adapted from Maurino and Peterhänsel (2010).

RubisCO evolved 3 billion years ago when the concentration of CO_2 in the atmosphere was much higher than it is now and the concentration of oxygen was almost zero, thus oxygenation activity was very low (Fernie et al. 2012). Atmospheric concentrations of oxygen are much higher now and it supports the RubisCO oxygenase activity and this increases when the temperature rises, causing a high rate of photorespiration (Peterhänsel et al. 2010). In photorespiration, oxygenation is a wasteful side reaction of RubisCO in many ways as it uses active site of carboxylation, consumes RuBP, and the recovery of carbon in phosphoglycolate consumes ATP and reducing equivalents while releasing previously fixed CO_2 (Sage 2001).

1.2.3 C$_4$ photosynthesis

Compared to C$_3$ plants, C$_4$ species are relatively few; about 7,500 species of the 250,000 higher plant species use the C$_4$ photosynthetic pathway. Although fewer in number, they account for approximately a quarter of the primary productivity on the planet, and dominate the grassland and savannah biomes of warm-temperate to tropical latitudes (Sage and Zhu 2011).

C$_4$ photosynthesis is the most efficient form of photosynthesis due to its ability to concentrate CO$_2$ around RubisCO and thus suppresses ribulose 1,5-bisphosphate (RuBP) oxygenation and photorespiration (Sage and Zhu 2011). The CO$_2$ concentrating steps are spatially separated and require the coordination of two morphologically distinct cell types: bundle sheath (BSC) and mesophyll cells (MC) (Langdale 2011). These cells are arranged in layers concentrically around the vascular tissue. BSC constitutes the inner layer and the MC forms the outer layer. This arrangement of cells is known as the Kranz anatomy and is illustrated in **Figure 1.4** (Hatch 1992).

Mesophyll cells carry out the initial steps of CO$_2$ fixation using phosphoenolpyruvate carboxylase (PEPC) producing a 4-carbon organic acid, oxaloacetate (rather than a 3 carbon sugar phosphate in C$_3$ plants), hence the designation C$_4$ photosynthesis. Oxaloacetate (OOA) is then converted to either malate or aspartate, which diffuse to BSC. There, the four-carbon acid is decarboxylated and the resulting CO$_2$ is fixed by RubisCO. Pyruvate (PYR), a 3-carbon product, returns to mesophyll cells to be recycled to phosphoenolpyruvate (PEP) for the carboxylation reaction (Furbank 2011).
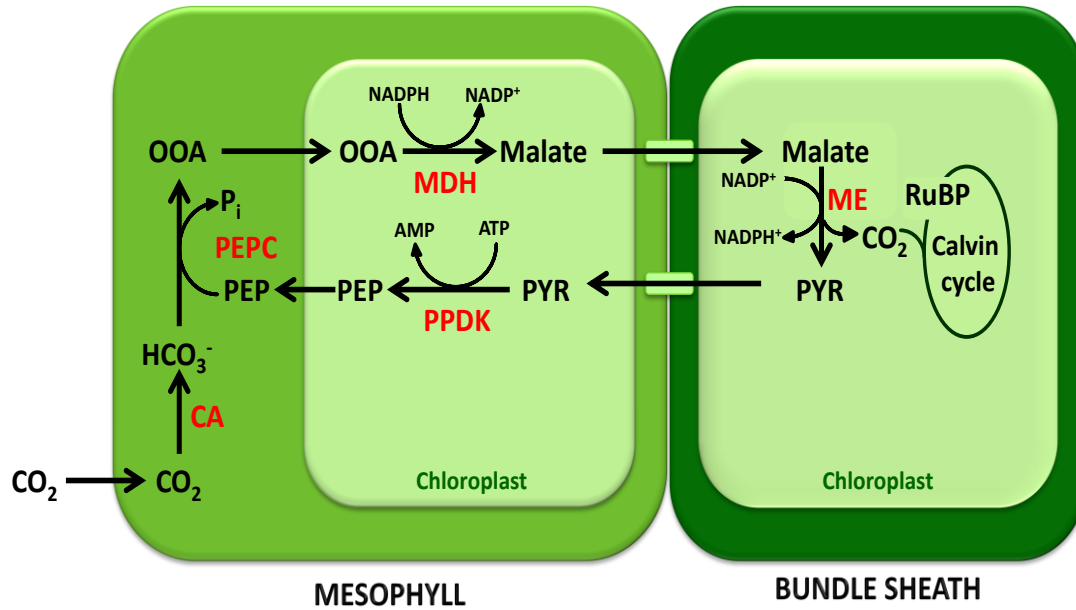
Figure 1.4-Schematic representation of C₄ photosynthesis. CO_2 enters into the MC cytosol where it will be converted into HCO_3^-. Then, HCO_3^- reacts with PEP, forming the 3 carbon compound oxaloacetate that diffuses into the MC chloroplast. OOA is converted into malate in the MC. Malate diffuses into the chloroplast of the BSC where it is decarboxylated to form pyruvate and CO_2, which is released in the vicinity of RubisCO. Pyruvate diffuses back to the MC chloroplast where it is converted into phosphoenolpyruvate, to be used in a new cycle of CO_2 fixation. The released CO_2 in the BSC chloroplast is used for carbohydrate synthesis through the Calvin cycle. **MC:** mesophyll cell; **BSC:** bundle sheath cell; **CA:** carbonic anhydrase, **PEP:** Phosphoenol pyruvate; **PEPC:** phosphoenolpyruvate carboxylase, **OOA:** oxaloacetat, **MDH:** Malate dehydrogenase, **ME:** malic enzyme, **PYR:** pyruvate; **PPDK:** pyruvate orthophosphate dikinase. Adapted from Kajala et al. (2011).

1.2.4 Single cell C₄ photosynthesis

Most terrestrial C₄ plants exhibit "Kranz anatomy" to concentrate CO_2 around RubisCO (Sage 2004). However, recent studies demonstrated that some terrestrial and aquatic plants perform C4 photosynthesis without any segregation between the two cells types (Edwards et al. 2004).

Hydrilla verticilliata is by far the best documented aquatic monocotyledonous plant with a facultative C₄ photosynthesis. Hydrilla is able to shift from C₃ to C₄ photosynthesis when the concentration of CO_2 is low (Edwards et al. 2004). This monocot typically performs C₃ photosynthesis; however, when the CO_2 concentration is low a C₄-based CO_2-concentrating mechanism is induced (Rao et al. 2002). As CO_2 enters into the leaf, cytosolic carbonic anhydrase converts it to bicarbonate which is then fixed by PEPC to produce OOA which is then imported into the chloroplasts, where it is reduced to malate by MDH utilizing NADPH. Malate is then decarboxylated

by NADP-ME to generate CO_2, pyruvate and NADPH. Pyruvate is converted to PEP by PPDK and PEP is exported to the cytosol to serve as substrate for PEPC (Leegood 2002) (**Figure 1.5**).
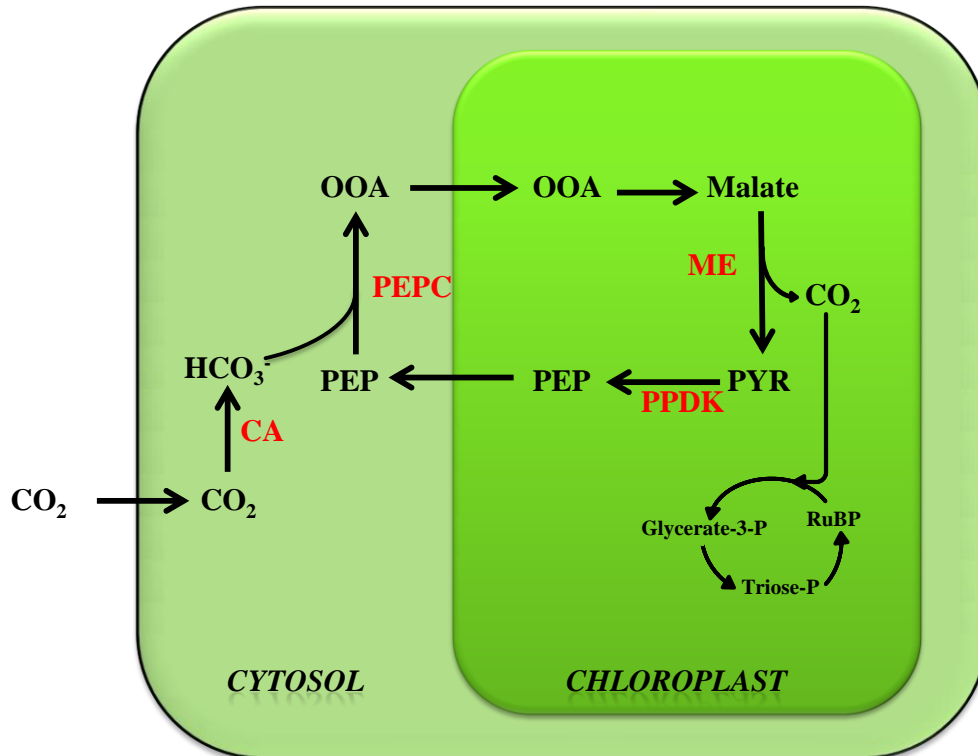
Figure 1.5-The C$_4$-like CO$_2$-concentrating mechanism in *Hydrilla verticillata*. PEP carboxylase (PEPC) in the cytosol fixes bicarbonate, presumably generated from CO_2 by carbonic anhydrase (CA), and produces malate, which is decarboxylated in the chloroplast by malic enzyme (ME) and the released CO_2 is fixed by RubisCO by the Calvin cycle. PEP is regenerated from pyruvate by pyruvate P$_i$ dikinase (PPDK). **CA**: carbonic anhydrase; **PEPC:** phosphoenolpyruvate carboxylase; **OOA:** oxaloacetat; **ME**: malic enzyme; **PYR:** pyruvate; **PPDK:** pyruvate orthophosphate dikinase; **PEP**: Phosphoenol pyruvate. Adapted from Leegood (2002).

1.2.5 Crassulacean acid metabolism (CAM)

Crassulacean acid metabolism (CAM) is an evolutionary adaptation to elevate CO_2 concentration around RubisCO and thus suppress photorespiration (Winter et al. 2015). In CAM, the CO_2-concentrating mechanism is achieved in two phases separated in time. The first phase process starts at night, when chloroplast-containing cells fix CO_2 using PEP and synthesizing phosphoenolpyruvate carboxylase (PEPC) located in the cytosol (Cushman 2001). CO_2 fixation via PEPC at night is exclusive to CAM, as are the fixation of CO_2 and the accumulation of malic acid (Winter et al. 2015). The malate accumulated is stored in the vacuoles. The second phase takes place during the day

while the stomata are closed, when the decarboxylation of malate generates pyruvate and liberates high intercellular [CO_2], creating an internal CO_2 source that is re-assimilated by RubisCO in the chloroplast via the Calvin cycle (Nimmo 2000). Pyruvate is then transported back to the chloroplast where it is converted to PEP by PPDK. Finally, PEP is converted to 3-PGA for carbohydrate biosynthesis. This process minimizes water loss in the middle of the day when evaporating demand is highest (Winter et al. 2015) (**Figure 1.6**).
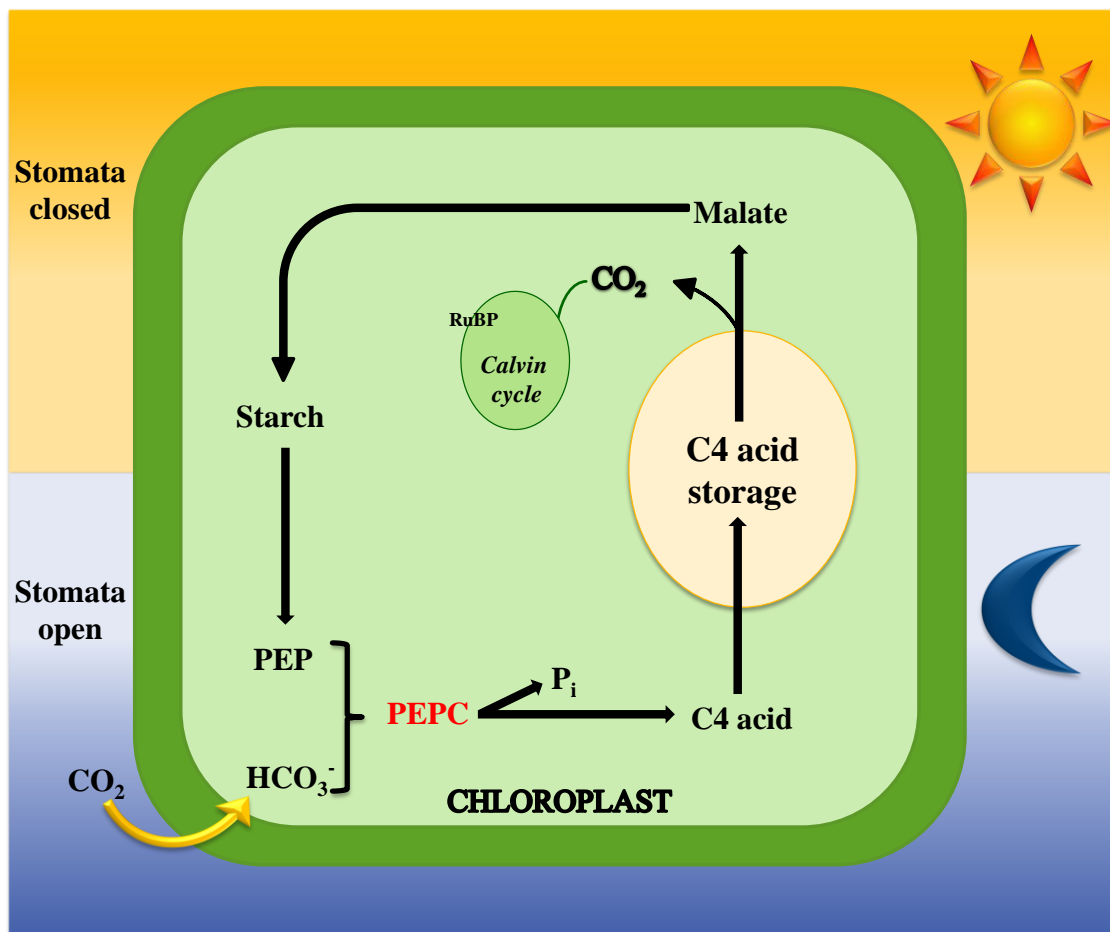
Figure 1.6-The Crassulacean acid metabolism. Crassulacean acid metabolism (CAM) bears similarities to C_4 photosynthesis; however, the division of labor is temporal rather than between cell types: PEP-carboxylase (PEPC) activity occurs at night, when cooler conditions mean less water is lost via open stomata. Malate produced via PEP carboxylation is stored in the vacuole until daytime, when a decarboxylase and RubisCO become active and the stored malate is converted to CO_2 and pyruvate. The released CO_2 is carboxylated by RubisCO in the Calvin cycle, while pyruvate is converted to starch. Stomata are closed during the day, greatly reducing water loss and allowing plants to survive in extremely water-limiting conditions. Adapted from Sage and Stata (2015).

1.3 Approaches to enhance photosynthesis in C_3 plants by introducing C_4 enzymes

A number of strategies have been put forward to improve the inefficiency of C_3 plants. These include: improving the specificity of RubisCO, modifying photorespiration or introducing CO_2-concentrating mechanisms (Leegood 2012).

Experimental evidence suggests that it is unlikely to engineer RubisCO for increased carboxylation efficiency under current environmental conditions (Zhu et al. 2004). Ongoing research to increase the CO_2/O_2 specificity of RubisCO by altering its active site or by inserting more efficient RubisCO genes into higher plants have not been successful so far (Parry et al. 2013).

Transferring C_4 traits into C_3 plants in order to improve their photosynthetic efficiency has been the most common strategy to increase the supply of CO_2 for RubisCO (Häusler et al. 2002, Leegood 2002, Kajala 2011). Hence, individual or multiple enzymes (PEPC, PPDK, ME and MDH) of the C_4 pathway have been overexpressed in different C_3 plants (Ruan et al. 2012). For example, the introduction of maize PEPC in rice did not contribute significantly to CO_2 fixation; rather, it slightly lowered the CO_2 assimilation rate by ca: 7.3% (Suzuki et al. 2006). Ku et al. (2001) introduced maize PEPC and PPDK into rice obtaining photosynthetic rates up to 35% higher than wild type plants. However, other attempts to introduce the same enzymes into rice did not result in yield enhancement (Zhang et al. 2008, Matsuoka et al. 2000). When the full C_4 pathway was introduced into rice the plants exhibited slight stunting (Taniguchi et al. 2008). Unfortunately, most attempts have not produced promising results, and it is uncertain whether this approach will be sufficient to improve photosynthetic rates (Ruan et al. 2012). Overexpression of sedoheptulose-bisphosphatase (SBPase), an enzyme related with the regeneration of sugars during the Calvin cycle, in tobacco and rice showed improved photosynthesis and accumulation of biomass (Tamoi et al. 2006). Expression of maize PEPC in tobacco increased PEPC activity compared to wild type plants. However, transcript and protein levels were very low (Hudspeth et al. 1192). When, the same gene was expressed in rice it showed reduction in oxygen inhibition of photosynthesis and photosynthetic rate was reduced as well (Ku et al. 1999). When maize PEPC and PPDK, sorghum MDH, and rice ME were overexpressed in rice plants, the results showed that there was only slight CO_2 assimilation rate (Taniguchi et al. 2008).

More recent studies focused on the catalytic properties of RubisCO across different species, which provides a repertoire of information that could be used to improve RubisCO in C_3 crops. For example, it is expected that the substitution of C_3 RubisCO by its orthologue from some red algae would increase the catalytic properties of the enzyme. This is due to the fact that algal RubisCOs have CO_2/O_2 ratio specificity while maintaining adequate carboxylation rate and CO_2 affinity (Singh et al. 2014; Parry et al. 2013). However, the problem is that RubisCO is a complex protein, composed of a large and a small subunit. The large subunit is encoded in chloroplast DNA and the small subunit in the nucleus (Portis 2001). The small subunit is synthesized in the cytoplasm, processed and transported to the chloroplast, where it binds with the large subunit (Leegood 2013; Pessarakli 2005).The assembly and further posttranslational modifications of RubisCO are not completely understood (Whitney et al. 2011).

The structure in the leaves of the two different cell types, the Kranz anatomy in C_4 plants makes it difficult to introduce the C_4 pathway into C_3 plants. C_4 plants have a smaller interveinal distance between mesophyll and bundle sheath cells in the leaves, so metabolite transport might be compromised in C_3 plants (Leegood 2012). It is also possible that there could be limitations imposed by the C_3 transporters on transport of intermediates of the C_4 cycle between the cytosol and the chloroplast (Leegood 2001). In C_3 plants, all photosynthetic enzymes are confined in MC, while in C_4 plants they are localized in MC and/or BSC. The venation system of C_4 plants is extensive, and BCS surround each vein and an outer ring of MC surrounds the bundle sheath. The fact that the mechanisms causing the development and organization of the cells are not completely understood makes it challenging to predict the changes that are need to convert a C_3 to the typical C_4 leaf anatomy (Gowik and Westhoff 2011)

Matsuoka et al. (2001) suggested that it might be easier to over-express enzymes of the C_4 pathway either individually or in concert, than it is to introduce the complex Kranz leaf anatomy of C_4 photosynthesis, with its division of labor between thin-walled mesophyll cells surrounding the thick-walled chlorenchymatous bundle-sheath.For these reasons, considerable interest has focused on engineering single-celled C_4-type CO_2-concentrating mechanism such as that found in the aquatic plant *Hydrilla verticillata* in C_3 plants (Leegood 2002).

1.4 Introduction of photorespiratory bypass pathways into C_3 plants

The negative view of photorespiration changed when it was recognized as an essential element of the primary carbon metabolism that interacts with many other pathways. Photorespiration is essential for the survival of all organisms that use RubisCO for CO_2 fixation (Maurino and Peterhänsel 2010). Due to the fact that photorespiration is an essential biological process, three different bypasses for the reduction of photorespiratory losses by genetic engineering were suggested (Peterhänsel et al. 2012) (**Figure 1.7**).

The first bypass pathway starts with glycolate being oxidized by a glycolate dehydrogenase from *Escherichia coli* to produce glyoxylate. Then the acitivity of glyoxylate carboligase also derived from *E. coli* produces tartronic semialdehyde, releasing CO_2 in the vicinity of RubisCO. The product of the decarboxylation reaction is then reduced to glycerate by tartronic semialdehyde reductase. The major difference of this bypass pathway is that ammonia release is avoided and therefore re-fixation of NH_3 is not required (Peterhänsel et al. 2012; Kebeish et al. 2007; Xin et al. 2015).

The second bypass pathway also starts with glyoxylate which is decarboxylated in the peroxisome by glyoxylate carboligase, as in the first pathway (Carvalho et al. 2012). The resulting tartronic semialdehyde is fed back into photorespiration by an isomerase (HPR). As it happens with the first bypass, ammonia release is also avoided.

The third bypass completely oxidizes glycolate to CO_2 by a combination of endogenous and heterologous enzymes. Glycolate oxidation in this pathway is catalyzed by a plant glycolate oxidase that was relocated from the peroxisome to the chloroplast. An additional catalase is needed as this enzyme produces equimolar amounts of H_2O_2 during glycolate oxidation. Together with acetyl-CoA, glyoxylate resulting from glycolate oxidation is converted to malate by malate synthase. The remainder part of the pathway is catalyzed by endogenous enzymes which shift CO_2 release reactions from the mitochondria to the chloroplast (Maier et al. 2012).
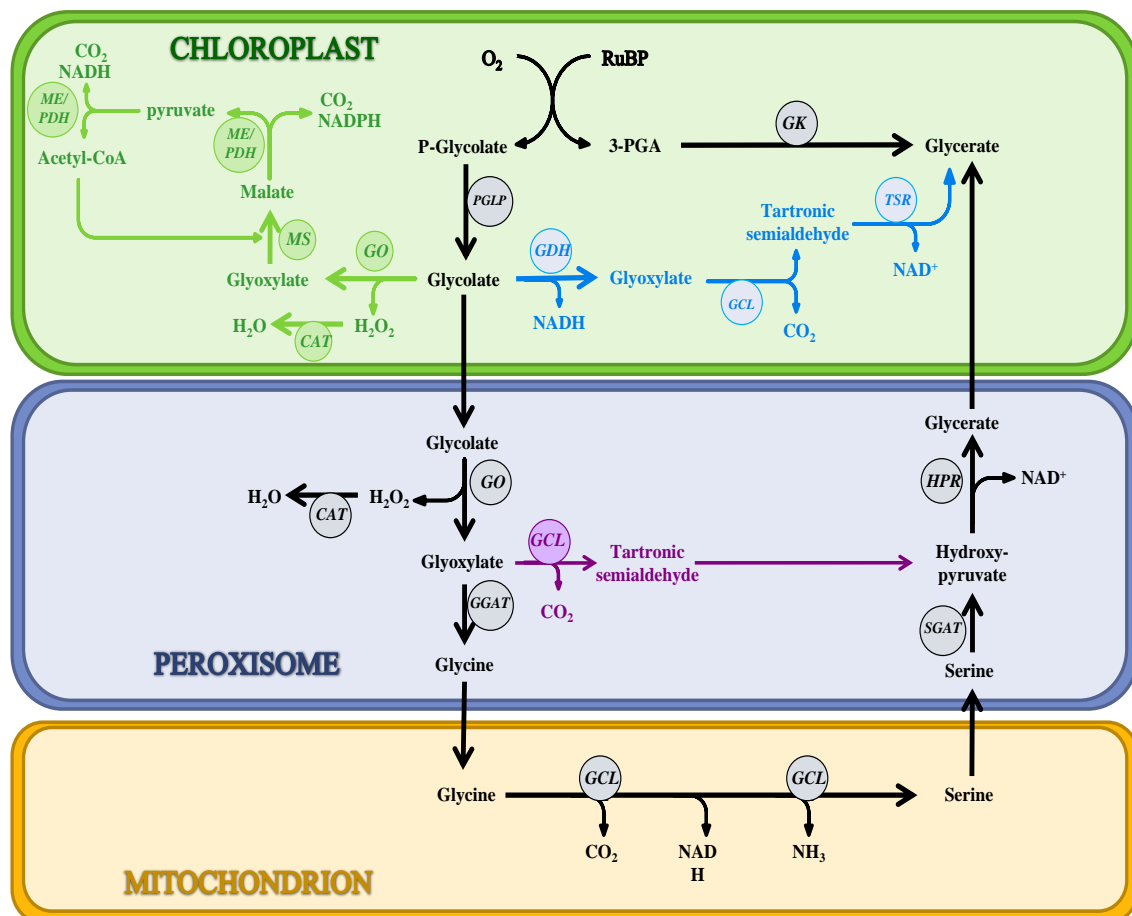
Figure 1.7-Scheme of the photorespiratory pathway and the three bypass pathways. The first bypass (Kebeish et al.2007) (colored in blue) starts with oxidation of glycolate by a glycolate dehydrogenase, from *E. coli,* to glyoxylate. Then glyoxylate carboligase also derived from *E. coli* produces tartronic semialdehyde, releasing CO_2 in the vicinity of RubisCO. The product of the decarboxylation reaction is then reduced to glycerate by tartronic semialdehyde reductase. The second bypass (Carvalho et al. 2012) (colored in purple) starts with a photorespiratory intermediate and produces tartronic semialdehyde. The decarboxylation reaction is catalysed by the same enzyme as in bypass 1, glycolate dehydrogenase, but targeted to the peroxisome.The third bypass (Maier et al. 2012 (colored in green) completely oxidizes glycolate to CO_2 by plant glycolate oxidase (GO) that was relocated from the peroxisome to the chloroplast. In this pathway, an additional catalase (CAT,) is required for detoxification of H_2O_2 produced by GO. Together with acetyl-coA, glyoxylate resulting from glycolate oxidation is converted to malate by malate synthase (MS), thus, two C2 compounds are metabolized to one C4 compound. **PGLP**: phosphoglycolate phosphatase; **GO**: glycolate oxidase; **CAT**: catalase; **GGAT**: glutamate:glyoxylate aminotransferase; **SGAT**: serine:glyoxylate aminotransferase; **HPR**: hydroxypyruvate reductase; **GK**: glycerate kinase; **GDH**: glycolate dehydrogenase; **GCL**: glyoxylate carboxylase; **TSR**: tartronic semiadehyde; **ME**: malic enzyme; **PDH**: pyruvate dehydrogenase; **MS**: malate synthase. Adapted from Peterhänsel et al. (2012).

The main benefits of these bypass pathways have been demonstrated experimentally in the case of the first two bypasses. The first and third bypass pathways release CO_2 into the chloroplast, increasing chloroplastic CO_2 concentration and reducing further oxygenation reactions (Xin et al. 2015). Ammonia release is avoided, reducing the waste of energy that this process requires. Using a glycolate dehydrogenase which synthesizes reducing equivalents instead of transferring electrons to oxygen is one of the main benefits of bypass pathway 1. In the case of bypass pathway 3, there are no costs for re-reduction of 3-PGA in the Calvin cycle as this compound is not part of the glycolate recycling process (Maurino and Peterhänsel 2010).

Bypass pathways 1 and 3 have been introduced into *Arabidopsis thaliana* and led to enhanced growth resulting in ca: 30-50% increases in biomass (Kebeish et al. 2007, Fahnenstich et al. 2008, Maier et al. 2012).

1.5 Engineering carbonic anhdrases

Introduction of a CCM into C_3 species has been suggested to improve the performance of RubisCO in C_3 plant chloroplasts (Lin et al. 2014; Price et al. 2013). The rate of carboxylation and oxygenation activity of RubisCO is dependent on the ratio of $[CO_2]$ over $[O_2]$, thus it is expected that photorespiration can be suppressed by increasing $[CO_2]$ in the stroma of the chloroplasts (Driever and Kromdijk 2013). Different selection pressures have pushed evolution to give rise to a large variety of CO_2-concentrating mechanisms (CCMs), all succeeding in reducing photorespiration by increasing $[CO_2]/[O_2]$ in the vicinity of RubisCO (Sage et al. 2012). The simplest form of CCMs consists on a compartment for CO_2 accumulation, inorganic carbon transporters and carbonic anhydrases which are responsible for increasing the ratio of $[CO_2]/[O_2]$ near the site of RubisCO activity (Sidhu et al. 2014). Cyanobacteria achieve the same effect in a single cell by localizing RubisCO to specialized sub-cellular compartments called carboxysomes. Carboxysomes are polyhedral bodies with a protein shell that encloses carbonic anhydrase (CA) and RubisCO packed in an ordered or semi-ordered array (McGrath and Long 2014).

The CCM is also a fundamental element of algal photosynthesis, metabolism, growth and biomass production (Wang et al. 2015). Many microalgae, such as *Chlamydomonas reinhardtii*, have been used for several decades to exemplify the active C_i transport in eukaryotic algae (Wang et al. 2011). Recent advances in genetic and molecular

approaches have suggested the use of *Chlamydomonas reinhardtii* CCM to reduce the leakage of CO_2 from plants and allow efficient recycling of mitochondrial CO_2 for carbon fixation in chloroplasts (Zabaleta et al. 2012). In Chlamydomonas spp. bicarbonate is concentrated in the chloroplast stroma via several C_i transporters and α and β carbonic anhydrases. Carbonic anhydrases are Zn-metalloenzymes that catalyze the reversible hydration of carbon dioxide into bicarbonate (Zabaleta et al. 2012). RubisCO is confined within a small comparment (the pyrenoid) which is surrounded by a dense sheath of non-overlapping starch plates (Engel et al. 2015).

Mathematical modeling shows that substantial increases in substrate-saturated CO_2 assimilation rate and yield can be achieved by incorporation of an algal CCM in C_3 chloroplasts (McGrath and Long 2014). Another fact that makes this model more suitable to increase photosynthetic and yield efficiency of crops is the fact that it does not require any tissue specific differentiation and enzyme and transporter localization as it happens when converting a C_3 to a C_4 plant (McGrath and Long 2014). Recently, the expression of a bicarbonate transporter (LCIA or LCIB) or a CA (CAH1 or CAH3), has been reported in tobacco plants (Nölke et al. 2016). Those plants that express one of the components (LCIA, LCIB, CAH1 or CAH3) had a faster growth rate and/or faster vegetative phase, which is the period of growth between germination and flowering.
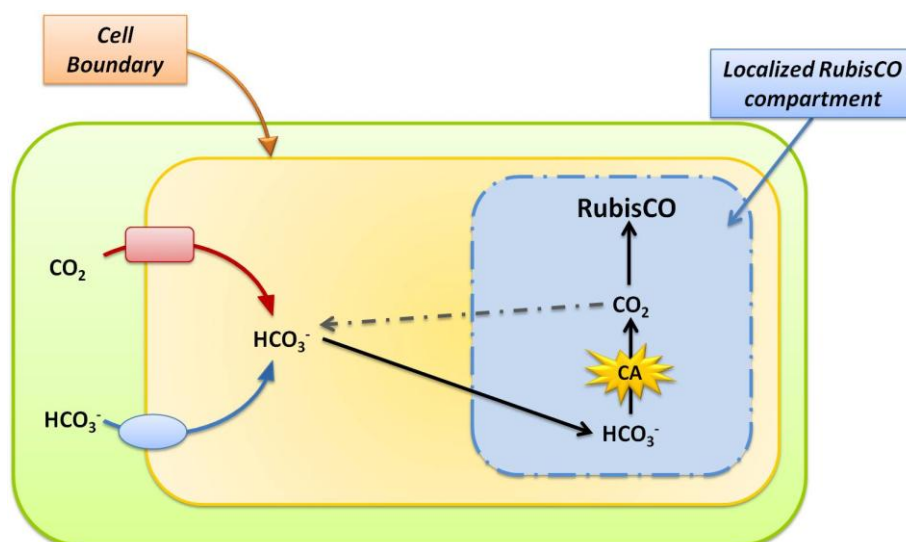
Figure 1.8-Simplified model of the cyanobacterial and algal CCM. CO_2 easily penetrates cell membranes and is converted into bicarbonate (HCO_3^-) in the periplasmic space by carbonic anhydrases and thus preventing CO_2 to leak from the plant cell. Inside the localized RubisCO compartment, the pyrenoid, a CA converts HCO_3^- to CO_2 in the vicinity of RubisCO. In the case of cyanobacteria this specialized compartment is called carboxysome, while in algae it is referred to as pyrenoid. Adapted from Wang et al. (2015).

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AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

The major aim of my thesis was to explore strategies to increase rice productivity through the engineering of a photorespiratory bypass pathway. A further aim was to generate transgenic rice plants with carbonic anhydrases which are enzymes that may also increase plant biomass.

My specific objectives were:

1. To generate a population of transgenic rice lines expressing the *Escherichia coli* glycolate catabolic pathway.

2. To evaluate the effect of the introduced bypass pathway in rice and to investigate the potential of this strategy to reduce the loss of fixed CO_2 during photorespiration thus enhancing photosynthesis, and yield.

3. To generate transgenic lines expressing one or two carbonic anhydrases under the control of two different promoters and to investigate if there were differences in transcript abundance depending on the promoter used.

4. To ascertain the potential of carbonic anhydrases as a new tool to increase the amount of CO_2 in proximity of RubisCO and thus increase plant productivity.

CHAPTER 2

Generation and molecular characterization of transgenic rice plants engineered with the glycolate catabolic bypass pathway

CHAPTER 2: GENERATION AND MOLECULAR CHARACTERIZATION OF TRANSGENIC RICE PLANTS ENGINEERED WITH THE GLYCOLATE CATABOLIC BYPASS PATHWAY

2.1 ABSTRACT

Increasing rice yield and productivity of staple crops such as rice is necessary to meet the global food demands. Photosynthesis is the primary determinant for plant biomass; consequently many efforts have been made to improve it. One way to improve photosynthesis is to reduce the losses of carbon dioxide when phosphoglycolate is recycled by photorespiration in C_3 plants. The aim of the present study was to establish a bypass pathway inside rice chloroplasts which was able to deal with the glycolate metabolism. I transformed rice plants with a glycolate catabolic bypass pathway from *Escherichia coli* to reduce the losses of carbon dioxide during photorespiration. This pathway requires the enzymatic activity of glycolate dehydrogenase (GDH, comprising three subunits: *glcD glcE* and *glcF*); glycolate carboligase (*gcl*) and tartronic semialdehyde (*tsr*). Regenerated plants were analyzed to confirm the expression of the input transgenes. Fertile lines with the combination of genes of interested were selected for in-depth analyses.

2.2 INTRODUCTION

Rice is the most important staple food crop for more than half of the world's population, particularly for the poor in developing countries in Asia. There is an urgent need to increase rice yield and productivity (Fisher 2014). During the last decade, major effort has been devoted to identify targets to improve its photosynthetic performance (Furbank et al. 2015).

Photosynthesis, the main process that allows plants to use the energy of sunlight to generate carbohydrates, emerges as the key route to increase the genetic yield potential of our major food crops (Long et al. 2015). Photorespiration is essential for C_3 plants but operates at the expense of fixed carbon dioxide and energy. It is also estimated to reduce the theoretically attainable efficiency of gross C_3 photosynthesis by 48% at ambient conditions (Walker et al. 2016). Three bypass pathways described in the general introduction have been investigated in order to bypass photorespiration, and thus, increase yield. In this chapter I will focus on the generation and molecular characterization of transgenic rice plants expressing the *E.coli* glycolate catabolic pathway.

The photorespiratory pathway starts when ribulose-1,5-bisphosphate (RuBP) reacts with oxygen rather than carbon dioxide. This reaction is catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). This reaction produces one molecule of 3-phosphoglycerate and one molecule of phosphoglycolate. As phosphoglycolate is toxic in the chloroplast, it has to be converted to glycolate, and this is a net waste of fixed carbon (Datal et al. 2015). Photorespiration is important as it detoxifies glycolate and recycles 75% of the carbon into phosphoglycerate, which can re-enter the C_3 cycle. However, the recycling process releases carbon dioxide and requires ATP and NADPH, thereby significantly reducing the efficiency of C_3 photosynthesis (Walker et al. 2016).

Transgenic *Arabidopsis thaliana* was generated (Kebeish et al. 2007) by expressing the three *Escherichia coli* enzymes glycolate dehydrogenase (GDH; Lord, 1972; Pellicer et al. 1996), glycolate carboligase (*gcl*; Chang et al. 1993) and tartronic semialdehyde reductase (*tsr*; Gotto and Kombert 1961). **Figure 2.1** shows the pathway in the chloroplast of *Arabidopsis thaliana.* This pathway operates in the chloroplasts, where it

involves the conversion of glycolate to glycerate, shifting CO_2 release from the mitochondria to the chloroplasts and avoiding the release of NH_3 (Kebeish et al. 2007).

The energy balance calculated for the bacterial pathway is superior to photorespiration, reflecting the fact that bacterial GDH does not use oxygen as an electron acceptor unlike glycolate oxidase in plants. This photorespiratory bypass reduced the flux through the photorespiratory pathway, enhancing photosynthesis and biomass production (Kebeish et al. 2007).
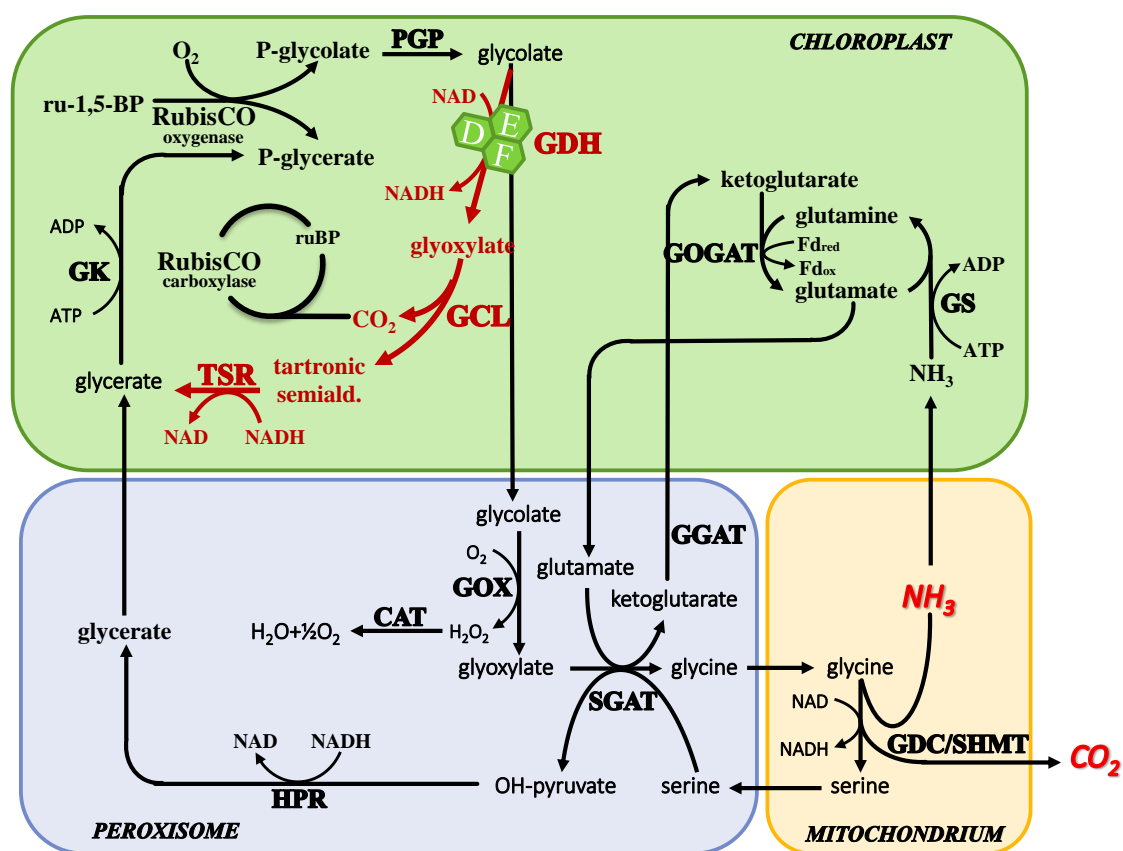
Figure 2.1-The photorespiratory pathway (in black) in C_3 plants and the suggested pathway (red) for the conversion of glycolate to glycerate. The oxygenase reaction of RubisCO forms P-glycerate and P-glycolate; the latter is dephosphorylated by PGP to form glycolate, which is in turn oxidized by GDH to form glyoxylate. Two molecules of glyoxylate are condensed by *gcl* forming tartronic semialdehyde and CO_2, which is released in the chloroplast. Tartronic semialdehyde is then reduced by *tsr* to glycerate, which is phosphorylated by GK to form P-glycerate that is used in the Calvin cycle. **RubisCO:** ribulose-1,5-bisphophate carboxylae/oxygenase; **RuBP:** ribulose-1,5-bisphosphate; **PGP:** phosphoglycolate phosphatase; **GOX:** glycolate oxidase; **CAT:** catalase; **GGAT:** glyoxylate/gluatamate aminotransferase; **GDC/SHMT:** glycine decarboxylase/serine hydroxymethyl aminotransferase; **HPR:** hydroxypyruvate reductase; **GK:** glycerate kinase; **GS:** glutamine synthetase; **GOGAT:** glutamate/oxoglutarate aminotransferase; **Fd$_{red}$:** reduced ferredoxin; **Fd$_{ox}$:** oxidized ferredoxin; **GDH:** glycolate dehydrogenase; *gcl*: glycolate carboligase; *tsr*: tartronic semialdehyde. Adapted from Kebeish et al. (2007).

I used co-transformation by direct DNA transfer to introduce simultaneously several unlinked genes involved in the glycolate catabolic bypass pathway into rice at a single locus, thus preventing segregation in subsequent generations (Altpeter et al. 2005). I generated a population of plants transformed with different combinations of the *Escherichia coli* glycolate catabolic pathway in rice chloroplasts with the objective of reducing the loss of fixed carbon by photorespiration, and therefore increase the biomass accumulation and efficiency of photosynthesis.

2.3 AIMS AND OBJECTIVES

The overall aim in this chapter was to engineering the glycolate catabolic pathway in rice.

The specific objectives were to:

- Introduce the genes involved in the glycolate catabolic pathway of *Escherichia coli* in rice in different combinations (5 genes: *glcD*, *glcE*, *glcF*, GDH, *tsr*; 3 genes: *glcD*, *glcE* and *glcF*; 2 genes: *gcl* and *tsr*) together with a selectable marker gene for hygromycin resistance to generate a population of rice plants containing and expressing these genes.
- Select and regenerate putative transgenic plants.
- Analyze mRNA expression levels and transcript abundance in transgenic plants.

2.4 MATERIALS AND METHODS

2.4.1 Transformation vectors

Genes encoding *Escherichia coli glcD*, *glcE* and *glcF* (the three GDH subunits) as well as *tsr* and *gcl* were cloned earlier in the laboratory (D. Yuan PhD thesis 2012). The corresponding cDNAs were transferred individually into pTRAux_Cab7 vectors derivative of pPAM (Rademacher et al. 2002). All genes were under the control of the *Oryza sativa* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter and GAPDH-3'UTR terminator. A sixth plasmid containing the hygromycin phosphotransferase (*hpt*) selectable marker gene (Sudhakar et al. 1998) was used for the generation of transgenic rice plants.

2.4.2 Rice transformation and plant regeneration

Mature rice seeds (*Oryza sativa* L. *cv.* EYI 105) were excised and cultured as previously described (Sudhakar et al. 1998; Valdez et al. 1998). After 7 days, rice 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 five glycolate catabolic pathway plasmids and the *hpt* selectable marker gene. Rice embryos were incubated on high-osmoticum MS medium (0.2 M mannitol, 0.2 M sorbitol) for 4 h prior bombardment. Bombarded embryos were cultured on MS medium supplemented with 30 mg/l hygromycin (Roche, Mannheim, Germany) and 2,4-dichlorophenoxyacetid acid (2,4-D) in the dark. Embyogenic callus were sequentially transferred to shooting and rooting media containing hygromycin. Regenerated plantlets were transferred to pots containing soil (Traysubstract, Klasmann-Deilmann GmbH, Geeste, Germany) and were grown under flooded conditions in a growth chamber at 26 ± 2ºC, with a 12h photoperiod (900 µmohn/m^2/s photosyntheticlly-active radiation) and 80% relative humidity. Plants were irrigated with a soluble Fe solution (Sequestrene 138 Fe G-100; Syngenta Agro SA, Madrid, Spain) until seed maturity (**Figure 2.2**; **Table 2.1**).
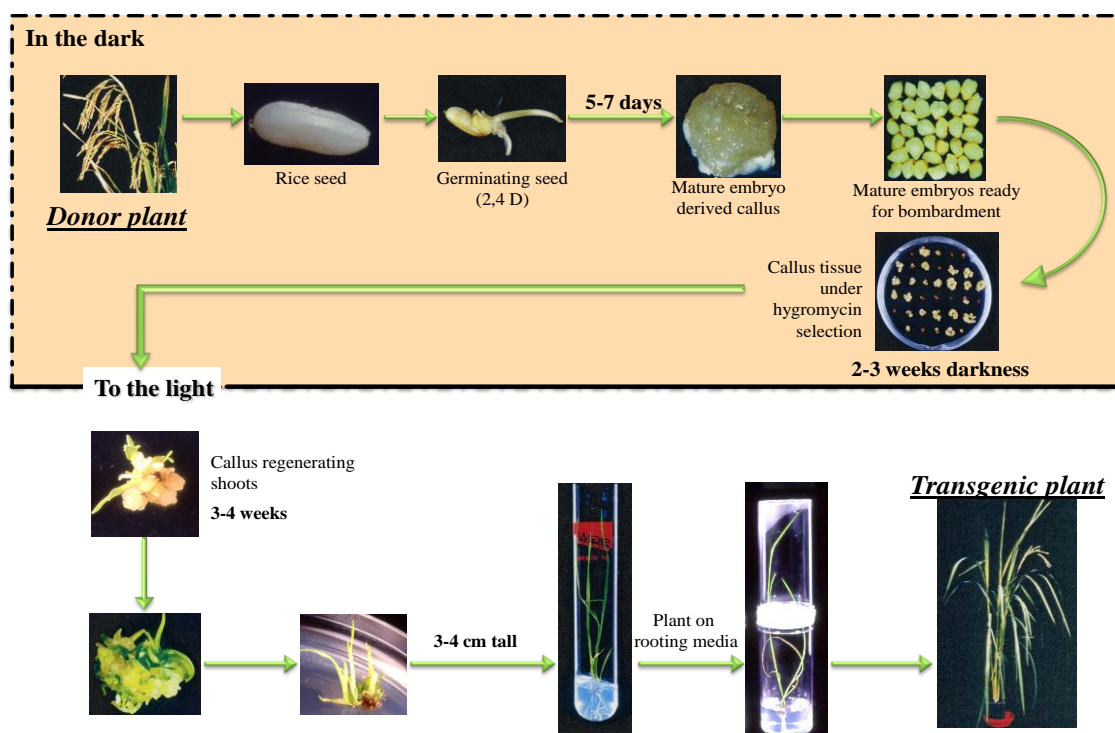
Figure 2.2-Representation of the rice transformation process showing the preparation of the embryos before bombardment, the selection of transformed tissues under dark and regeneration of putative transgenic plants under light conditions.

	CALLUS INDUCTION	OSMOTICUM MEDIUM	SELECTION MEDIUM	SHOOT INDUCTION MEDIUM	ROOT INDUCTION MEDIUM
Approx. time in culture	5 to 7 days	24h	Four weeks (two week subcultures)	21-25 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					
Phytalgel (Sygma)	5 g	3 g	5 g	4 g	3 g + 2 g agar
Autoclave at 121ºC for 20 min					
BAP (Sigma, 1mg/ml)	--	--	--	3 mg (3 ml)	--
NAA (Sigma, 1mg/ml)	--	--	--	0.5 mg (0.5 ml)	--
Hygromycin (Roche, 50mg/ml)	--	--	600 µl	600 µl	600 µl
Vitamin B5 (200x) **	--	--	--	--	2.5 ml
* MS Medium with Gamborg's B5 vitamins (Duchefa Biochemie, Haarlem, The Nederlands) ** 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					

Table 2.1- Medium composition (1l) for rice transformation and regeneration.

2.4.3. RNA extraction

Total RNA was extracted from 100-150 mg of frozen, ground leaf material using a modification of the method described by Vicient and Delseny (1999). The 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 minutes before extracting with one volume of 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma-Aldrich; St. Louis, US). After incubation samples were centrifuged at 10,000x g for 10

min at 4ºC and the supernatant was recovered. After two rounds of extraction, the supernatant was mixed with one volume of 4 M LiCl and incubated at −20ºC overnight. RNA was precipitated by centrifuging at 10,000x g for 30 min at 4ºC and the resulting pellet was washed with 500 µl 75% ethanol for 1 h on ice. After centrifugation at 10,000x g for 10 min at 4ºC, the RNA pellet was air-dried and resuspended in 75 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).

2.4.4 mRNA blot analysis

Total RNA (15 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 2.2**) 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.

GENE	PROBE PRIMERS	
	Position	*Sequence*
glcD	**Forward**	5'- GGTGTGTTGTTGGTGATGGCGCGCTTT - 3'
	Reverse	5'- CTCCACGCCGTCCAGCTCGCATA - 3'
glcE	**Forward**	5' - GCGCTGCTGGAGCAGGTGAAT - 3'
	Reverse	5' - CACTCATGGCTTCTTGCAGGCTGATT - 3'
glcF	**Forward**	5' - GCCTGTGTTCACTGCGGATT - 3'
	Reverse	5' - CGGGCCACCAGGCATCAATA - 3'
gcl	**Forward**	5' - CTCAGCGATGCGTAAGCACGGCGGTATT - 3'
	Reverse	5' - CACCATGTCAGACGCCAGCAGCGTT- 3'
tsr	**Forward**	5' - GGCATTATGGGTACACCGATGGCCATTA - 3'
	Reverse	5' - CGCACTTTGCAGTGCCAGGTTGAGAT- 3'

Table 2.2-Primers used to synthesize DIG-labelled probes for mRNA blot analysis.

2.4.5 cDNA synthesis

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 2.4.3 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).

2.4.6 Real-time qRT-PCR

Quantitative real-time RT-PCR was carried out using a BioRad CFX96TM 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 mastermix (BioRad), 1 µl of the 5 µM specific forward and reverse primers (**Table 2.3**), and 0.5 ng of the cDNA template. The amplification program started with a denaturation step at 98ºC for 2 min, followed by 35 cycles of 98ºC for 5 s, 59.4ºC for 30 s. Specificity was confirmed by product melt curve analysis over the temperature range 65–95ºC with fluorescence acquired after every 0.5ºC increase, and the fluorescence

threshold value and gene expression data were calculated with BioRad CFX96TM software.

GENE	qRT-PCR PRIMERS	
	Position	*Sequence*
*glcD*_RT	**Forward**	5'- CAGCATCAGTGGCGAACAT - 3'
	Reverse	5'- CGGTGTAGCGTGGGAATGT - 3'
*glcE*_RT	**Forward**	5' - CGCTATCGGCTCCTTTATTC - 3'
	Reverse	5' - TAAACACGCCGCAAGGGTC - 3'
*glcF*_RT	**Forward**	5' - CCATCTGTGCTGCGGTTCA - 3'
	Reverse	5' - TCATTTTGTTATCCCGCAGC - 3'
*gcl*_RT	**Forward**	5' - CAACGCTTATCTGGGGCTG - 3'
	Reverse	5' - GTCAACACCGTAGCCATTCA - 3'
*tsr*_RT	**Forward**	5' - GGCTTCAAAATCGCTCTGC - 3'
	Reverse	5' - AGGTCGCAGTGTTTGGCAGG- 3'
Ubi_RT	**Forward**	5' - ACCACTTCGACCGCCACTACT - 3'
	Reverse	5' - ACGCCTAAGCCTGCTGGTT- 3'

Table 2.3-Oligonucleotide sequences of rice ubiquitin and transgenes for Real-Time PCR analysis.

2.5 RESULTS

2.5.1 Generation of transgenic lines expressing different combinations of the genes constituting the glycolate catabolic pathway

Particle bombardment was used to co-transform mature seed-derived-callus with three different combinations of transgenes. In one combination the five genes of the glycolate catabolic pathway and the *hpt* selectable marker gene were co-bombarded. A second combination only used the genes encoding the three subunits of the glycolate dehydrogenase enzyme simultaneously with *hpt*. The last two enzymes of the glycolate catabolic pathway, *gcl* and *tsr*, and *hpt* were transformed into rice in a third experiment. A total of 128 independent hygromycin-resistant transgenic lines were recovered. Only 24 lines were fertile. Regenerated plants were grown to maturity in a growth chamber. Plants exhibited high sterility and in some cases they died before reaching maturity, so

no seeds were recovered which could have been used to analyze further generations for a lot of the transgenic plants.

2.5.2 Molecular characterization of putative transgenic plants

Transgene expression was verified by mRNA blot analysis using leaves from the putative transgenic plantlets. Gene-specific probes were used to confirm transcript accumulation. mRNA blots confirmed accumulation of the transcripts of each of the input transgenes, and revealed some heterogeneity in transgene expression at the level of transcription (**Figure 2.3**). Different combinations of expressed transgenes were identified in different plants. In some cases none of the genes of interest were found.

I recovered fifty nine lines which were bombarded with the five genes encoding the glycolate catabolic pathway. Eleven lines expressed the full glycolate catabolic pathway; however, only 5 of those lines were fertile. Forty nine lines were recovered from experiments in which the three subunits of glycolate dehydrogenase were bombarded. Seven lines co-expressed the three input transgenes, but only three of these lines were fertile. Twenty lines were recovered with the combination of *gcl* and *tsr*. Ten lines contained both transgenes but only five were fertile. **Table 2.4** provides a summary of the total number of transgenes expressed in each fertile line, showing that occasional non-expression was not specific to any transgene in particular. The sterility of the transgenic lines was not correlated with the expression of any specific transgene. Two fertile lines that expressed *gcl* and *tsr* in the T_0 generation did not express the transgenes in the T_1 generation, so those plants were not used for subsequent experiments. Line 5, which expressed the full glycolate catabolic pathway, was fertile at the T_0 generation; however, when the few seeds obtained where grown on selection media, none of the seeds grew, so it was not possible to be used in subsequent experiments.

Transcript abundance was also analyzed in a number of lines to investigate if there was any relationship between the amount of transcript and the sterility of the lines. In the case of Line 1, where the expression of the transgenes was confirmed by mRNA blot analyses, qRT-PCR revealed that *glcD*, *glcE* and *tsr* had a similar transcript accumulation, compared to *glcF* and *gcl*, which had a lower transcript accumulation. This data was consistent with the intensity of the band observed when the expression

was analyzed by mRNA blot. Line 16, which was bombarded with the combination of the three GDH subunits, showed that the *glcE* subunit had a higher transcript accumulation compared to *glcD* and *glcF*. *glcF* transcript accumulation was lower than that of *glcD* (**Figure 2.4**). The data suggested that there was no relationship between the level of the expression of the genes and the sterility of plants, as in both cases the lines were fertile. In the case of lines 55 and 57, the qRT-PCR analysis showed that both genes where highly expressed, compared to the other lines. Phenotypic analyses of transgenic plants that expressed only the two last genes of the glycolate catabolic pathway, *gcl* and *tsr*, showed that the plants had less biomass and higher sterility compared to other lines which expressed the full pathway or only GDH. These data might suggest that the expression of those genes was toxic for the plants. Low sterility was found in general in all regenerated plants, so there was no direct correlation between the expression of the genes and sterility; however more lines should be analyzed in order to draw a definitive conclusion of the effects of the genes on sterility.

A)

B)

C)

D)

E)
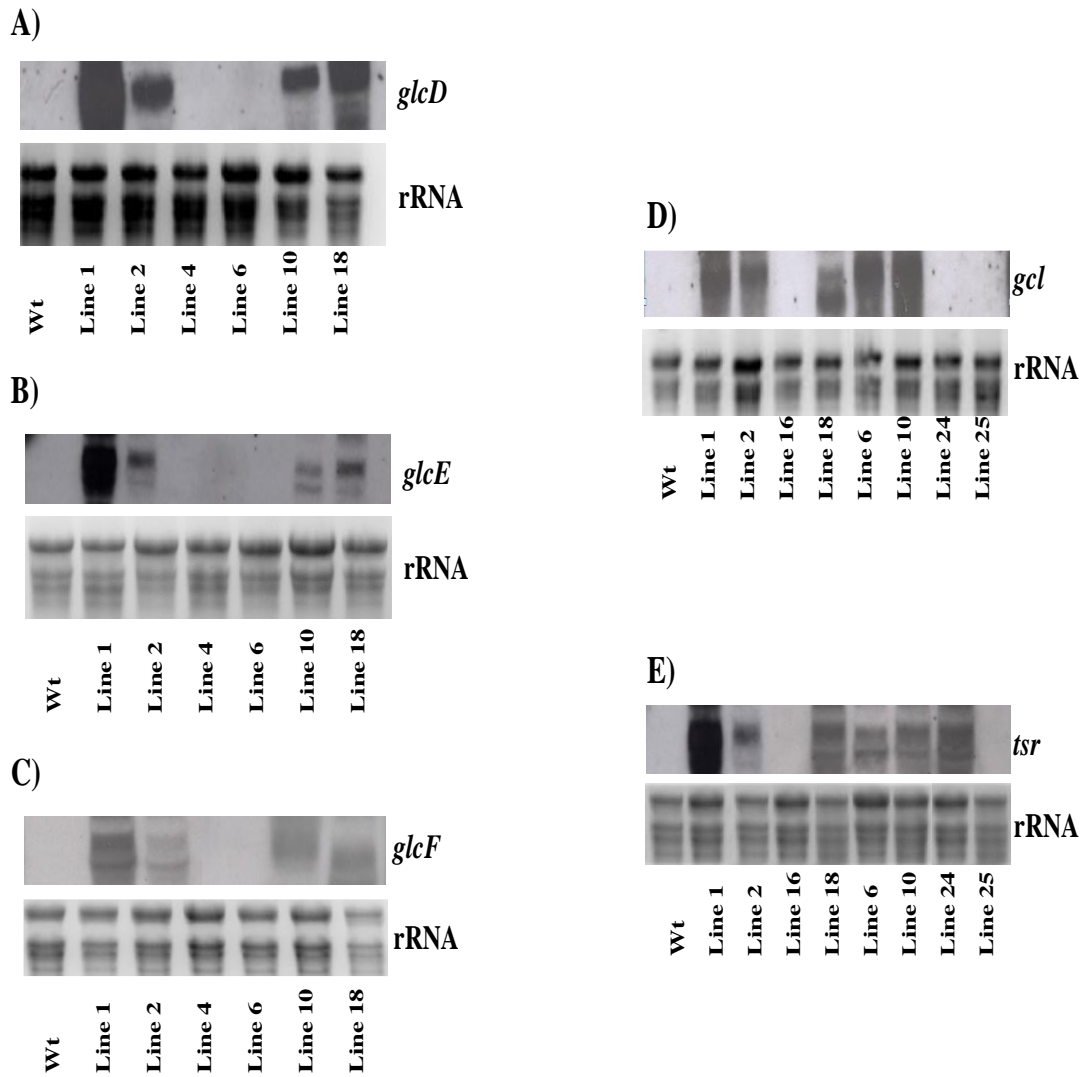
Figure 2.3-Representative mRNA blots showing expression of *glcD* (A), *glcE* (B), *glcF* (C), *gcl* (D) and *tsr* (E) in leaves of T$_0$ transgenic plants. The gaps in the membranes represent no expression of input transgenes in particular lines.

Line	Number of input genes	*glcD*	*glcE*	*glcF*	*gcl*	*tsr*
1	5	✓	✓	✓	✓	✓
3	5	✓	✓	✓	✓	✓
6	5	✗	✗	✗	✓	✓
12	5	✗	✗	✗	✗	✗
13	5	✓	✓	✗	✗	✗
16	5	✗	✗	✗	✗	✗
18	5	✓	✓	✓	✓	✓
24	5	✓	✓	✗	✗	✓
26	5	✗	✓	✗	✗	✓
32	5	✓	✓	✓	✗	✓
35	5	✗	✗	✗	✗	✓
36	5	✗	✓	✗	✗	✓
41	5	✓	✓	✗	✗	✓
45	5	✓	✓	✓	✓	✓
50	5	✓	✓	✗	✗	✓
53	5	✗	✗	✗	✗	✓
57	3	✓	✗	✗	-	-
60	3	✗	✓	✓	-	-
61	3	✓	✓	✓	-	-
65	3	✓	✓	✗	-	-
68	3	✓	✓	✓	-	-
71	3	✗	✓	✓	-	-
76	3	✗	✗	✗	-	-
83	3	✗	✓	✓	-	-
84	3	✗	✗	✗	-	-
87	3	✗	✓	✓	-	-
94	3	✗	✓	✓	-	-
96	3	✓	✓	✓	-	-
102	2	-	-	-	✓	✓
105	2	-	-	-	✓	✓
106	2	-	-	-	✓	✓
121	2	-	-	-	✓	✓
122	2	-	-	-	✓	✓
126	2	-	-	-	✓	✓

Table 2.4-Summary of transgene expression in fertile lines.
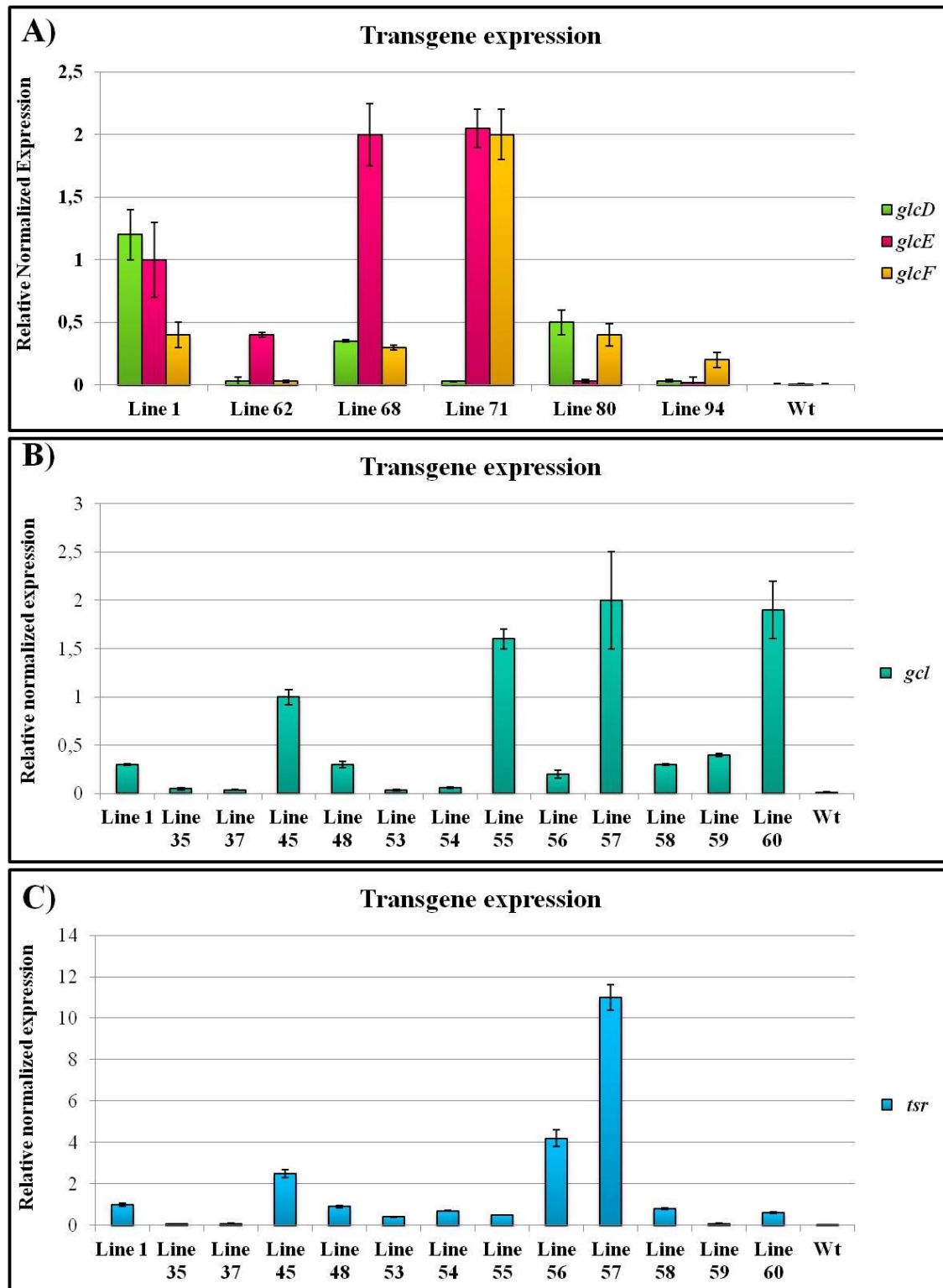
Figure 2.4-Transcript accumulation normalized against Ubiquitin in wild type (WT) and putative transgenic lines presented as mean of three technical replicates. (A) *glcD*, *glcE* and *glcF* transcript accumulation in 6 independent lines showing that lines 1 and 68 express the three input transgenes. **(B)** *gcl* transcript accumulation in 13 independent lines. Lines 45, 55, 57 and 60 showed the highest *gcl* transcript accumulation compared to the other lines. Lines 1, 48, 56, 58 and 59 accumulated lower transcript levels. **(C)** *tsr* transcript accumulation in 13 independent lines. Lines 45, 56 and 57 showed the highest transcript accumulation.

2.6 DISCUSSION

Photorespiration is an essential physiological process that causes the loss of ca: 30% of the carbon dioxide fixed during photosynthesis in C_3 plants (Monteith 1977; Giordano et al. 2005). However, photorespiration is essential for all organisms that perform oxygenic photosynthesis as mutations of genes that encode key enzymes of the photorespiratory pathway frequently result in lethality (Somerville 2001). The only way to decrease photorespiration is a reduction in the oxygenation activity of RubisCO (Long et al. 2006). Most of the strategies used to reduce the leakage of CO_2 during the photorespiratory pathway in C_3 plants usually aimed at concentrating CO_2 in the vicinity of RubisCO by introducing a C_4-like CO_2-concentrating mechanism (Zhu et al. 2010). However, the C_4 pathway necessitates the compartmentalization of bundle sheath and mesophyll cells to increase the diffusion of CO_2 at the active site of RubisCO. The molecular basis of this differentiation is not fully understood (Edwards et al. 2004). As a result converting C_3 into C_4 plants is very challenging (Edwards et al. 2001). Several researchers have attempted to establish components of the C_4 CO_2-conncentrating mechanism in Arabidopsis and other C_3 plants in order to reduce photorespiration (Peterhänsel et al. 2010; Haeusler et al. 2002) but limited success has been reported in reducing photorespiration by this approach thus far (Peterhänsel et al. 2010).

A novel pathway based on redirecting photorespiration instead of reducing it has been reported in *Arabidopsis thaliana* (Kebeish et al. 2007), *Solanum tuberosum* (Nölke et al. 2014) and *Camelina sativa* (Dalal et al. 2015). This pathway converts glycolate into glycerate inside the chloroplast and photorespiration is thus reduced. This reduction is achieved by installing a bacterial glycolate catabolic pathway in the chloroplast. Three enzymes are required to complete the conversion of glycolate to glycerate in the chloroplast, with the concomitant release of CO_2 (Pellicer et al. 1996; Lord 1972). These enzymes are glycolate dehydrogenase (GDH, consisting of three subunits, namely *glcD*, *glcE* and *glcF*); glyoxylate carboligase (*gcl*) and tartronic semialdehyde reductase (*tsr*). This strategy has two advantages. Firstly NH_3 is not released due to the activity of the plastidial glycolate pathway, and thus it does not have to be re-fixed. Consequently no energy is wasted as in photorespiration. A second advantage is that there is no waste of ATP used to regenerate ribulose-1,5-bisphosphate (RuBP) which

enables the Calvin cycle to be receptive for more CO_2 to be fixed (Kebeish et al. 2007; Moroney et al. 2013).

I have used combinatorial genetic transformation with multiple unlinked vectors to generate a population of rice plants containing and expressing the genes involved in the *E.coli* glycolate catabolic pathway simultaneously with a selectable marker gene which confers hygromycin resistance. I was able to regenerate a total of 128 putative independent lines. High sterility was noted among the regenerated plants; however no correlation could be established between sterility and either the number of transgenes present or the level of transgene expression e.g. Line 1 and Line 5 contained all input transgenes but Line 5 was sterile, whereas Line 1 was not. The phenotype of T_0 plants also exhibited differences compared to wild type plants. Most T_0 plants were shorter and had more narrow leaves compared to wild type plants. It was not possible to analyze the expression of the genes in all regenerated plants due to the high mortality in regenerating plants. It might be that there was a toxicity effect due to the expression of the genes; however I could not obtain data for those plants as they died before they were big enough to collect samples and carry out such analyses. Fertility data is not available for transgenic *Arabidopsis, Solanum tuberosum* and *Camelina sativa*, so it is unclear if this phenomenon was observed in the earlier studies as well. When DNA is inserted into the plant's genome it is common that such insertions are accompanied by the insertion of additional DNA, deletions and/or rearrangements. These changes are generally known as insertional effects, and they have the potential to give rise to unintended traits in plants (Schnell et al. 2015). These effects could be the cause of the phenotypes that I could observe in the transgenic plants regenerated, however, a deeper analysis of the plants should be carried out to draw reliable conclusions.

Seventeen lines among all the lines recovered did not express any of the introduced transgenes. This effect could be related with gene silencing. Transcriptional silencing involves promoter methylation and structural changes in chromatin (Ye and Signer, 1996). Thus using the same promoter for controlling transgene expression in the present study could be a reason for the lower expression levels of transgene. It has been described in previous studies that the presence of one or more rearranged copies of DNA can potentially lead to transgene silencing even if some copies are intact and functional (Kohli et al. 1998). However, there have been several reports which describe

transgenic plants under the control of the same promoter showing strong and stable expression (Peremartí et al. 2010). Gene silencing was detected in two fertile lines expressing the *gcl-tsr* combination and in one line expressing the complete glycolate catabolic pathway. All T_1 seeds of Line 105 and Line 106 (expressing *gcl* and *tsr* in T_0) and T_1 seeds of Line 4 (expressing the full glycolate catabolic pathway in T_0) were unable to grow in the presence of hygromycin in the media. However, when those seeds were grown in the absence of hygromycin they germinated. Transgene integration and inheritance has been widely investigated in crop plants. It is predominantly a random process and depending on the genomic position of the integrated transgene and the structure of the transgenic locus, as well as the transgene copy number, expression may be stable or variable. The integration site of a transgene in the host genome may have different impacts on transgene expression (Ahuja and Fladung 2014). As transgenes are integrated randomly they might be stably expressed, or they may exhibit deletions and rearrangements at the site of transgene integration which might lead to transgene inactivation or silencing (Somers and Makarevitch 2004; Zeng et al. 2010).

I co-bombarded all genes on independent plasmids, each plasmid encoding one gene of the glycolate catabolic pathway and one additional plasmid with the *hpt* gene. In the case of *Solanum tuberosum* the strategy was to use a multi-subunit gene encoding the DEF polyprotein (Nölke et al. 2014) and in the case of *Camelina sativa* two plasmids were used: one plasmid containing the three GDH subunits (*glcD*, *glcE* and *glcF*) and a second plasmid containin *gcl* and *tsr* (Dalal et al. 2015). This strategy ensured the correct stoichiometric amounts of the three subunits. In retrospect it would have been useful to have used this strategy in my experiments also to ensure that no subunit was less abundant that another and thus limiting the assembly of complete DEF subunits.

The advantage of using the bacterial glycolate dehydrogenase is that it does not use reducing compounds as electron acceptors to oxidize glycolate. This allows the conservation of reducing power associated with the reaction as reducing equivalents (Peterhänsel et al. 2013). Consequently the possibility of accumulation of H_2O_2, as in the case of the endogenous glycolate oxidase in rice peroxisomes is avoided. No toxic compounds have been described in the literature regarding the activity of glycolate carboligase or tartronic semialdehyde; therefore it appears that no negative effects should have been observed in the plants.

2.7 CONCLUSIONS

I regenerated 24 fertile plant lines with stable expression of genes involved in the glycolate catabolic pathway. Out of these lines, which were bombarded with different combination of transgenes, only 13 lines expressed the combination of genes of interest. mRNA blot analysis confirmed the expression of input transgenes and the analysis of the abundance of transcripts did not reflect any relationship between the high ratio of sterility and levels of expression of specific transgenes. Four lines that expressed the full glycolate catabolic pathway, two lines that expressed GDH alone and two lines that expressed the *gcl-tsr* genes, were used for further analyses described in Chapter 2.

Regeneration of more transgenic plant lines that express the three transgene combinations is required to analyze in detail the consequences of the introduction of the glycolate catabolic pathway in rice in terms of enzymatic activity and DNA integrity.

This chapter provides the basis for selecting individual transgenic plants for more detailed investigations which are described in the subsequent chapter.

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CHAPTER 3

Phenotypic measurements of transgenic plants transformed with the glycolate catabolic bypass pathway

CHAPTER 3: PHENOTYPIC MEASUREMENTS OF TRANSGENIC PLANTS TRANSFORMED WITH THE GLYCOLATE CATABOLIC BYPASS PATHWAY

3.1 ABSTRACT

Improving rice yield through the use of a glycolate catabolic bypass has been achieved in plants such as *Arabidopsis thaliana*, *Solanum tuberosum* and *Camelina sativa.* In this chapter I describe the phenotypic effects I measured in transgenic rice plants that expressed three different combinations of genes involved in the glycolate catabolic bypass pathway. One combination was the introduction of the full bypass pathway; another combination was the three sub-units of glycolate dehydrogenase (GDH), the first enzyme of the pathway; and the third combination included the two last enzymes of the glycolate catabolic bypass pathway (GCL and TSR). I determined that expression of the full glycolate catabolic bypass pathway was effective in two of the four transgenic lines analyzed, in terms of plant performance and total grain yield. Introduction of the full glycolate catabolic bypass pathway GDH showed a positive effect in terms of the number of total grains, especially in one the transgenic lines. Introduction of the two last enzymes of the pathway did not result in a measurable effect in the transgenic plants.

3.2 INTRODUCTION

Increasing photosynthetic efficiency has been suggested as a way to increase productivity in plants, as carbon metabolism in higher plants drives growth and determines yield on a large extend (Long et al. 2006). Earlier studies have demonstrated that carbon metabolism can be improved and optimized through genetic engineering (Peterhänsel et al. 2008). Several attempts have been made to improve RubisCO performance through genetic engineering (Yamori 2013). For example, the introduction of a C_4-RubisCO small subunit (*RbcS*) gene from sorghum into rice resulted in transgenic plants with a greater catalytic turnover rate of RubisCO (Ishikawa et al. 2011). Increasing the levels of CO_2 in the vicinity of RubisCO has also shown that this strategy can improve yield as a direct consequence of elevated [CO_2], simultaneously decreasing oxygenation and photorespiration, leading to a higher rate of photosynthesis and yield (Long et al. 2006).

Photorespiration is necessary for growth and survival; however, it is an inefficient and wasteful process that decreases the overall efficiency of photosynthesis as 25% of the fixed carbon and nitrogen are lost in this process (Peterhänsel and Maurino 2011). Attempts to inhibit photorespiration completely were unsuccessful, as mutants lacking this pathway were lethal under ambient CO_2 conditions (Somerville 1984; Somerville 2001). This demonstrated that photorespiration is important to protect plants from the accumulation of toxic inhibitors of photosynthesis (Campbell and Ogren 1990), light excess effects (Wingler 2000) and to provide reducing power for nitrate assimilation (Bloom et al. 2010)

Enhancing the activity of enzymes of the Calvin cycle has also been investigated. In tobacco, photosynthesis and biomass were increased by overexpressing the Calvin cycle enzyme SBPase (Lefebvre et al. 2005; Miyagawa et al. 2001). Transgenic tobacco plants overexpressing SBPase showed enhanced photosynthetic efficiency and growth when grown under atmospheric conditions (360 p.p.m CO_2). Compared to wild type tobacco these plants had 1.5-fold more dry matter and were 1.24-fold higher. Furthermore, those plants showed a 1.2-fold increase in initial activity of RubisCO compared to wild type plants. This indicates that enhancing the efficiency of photosynthesis, and subsequently the amount of fixed carbon might be a useful approach to boost productivity in plants (Nölke et al. 2014).

Kebeish et al. (2007) introduced the glycolate catabolic pathway into *Arabidopsis thaliana* to create a bypass which increased biomass production and also improved photosynthesis. *E.coli* is able to grow using glycolate as a sole carbon source (Lord 1972). A glycolate dehydrogenase complex (GDH) oxidizes glycolate to glyoxylate without using oxygen. GDH comprises three different units: *glcD*, *glcE* and *glcF* (Lord 1972, Pellicer et al. 1996). Two molecules of glyoxylate are condensed by glyoxylate carboligase (GCL) to form tartronic semialdehyde and CO_2 (Chang et al. 1993).In the last step of the pathway, tartronic semialdehyde is reduced to glycerate by tartronic semialdehyde reductase (TSR) and subsequently glycerate kinase phosphorylates it to glycerate-3-phosphate (Gotto and Kornberg 1961). The full photorespiratory bypass pathway of *E.coli* (**Figure 3.1**) reconstitutued in *Arabidopsis thaliana* was able to bypass photorespiration avoiding the flow of metabolites through peroxisomes and mitochondria. The released CO_2 when two molecules of glycolate are ligated to tartonic semialdehyde is catalized in the vicinity of RubisCO, reducing the oxygenase activity and hence photorespiration (Kebeish et al. 2007).
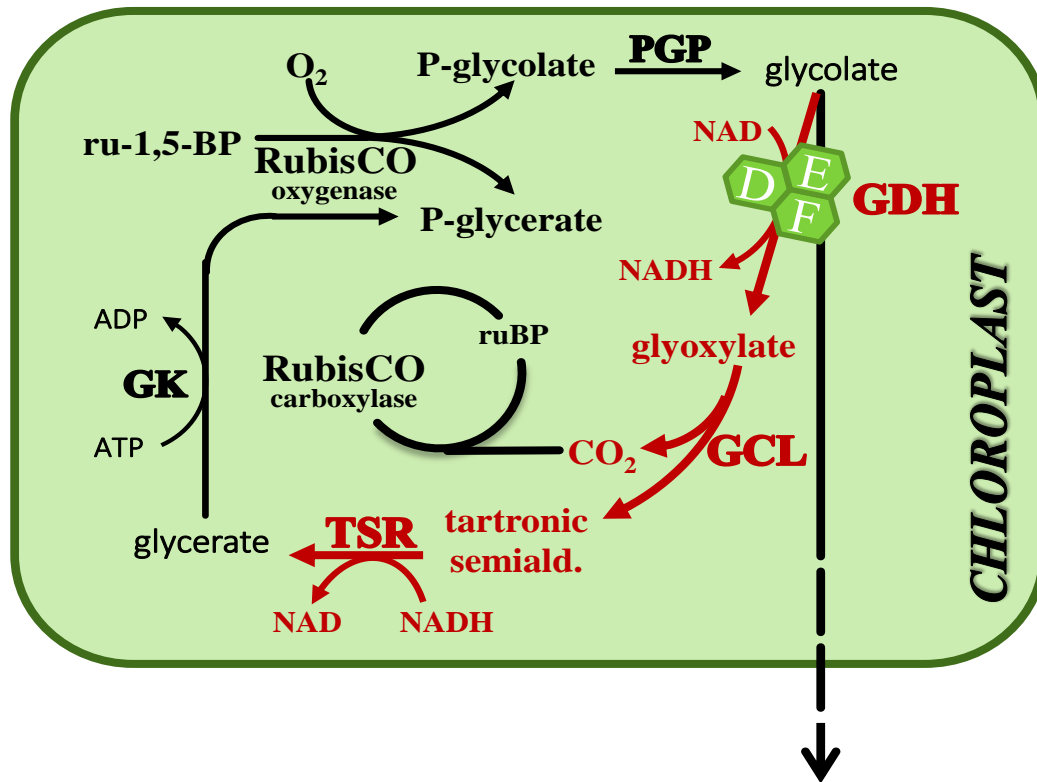
Figure 3.1-Overview of the photorespiratory bypass pathway. Glycolate is converted to glyoxylate by the activity of GDH. Two molecules of glyoxylate are ligated by *gcl* releasing CO_2 nearby RubisCO and one molecule of tartronic semialdehyde. The latter is then converted to glycerate by TSR. **GDH**: glycolate dehydrogenase; **GCL**: glyoxylate carboligase; **TSR**: tartronic semialdehyde reductase, **GK**: glycolate kinase, **PGP**: Phosphoglycolate phosphatase. Adapted from Kebeish et al. (2007).

RubisCO oxidizes ribulose-1,5-bisphosphate to P-glycolate and P-glycerate. In the subsequent step, PGP dephosphorylates phosphoglycolate to glycolate. GDH oxidizes glycolate to glyoxylate and GCL condense two molecules of glyoxylate to one molecule of tartronic semialdehyde and releasing CO_2 in the chloroplast. The produced tartronic semialdehyde is reduced by TSR to glycerate. Finally, glycerate is phosphorylated to glycerate-3-phosphate in a reaction catalyzed by glycerate kinase (GK) to P-glycerate which is used for carbohydrate biosynthesis in the Calvin cycle.

Transgenic *Arabidopsis thaliana* plants transformed with the glycolate catabolic bypass pathway grew faster, produce more shoot and root biomass, and contained more soluble sugars, reflecting reduced photorespiration and enhanced photosynthesis that correlated with an increased CO_2 concentration in the vicinity of RubisCO (Kebeish et al. 2007). Interestingly, the introduction of the partial photorespiratory bypass, using only GDH, resulted in *Arabidopsis plants* bigger in size compared to wild type and also with a

bigger rosette size compared to wild type plants. In potato, only the first enzyme of the photorespiratory bypass pathway was used. Results showed that transgenic plants were able to reduce photorespiration and improve CO_2 uptake with a significant impact on carbon metabolism. The levels of sugars were significantly higher compared to control plants and this resulted in a substantial increase in shoot and leaf biomass (Nölke et al. 2014). Transgenic potatoes were able to develop 25% more shoots per plant and leaf number was also increased compared to control plants. Potatoes transformed with glycolate dehydrogenase had a 2.3 fold increase in tuber yield. Another plant that has been transformed with the glycolate catabolic bypass pathway is *Camelina sativa*, an oilseed crop with great potential for biofuel production. Transgenic plants expressing the *E.coli* glycolate catabolic pathway had reduced photorespiration and increased photosynthesis in both partial and full bypass-expressing lines. In the case of the partial bypass, seed yield increased by 50-57%, and in the case of the full pathway the increase was 57-73%, with no loss of seed quality. Vegetative biomass also increased and the plants developed faster (Dalal et at. 2015).

The glycolate catabolic bypass pathway which was previously introduced into *Arabidopsis,* potato and camelina was used to transform rice plants in order to investigate if this technology might also increase rice biomass. Phenotypic parameters such as height of the plants, area of the last expanded leaf, chlorophyll content and fresh and dry weight were analyzed in rice plants expressing the full bypass pathway, the first enzyme of the pathway (GDH) and the last two enzymes of the pathway (GCL-TSR) in order to ascertain if this bypass pathway in rice might be able to increase biomass similarly to Arabidopsis, potato and camelina. I measured the parameters described above and also the number and weight of total seeds per plant to investigate if the introduced pathway could increase yield of the transgenic rice plants Total seeds starch was measured, as this is the immediate product of photosynthetic carbon assimilation.

3.3 AIMS AND OBJECTIVES

The overall aim in this chapter was to analyze the phenotypic effects of the introduction of the glycolate catabolic pathway in the transgenic plants regenerated in Chapter 2.

The specific objectives were to:

- – Measure the height of the plants, area of the last expanded leaf, chlorophyll content and fresh and dry weight of the transgenic plants expressing the genes involved in the glycolate catabolic pathway of *Escherichia coli* in rice in different combinations (5 genes: *glcD*, *glcE*, *glcF*, GDH, *tsr*; 3 genes: *glcD*, *glcE* and *glcF*; 2 genes: *gcl* and *tsr*) compared to wild type control plants.

- – Measure the total number of grains and the weight of the grains of the transgenic plants compared to wild type plants.

- – Analyze the starch content in the seeds of the transgenic plants which expressed different combination of genes compared to wild type controls.

3.4 MATERIALS AND METHODS

3.4.1 Plant material

Transgenic rice (*Oryza sativa* L. *cv* EYI-105) plant lines expressing different combinations of *glcD*, *glcE*, *glcF*, *gcl* and *tsr* were generated as described in Chapter 2.

3.4.2 Germination and growth conditions

Seeds from transgenic plants expressing each one of the three combinations of interest (the complete glycolate catabolic pathway, the three GDH subunits or the two last enzymes of the pathway) 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 ($26 \pm 2°C$, 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 $26 \pm 2°C$, with a 12-h photoperiod (900 µmohn/m2/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.

3.4.3 RNA extraction

RNA extraction was carried out following the procedure described in Chapter 2, section 2.4.3.

3.4.4 cDNA synthesis

cDNA synthesis was carried out following the protocol described in Chapter 2, section 2.4.5.

3.4.5 Real-time qRT-PCR

The procedure described in Chapter 2, section 2.4.6, was followed to carry out the qRT-PCR. The primers used for the qRT-PCR are described in detail in **Table 2.3**, Chapter 2.

3.4.6 Phenotypic analysis

The seeds from different transgenic lines were germinated and analyzed every two weeks to measure different parameters as described below. Plants were maintained as described in Chapter 2 until seed maturity.

3.4.6.1 Measurement of chlorophyll content

Transgenic plants expressing 5 (*glcD*, *glcE*, *glcF*, *gcl* and *tsr*), 3 (*glcD*, *glcE*, *glcF*) or 2 (*gcl* and *tsr*) genes were used to measure leaf chlorophyll content with a SPAD meter. Six measurements were performed in the last expanded leaf of each plant every two weeks until seed maturity.

3.4.6.2 Area of the last expanded leaf

Length and maximum width from the last expanded leaf were measured and leaf area was calculated using the formula:

Leaf area = *K* x length x width

where K is the "adjustment factor". K varies with the shape of the leaf which in turn is affected by the variety, nutritional status, and growth stage of the leaf. In the case of rice K=0.75 for all stages (Tsunoda 1964; Murata 1967).

3.4.6.3 Fresh/ Dry weight

Plants were allowed to grow until seed maturity under ambient conditions. Once the seeds were mature enough to be collected, the plants were harvested. The weight of all the shoots was measured (fresh weight) previous to drying them at 65ºC for 5 days and then dry weight was measured.

3.4.6.4 Seeds number and weight

Mature seeds from transgenic plants were collected and dried at 37ºC for 3 days. Seed number was counted using the Contador Seed Number (Hoffman Manufacturing, Inc). 50 seeds of each line were used to estimate the weight of the individual seed using a precision balance.

3.4.7 Starch extraction and quantification

100 mg of seeds were ground into a fine powder and dried at 55ºC overnight for the total starch assay. Starch extraction and quantification was performed according to the manufacturer's instructions, using Megazyme total starch kit (Megazyme, Wicklow, Ireland).

3.4.8 Statistical Analysis

A linear model (LM) was used to determine statistically significant differences in the transgenic rice plants expressing the different combinations of the glycolate catabolic bypass pathway. Traits were compared by Tukey's mean separation procedure ($p<0.05$). All the analyses were performed using the JMP Pro (JMP®, Version 11.0.0. SAS Institute Inc., Cary, NC, 2013).

3.5 RESULTS

3.5.1 Transgene expression in engineered lines

Transgene expression in plants was determined using quantitative real-time RT-PCR. As shown in **Figure 3.2**, all transgenes were expressed in the transgenic lines which were used to carry out further phenotypic and biochemical analyses.

A) *glcD* transgene expression

B) *glcE* transgene expression

C) *glcF* transgene expression
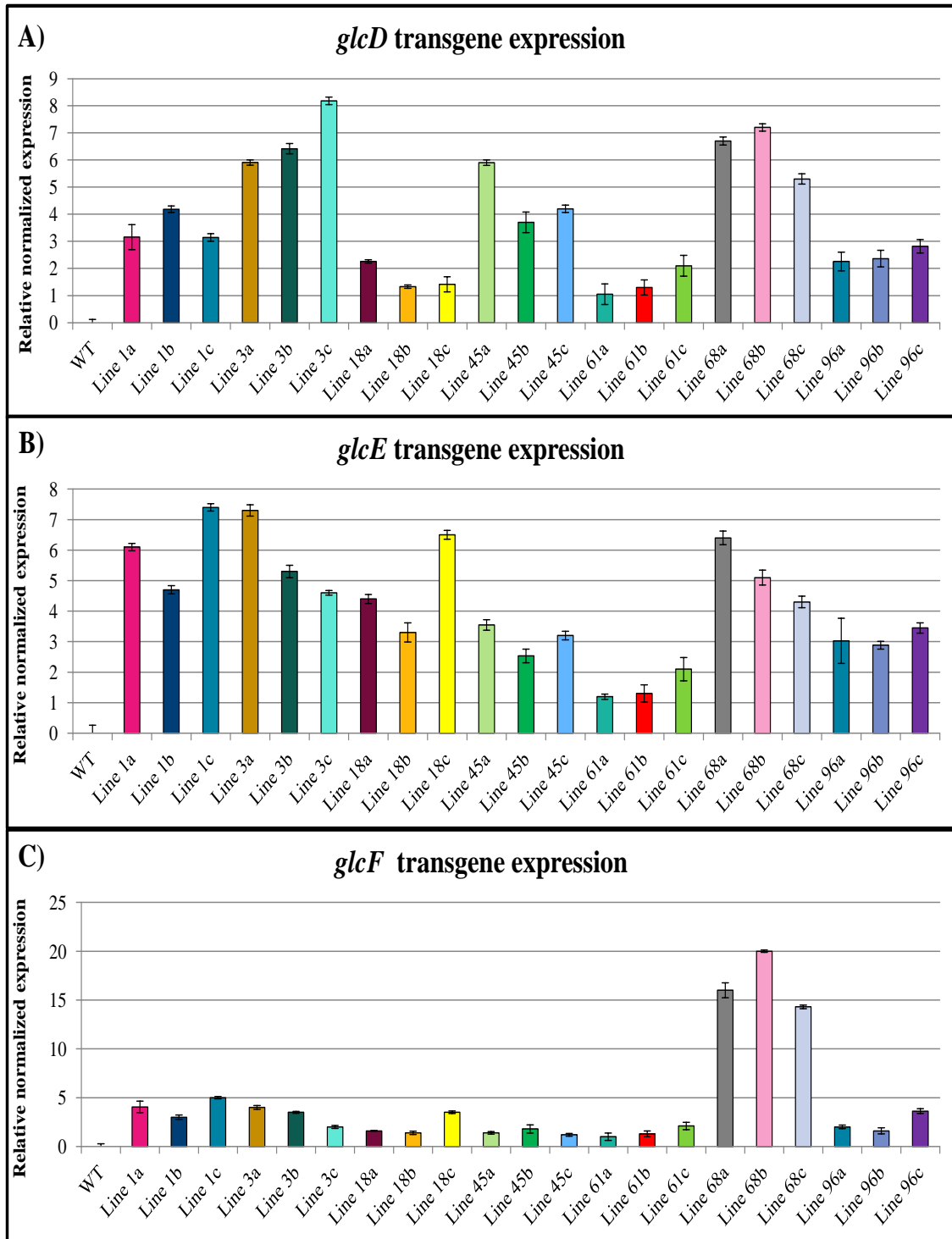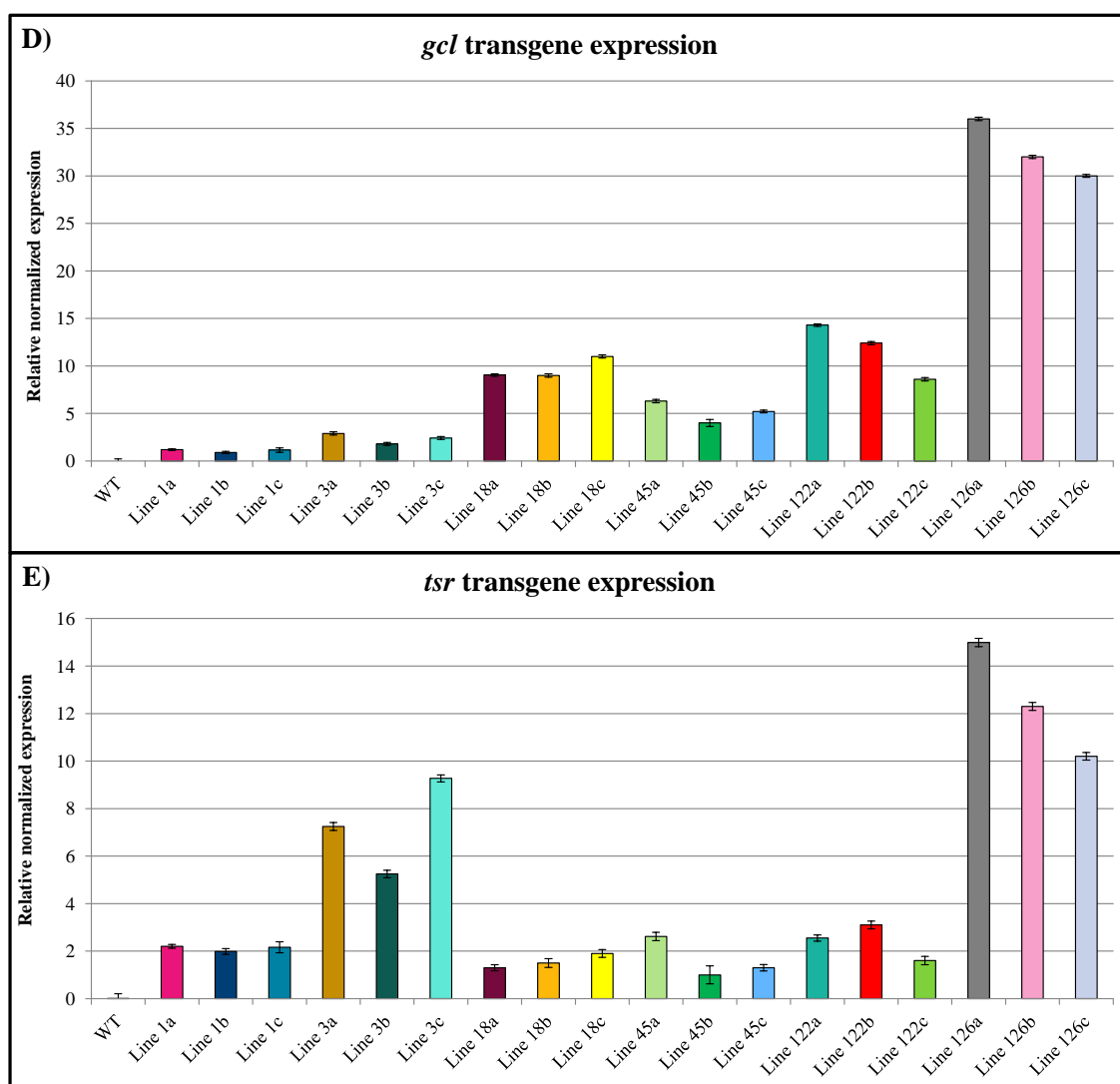
Figure 3.2-Relative mRNA expression of the transgenes in rice plants: (A) *glcD*, (B) *glcE*, (C) *glcF*, (D) *gcl* and (E) *tsr* in leaves, normalized against ubiquitin mRNA and presented as the mean of three technical replicates ± SE. The numbers in the axes represent the independent transgenic plants used for the experiment. For each line three biological replicates were used. Lines 1, 3, 18 and 45 expressed 5 genes (*glcD*, *glcE*, *glcF*, *gcl* and *tsr*); Lines 61, 68 and 96 expressed 3 genes (*glcD*, *glcE*, *glcF*; GDH) and Lines 122 and 126 expressed two genes (*gcl* and *tsr*).

3.5.2 Phenotypic analysis of plants expressing the glycolate catabolic bypass pathway

The height of the plants, the area of the last expanded leaf and the difference between fresh and dry weight, as well as the total number of seeds were measured in plants expressing the different combinations of the glycolate catabolic bypass pathway genes. Three siblings from each line were analyzed every two weeks from the date the plants

were transferred to soil. The specific generation and the genes expressed in the lines are summarized in **Table 3.1**.

PLANT LINE	GENES EXPRESSED	GENERATION
1	*glcD, glcE, glcF, gcl, tsr*	T_5
3	*glcD, glcE, glcF, gcl, tsr*	T_4
18	*glcD, glcE, glcF, gcl, tsr*	T_2
45	*glcD, glcE, glcF, gcl, tsr*	T_1
61	*glcD, glcE, glcF*	T_1
68	*glcD, glcE, glcF*	T_1
96	*glcD, glcE, glcF*	T_1
122	*gcl, tsr*	T_3
126	*gcl, tsr*	T_3

Table 3.1-Genes expressed in each independent line and the generation of the plants when the experiment was carried out.

3.5.2.1 Height of the plants expressing different gene combinations of the glycolate catabolic bypass pathway

The linear model (LM) used to analyze the transgenic rice plants expressing the different combinations of the glycolate catabolic bypass pathway revealed that the introduction of the different combination of genes has a significant effect on plant height at weeks 4, 8 and 12. Interestingly, differences measured in Line 1 and Line 3 compared to Line 18 and Line 45, which were lines that expressed the same combination of transgenes.

In the 8th week of measurements I also found that Line 96, which expressed GDH alone, was significantly taller than Line 61, wich expressed the same combination of transgenes. Additionally, it was significantly taller than Line 122 and Line 126, which expressed *gcl-tsr*, to Line18, which expressed the full glycolate catabolic bypass pathway and to the wild type control. The height of this line was stabilized at week 8th and no more statistically significant differences were found.

The summary of the statistical analyses is shown in **Table 3.2.** The growth pattern of the different transgenic lines during the experiment is shown in **Figure 3.3**.

PLANTS HEIGHT

GENERATION	EXPRESSED TRANSGENES	PLANT LINE	WEEK 2	WEEK 4	WEEK 6	WEEK 8	WEEK 10	WEEK 12
5		**1**	15.83	29.8a	34.2	37.8abc	57.6	63.7ab
4		**3**	15.3	27.8a	34.5	38.8ab	55.3	63.7a
2	*glcD, glcE, glcF, gcl* and *tsr*	**18**	13.2	22.5bc	25.5	25.5c	34.3	47.7c
1		**45**	13.1	22.5bc	30.7	38.7ab	43	42c
1		**61**	12.8	25.3abc	33.7	34.5bc	50.3	57abc
1	*glcD, glcE* and *glcF*	**68**	12	28.2a	31.7	37.3abc	48.2	54.7abc
1		**96**	11.8	28.5a	34.2	49.3a	49.8	49.8bc
3	*gcl* and *tsr*	**122**	10.4	25.2abc	30.7	35.8bc	46	54.3abc
3		**126**	10.4	20.8c	26.5	35.5bc	52	51abc
		WT	10.1	25.7ab	33.8	34.8bc	44.5	57abc
		df				9		
		F value	0.588	0.9713	0.2978	0.4172	0.414	0.4124
		p value	0.0576	<0.001	0.0749	0.0006	0.0619	0.0006

Table 3.2-Results of the linear model (LM) of the transgenic and wild type (WT) plants during the experiment. Means not sharing the same letter are significantly different at *p value*<0.05. Abbreviations: *df*: degrees of freedom; n=3 plants per line.
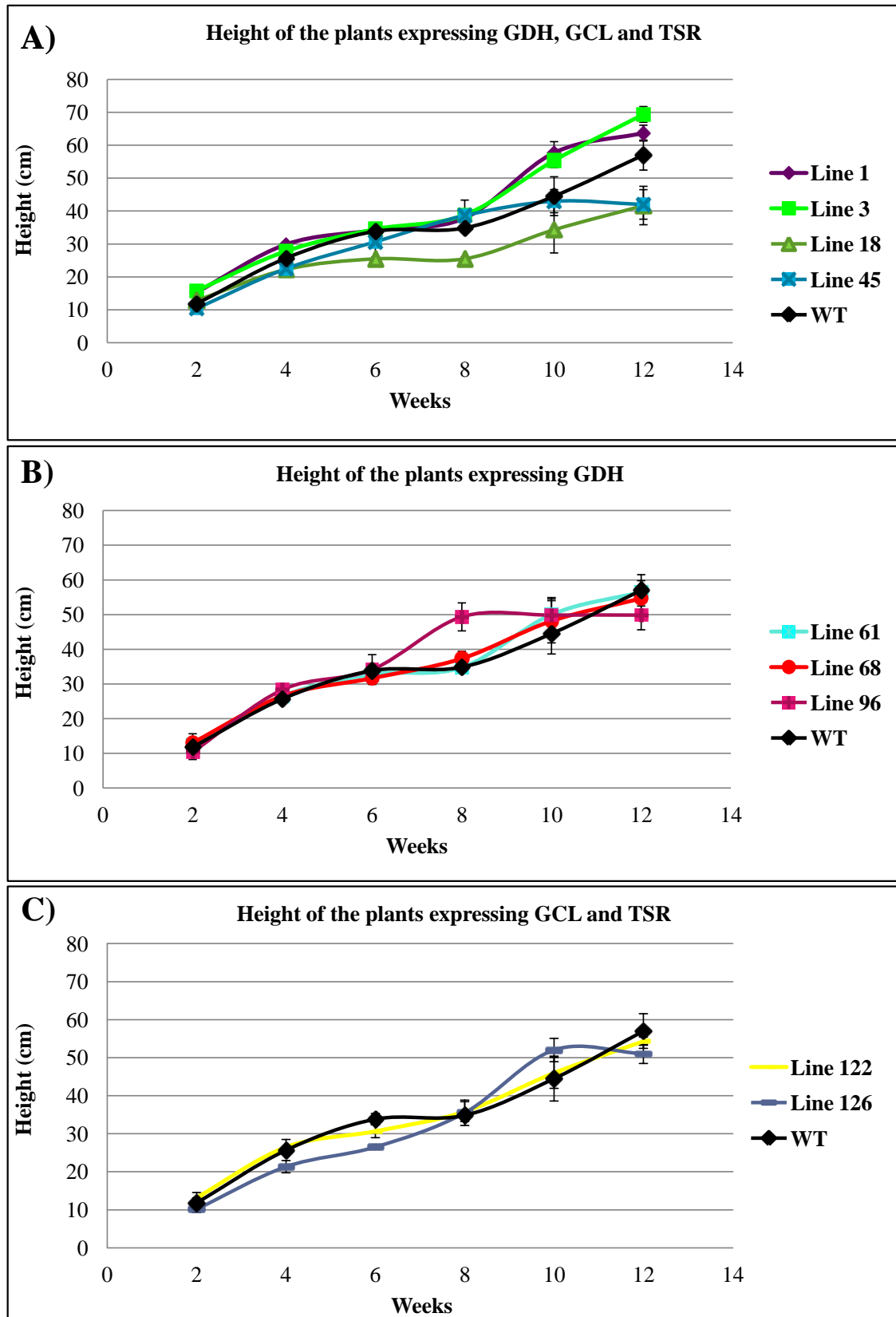
Figure 3.3-Height of transgenic and wild type (WT) plants in cm (±SE). n=3 plants per line**.**

3.5.2.2 Analyses of the area of the last expanded leaf of the plants expressing different gene combinations of the glycolate catabolic bypass pathway

The linear model (LM) used to analyze the area of the last expanded leaf in the transgenic rice plants expressing the different gene combinations of the glycolate catabolic bypass pathway and the wild type plants as controls, revealed that there were statistically significant differences for all measurements.

Similarly to plant height, differences in leaf area were found between Line 1 and Line 3 compared to Line 18 and Line 45. From the 2nd until the 10th week of growth differences were measured for Line 1 and Line 3 compared to Line 18 and Line 45. At the end of the experiment (week 12th), only Line 1 was significantly different compared to the other two lines which also expressed the same combination of transgenes.

The area of the last expanded leaves of Line 122 and Line 126, which expressed *gcl* and *tsr*, was similar as Line 18 and Line 45, which expressed the full glycolate catabolic bypass pathway, until the 12th week. Only in the 8th week I could observe that the area of the last expanded leaf of Line 122 was significantly larger than Line 18 and Line 45.

The summary of the statistical analyses is shown in **Table 3.3**. The growth rate of the different transgenic lines during the experiment is shown in **Figure 3.4**.

AREA OF THE LAST EXPANDED LEAF

GENERATION	EXPRESSED TRANSGENES	PLANT LINE	WEEK 2	WEEK 4	WEEK 6	WEEK 8	WEEK 10	WEEK 12
5	*glcD, glcE, glcF, gcl* and *tsr*	1	20ab	41a	57.2ab	56.1abc	51.9ab	47.4a
4		3	16.5a	40.7a	66.1a	60.9a	57a	40.3ab
2		18	14.7cd	12.1d	33.3cd	29.3de	28.4c	20.1b
1		45	14.3bcd	19.1cd	31.7d	28.3de	27.3c	20.9b
1	*glcD, glcE* and *glcF*	61	13.9abcd	32.4ab	44bcd	50.7abc	58.9a	35.4ab
1		68	12.7abc	28.2bc	48.5bcd	58.8ab	56.7ab	39.9ab
1		96	10.2bcd	34.8ab	49abcd	25.6e	35c	34.4ab
3	*gcl* and *tsr*	122	9.2abcd	33.7ab	37.1cd	45.6bc	40.1bc	36.3ab
3		126	7.1d	20.8cd	37cd	42.3cd	29.6c	28.1ab
		WT	5.8abcd	32.3ab	50.9abc	51.2abc	56.1ab	40.1ab
df						9		
F value			6.1733	21.4002	9.871	19.8473	16.012	4.4673
p value			0.0004	<0.001	<0.001	<0.001	<0.001	0.0026

Table 3.3-Results of the linear model (LM) of the transgenic and wild type (WT) plants during the experiment. Means not sharing the same letter are significantly different at *p value*<0.05. Abbreviations: *df*: degrees of freedom; n=3 plants per line.
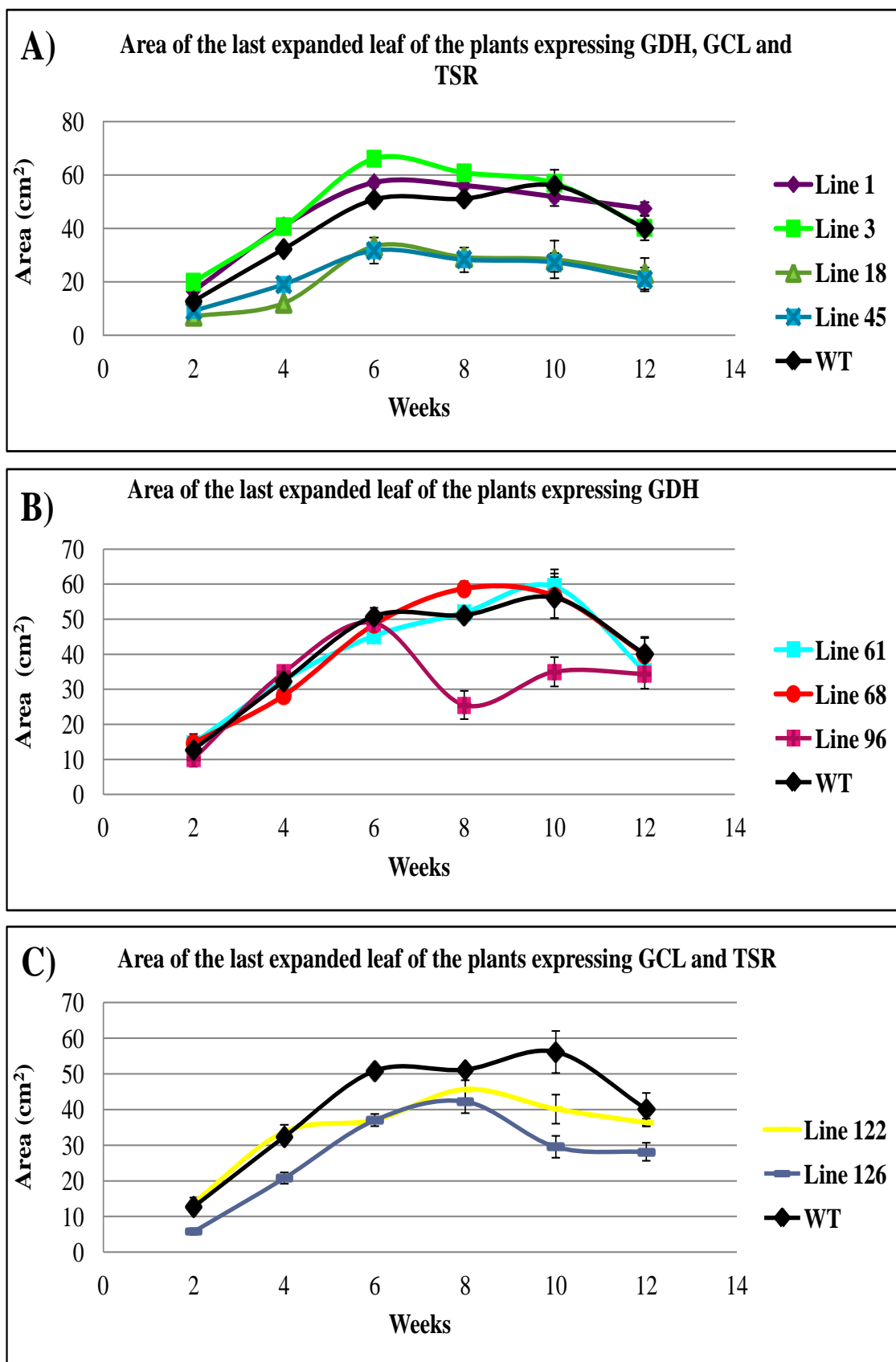
Figure 3.4-Measurements of the area of the last expanded leaves of transgenic and wild type (WT) plants in cm^2 ($\pm$SE). n=3 plants per line.

3.5.2.3 Number and weight of seeds of plants expressing the three different combinations of enzymes of the glycolate catabolic bypass pathway

Seeds of the transgenic plants were harvested to determine the yield of each individual line. Significant differences were found in the total number of seeds Line 1, which expressed the full glycolate catabolic bypass pathway. This line was the one that produced the highest amount of seeds. Lines 18 and 45, which expressed the full glycolate catabolic bypass, and Line 122, which expressed *gcl-tsr*, were the plants whith the lowest yield.

I also observed that the seeds of Line 3 were heavier compared to the seeds of Line 18 and Line 45, all of them expressing the full glycolate catabolic bypass pathway. The weight of the seeds of Line 122, which expressed *gcl* and *tsr*, were also significantly lower compared to the weight of the seeds of Line 3 (**Table 3.4; Figure 3.5**).

SEEDS ANALYSES

GENERATION	EXPRESSED TRANSGENES	PLANT LINE	TOTAL NUMBER OF SEEDS	WEIGHT OF 50 SEEDS	WEIGHT OF INDIVIDUAL SEED
5		**1**	1130a	0.84ab	0.016ab
4	*glcD, glcE, glcF, gcl* and *tsr*	**3**	662bc	0.98a	0.02a
2		**18**	76c	0.68b	0.014ab
1		**45**	94c	0.64b	0.013b
1		**61**	642bc	0.78ab	0.15ab
1	*glcD, glcE* and *glcF*	**68**	902b	0.73ab	0.014ab
1		**96**	302bc	0.81ab	0.016ab
3	*gcl* and *tsr*	**122**	137c	0.65b	0.013b
3		**126**	230bc	0.81ab	0.016ab
		WT	460bc	0.86ab	0.017ab
		df		9	
		F value	13.2715	3.1215	3.0516
		p value	<0.0001	0.0163	0.0181

Table 3.4- Statistical analyses of the total number of seeds, weight of 50 seeds and the weight of a single seed in the transgenic and wild type (WT) plants. Means not sharing the same letter are significantly different at *p value*<0.05. Abbreviations: *df*: degrees of freedom; n=3 plants per line.
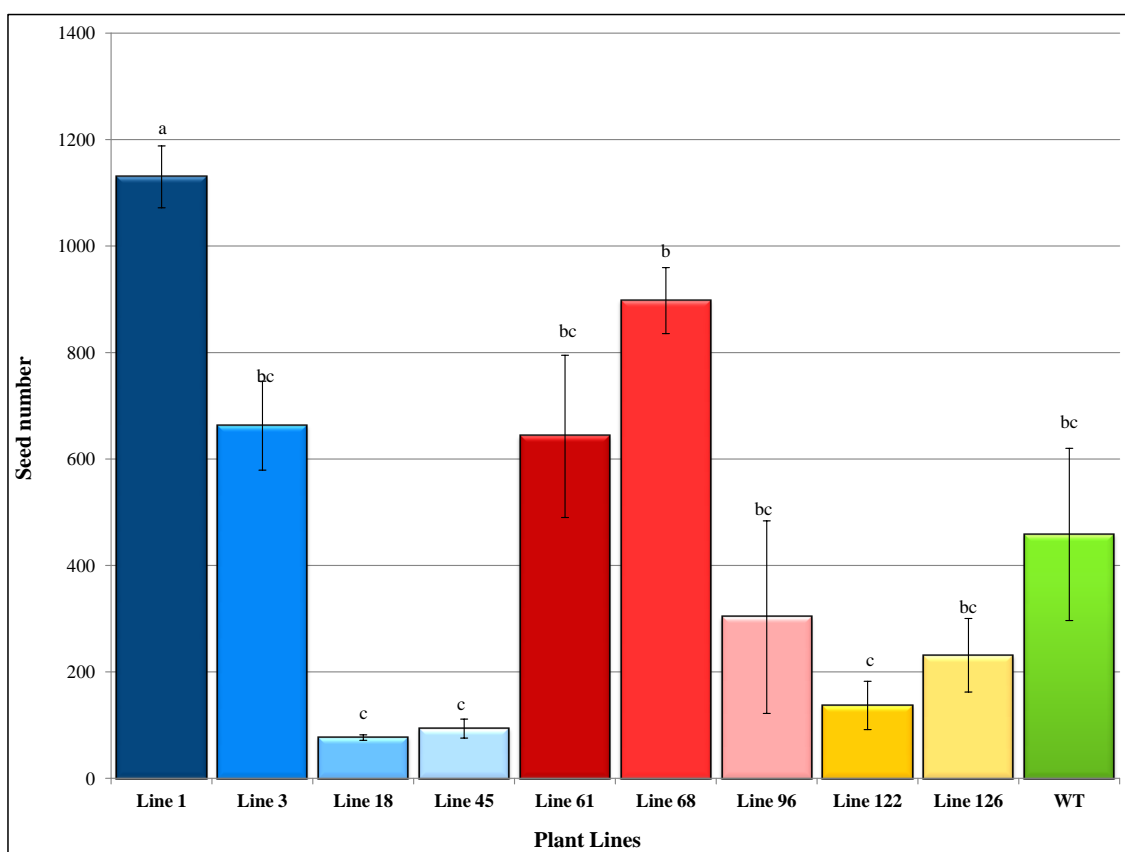
Figure 3.5-Mean grain yield of transgenic lines expressing different genes of the glycolate catabolic bypass pathway (±SE) and wild type (WT) as control. Lines 1, 3, 18 and 45 express the full glycolate catabolic pathway. Lines 61, 68 and 96 express GDH. Lines 122 and 126 express *gcl* and *tsr*. Means not sharing the same letter are significantly different at *p value*<0.05.

3.5.2.4 Chlorophyll content in plants expressing the three different gene combinations of the glycolate catabolic bypass pathway

The linear model (LM) showed that there were significant differences among the plants that expressed different combination of genes at weeks 2, 10 and 12. Significantly lower amount of chlorophyll content was measured at week 2 between Line 18, which expressed GDH-*gcl* and *tsr*, and Line 61, which expressed GDH alone.

The amount of chlorophyll at the 10th week was statistically lower in Line 18, compared to Line 1, Line 61 and Line 68. At the last end of the experiment (week 12th), the amount of chlorophyll of Line 1 and Line 3, was significantly higher compared to Line 18 and Line 45 (**Table 3.5; Figure 3.6**).

CHLOROPHYLL CONTENT

GENERATION	EXPRESSED TRANSGENES	PLANT LINE	WEEK 2	WEEK 4	WEEK 6	WEEK 8	WEEK 10	WEEK 12
5	*glcD, glcE, glcF, gcl* and *tsr*	**1**	28.6ab	38.7	40.1	39.1	42.3a	48.2a
4		**3**	31.6ab	36.4	37.2	40.2	40.6ab	47a
2		**18**	20.3b	27	33.8	31.4	30.5b	31.4c
1		**45**	25.1ab	33.7	31.7	37.1	35.4ab	33.1bc
1	*glcD, glcE* and *glcF*	**61**	32.9a	38.1	39.3	40.9	42.3a	45.2ab
1		**68**	30.5ab	38.1	36	40.8	43.9a	43.6abc
1		**96**	28.5ab	34.6	36.9	40	37.7ab	36.7abc
3	*gcl* and *tsr*	**122**	27.3ab	33.8	34.5	34.7	37.1ab	39abc
3		**126**	23.5ab	33.7	37.3	38.4	42.3ab	38.4abc
		WT	23.4ab	39	41.7	38.8	41.6ab	43.1abc
		df				9		
		F value	*2.8561*	*2.2631*	*1.7163*	*2.1077*	*3.1288*	*5.0122*
		p value	*0.0243*	*0.0616*	*0.1505*	*0.0792*	*0.0161*	*0.0013*

Table 3.5-Statistical analyses of the chlorophyll content in each measurement of the transgenic and wild type (WT) plants during the experiment. Means not sharing the same letter are significantly different at *p value*<0.05. Abbreviations: *df*: degrees of freedom; n=3 plants per line.
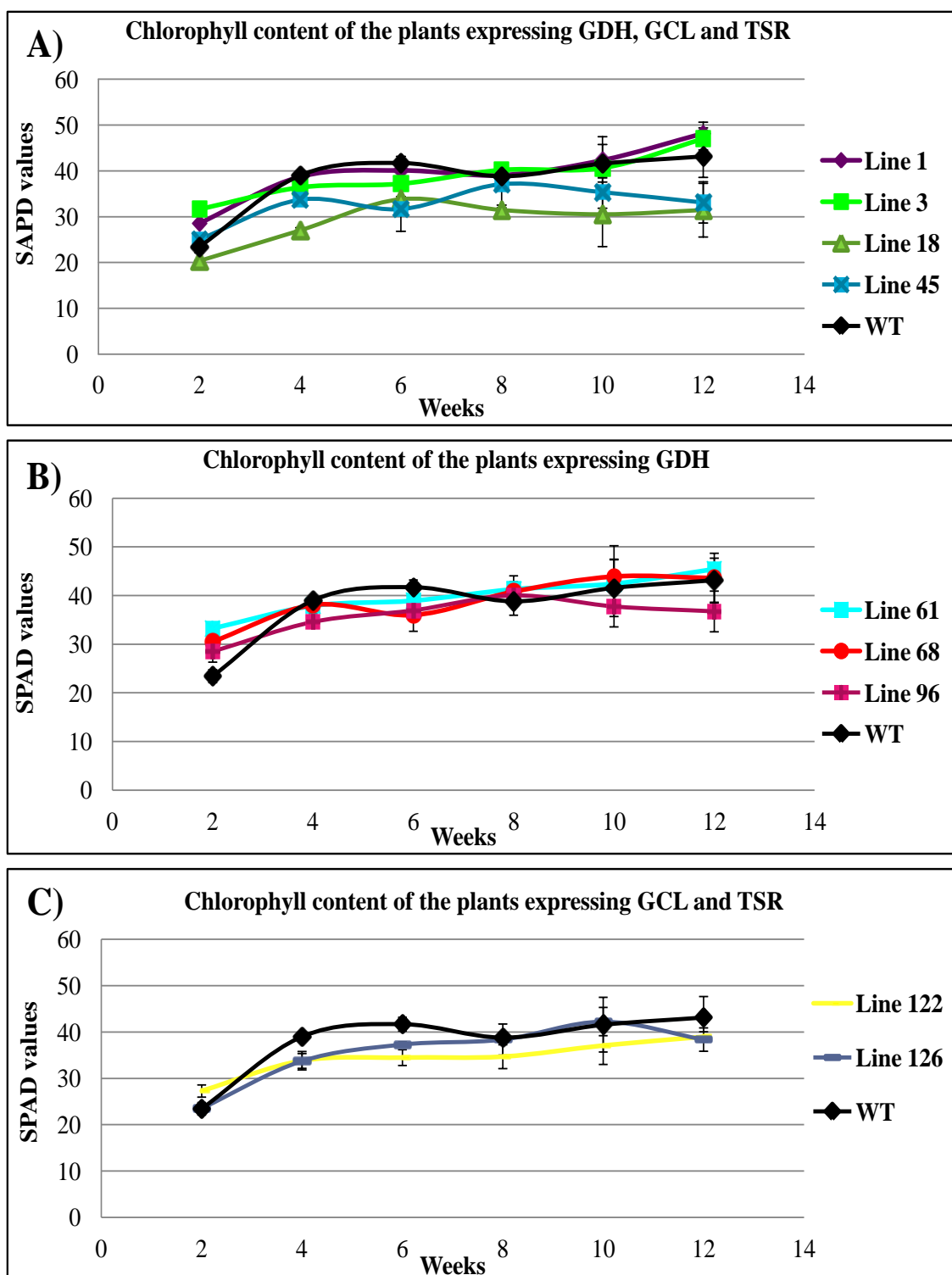
Figure 3.6- Mean of the chlorophyll content in transgenic lines expressing different genes of the glycolate catabolic bypass pathway and wild type (WT) as control (±SE). Lines 1, 3, 18 and 45 express the full glycolate catabolic pathway. Lines 61, 68 and 96 express GDH. Lines 122 and 126 express *gcl* and *tsr*.

3.5.2.5 Fresh and dry weight measurements

After the 12th week of the experiment I measured the fresh weight of the plants. I then dried the samples for 3 days at 65ºC and I measured the dry weight of each individual plant. Significant differences were found in the fresh weigth of Line 3 and Line 61, which expressed the full glycolate catabolic bypass pathway and GDH, respectively, compared to the wild type plants. The fresh weight of Line 3 and Line 61 was also significantly higher compared to Line 18 and Line 45, which also expressed the full glycolate catabolic bypass pathway, to Line 96, which expressed GDH and to Lines 122 and 126, which expressed *gcl* and *tsr*. Line 96, which expressed GDH, was the plant with the lowest fresh weight. Significant differences were found in the dry weight of Line 3, which expressed the full glycolate catabolic bypass pathway compared to Line 45, which expressed the same input transgenes, to Line 96 which expressed GDH, to Line 126, which expressed *gcl* and *tsr*, and to wild type plants.

Figure 3.7 shows the weight of the lines with the standard error and the difference of the fresh and the dry weight.

FRESH AND DRY WEIGHT OF THE PLANTS				
GENERATION	**EXPRESSED TRANSGENES**	**PLANT LINE**	**FRESH WEIGHT OF THE PLANTS**	**DRY WEIGHT OF THE PLANTS**
5	*glcD, glcE, glcF, gcl* and *tsr*	**1**	96.03abc	31.97ab
4		**3**	149.74a	53.63a
2		**18**	62.93bc	22.2ab
1		**45**	54.83bc	11.95b
1	*glcD, glcE* and *glcF*	**61**	149.87a	46.46ab
1		**68**	87.86abc	32.75ab
1		**96**	33.65c	13.99b
3	*gcl* and *tsr*	**122**	66.98bc	23.89ab
3		**126**	46.4bc	16.7b
		Wt	65.54b	31.81b
		df	9	
		F value	2.9685	4.0144
		p value	0.0217	0.0 50

Table 3.6-Statistical analyses of the fresh and dry weight in the transgenic and wild type (WT) plants at the end of the experiment. Means not sharing the same letter are significantly different at *p value*<0.05. Abbreviations: *df*: degrees of freedom; *FW*: fresh weight; *DW*: dry weight; n=3 plants per line.
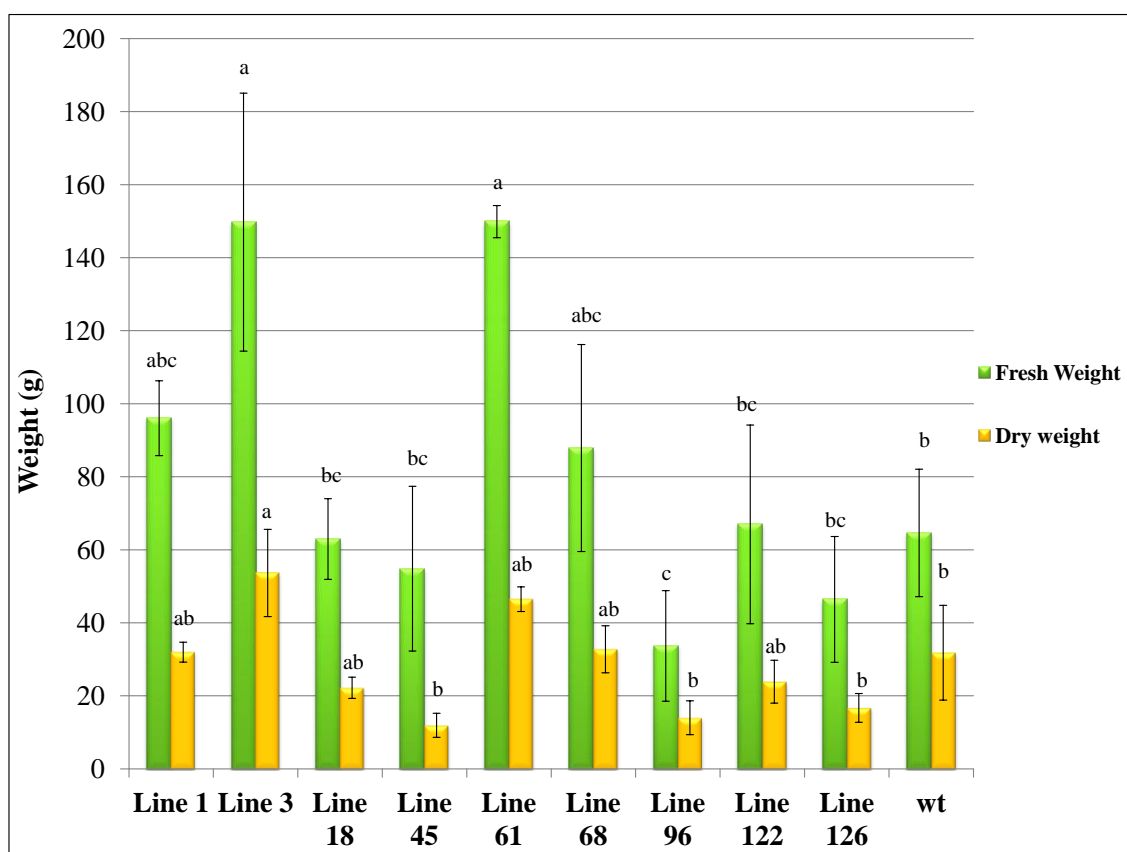
Figure 3.7-Fresh weight compared to dry weight [expressed in grams (g)] of the transgenic and the wild type (WT) plants. Lines 1, 3, 18 and 45 express the full glycolate catabolic pathway. Lines 61, 68 and 96 express GDH. Lines 122 and 126 express *gcl* and *tsr*. n=3 plants per line. Means not sharing the same letter are significantly different at *p value*<0.05.

3.5.2.6 The starch content of transgenic rice plants in not affected by the expression of the glycolate catabolic pathway

Total seed starch from transgenic plants that expressed the 3 different gene combinations of the glycolate catabolic bypass pathway was extracted with ethanol and treated with α-amylase and amyloglucosidase to obtain free glucose which reacts with GOPOD (glucose oxidase/peroxidase) to produce a pink product (quinone imine). Total starch was quantified by measuring the absorbance produced by the reaction in a spectrophotometer at 510 nm relative to the absorbance of 100 mg/l glucose as control.

The LM used to analyze the starch content in the transgenic and WT plants revealed that there were no significant differences between the plants. However, all transgenic plants which expressed the full glycolate catabolic bypass pathway had more starch than wild type plants. Lines 68 and 126, which expressed GDH alone and *gcl-tsr*, respectively; also accumulated more starch compared to wild type plants (**Table 3.7; Figure 3.8**).

STARCH CONTENT IN RICE SEEDS

GENERATION	EXPRESSED TRANSGENES	PLANT LINE	STARCH %
5		**1**	50.5
4	*glcD, glcE, glcF, gcl* and *tsr*	**3**	40.51
2		**18**	43
1		**45**	47.55
1		**61**	35.69
1	*glcD, glcE* and *glcF*	**68**	47.87
1		**96**	31.84
3	*gcl* and *tsr*	**122**	32.15
3		**126**	36.15
		WT	34.1
		df	*9*
		F value	*2.3816*
		p value	*0.0609*

Table 3.7-Linear model (LM) to analyze starch content in total seed mass in the transgenic rice and wild type (WT) seeds. Abbreviations: *df*: degrees of freedom; n=3 plants per line.
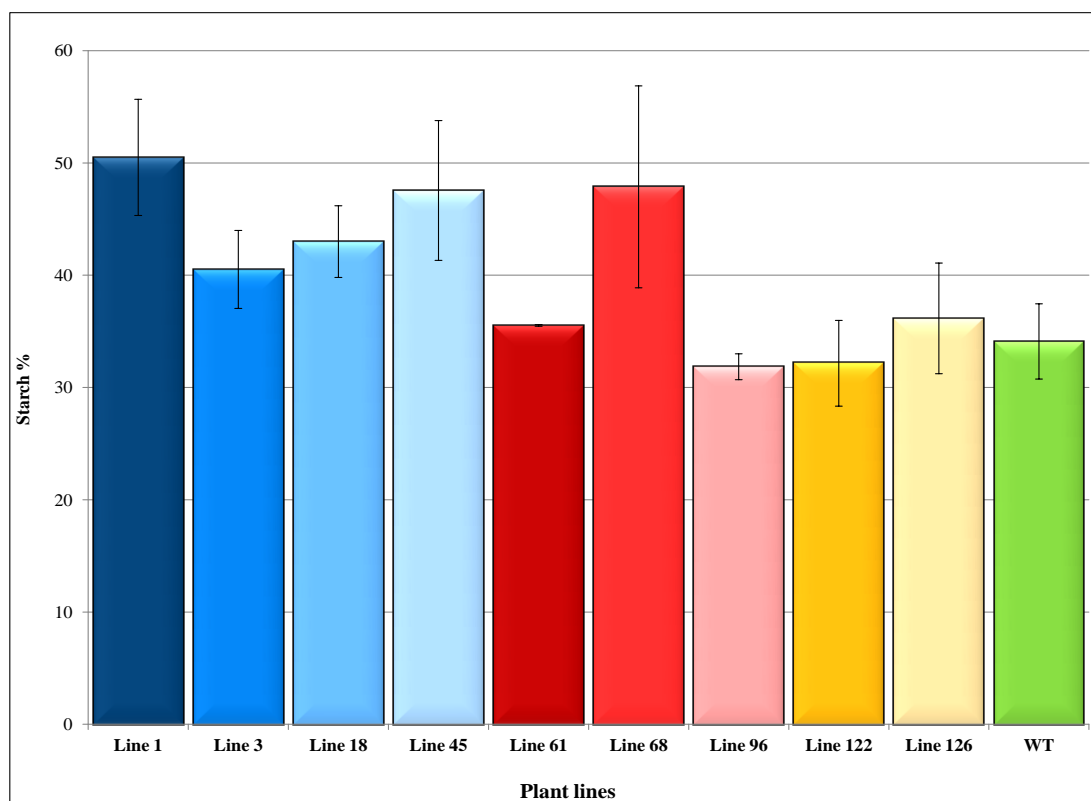
Figure 3.8-Total starch percentage in of seeds in transgenic rice seeds and wild type (WT) (±SE). Lines 1, 3, 18 and 45 express the full glycolate catabolic pathway. Lines 61, 68 and 96 express GDH. Lines 122 and 126 express *gcl* and *tsr*. n=3 plants per line.

3.6 DISCUSSION

Transgenic lines that expressed three different gene combinations of the *E.coli* glycolate catabolic pathway were selected to investigate the impact of introducing this pathway into rice plants. I used 4 independent lines expressing the full glycolate catabolic bypass pathway (Lines 1, 3, 18 and 45); three independent lines expressing the first enzyme, formed by three subunits (*glcD*, *glcE* and *glcF*), of the pathway (Lines 61, 68 and 96); and two independent lines expressing the last two enzymes of the pathway, *gcl* and *tsr* (Lines 122 and 126). qRT-PCR confirmed expression of the transgenes in all the lines selected to carry out in depth phenotypic characterization (**Figure 3.2**).

Increasing rice yield has been a major target in recent years, as more than half of the world's population uses rice as a staple crop (Wang et al. 2016). Rice consumption will increase as populations continue to grow, therefore there is a general agreement that rice yields must increase without increasing the demand for water and fertilizers (Mitchell and Sheehy 2006). Bypassing the photorespiratory pathway has been proposed as a way to increase carbon assimilation and thus biomass production in C_3 crops (Xin et al. 2015).

Previous reports demonstrated that the *E. coli* glycolate catabolic pathway is able to improve productivity in *Arabidopsis thaliana, Solanum tuberosum* and *Camelina sativa* compared to wild type plants. In all cases the yield and biomass of the transgenic plants increased (Kebeish et al. 2007; Dalal et al. 2015). The introduced bypass is able to concentrate CO_2 in the vicinity of RubisCO, partially suppressing the photorespiratory pathway (Kebeish et al. 2007). The energy that is not used in photorespiration may thus generate higher biomass (Kebeish et al. 2007).

I therefore engineered rice plants to express the same combination of genes and carried out in depth analyses to determine if the combination of genes used in other plant species had the same effect in rice.

I observed that in most of the analyses I performed, Line 1 and Line 3, had a better overall performance compared to Lines 18 and 45, which expressed the same combination of transgenes; however the differences I measured were not always statistically significant. The results obtained from Lines 1 and 3 are consistent with the results reported for *Arabidopsis thaliana* and *Camelina sativa* (Kebeish et al. 2007;

Dalal et al. 2015), where the transgenic plants expressing the full glycolate catabolic bypass pathway were taller compared to wild type plants. Lines expressing only the first enzyme of the pathway, GDH had a similar height to wild type plants (**Table 3.2**; **Figure 3.3**). This result does not agree with previous reports in other species expressing the same combination of genes; however, another round of experiments might be necessary to determine if these lines are able to perform better in subsequent generations.

Contrary to my expectations, Lines 18 and 45, which expressed the full glycolate catabolic pathway, exhibited the worst performance. These lines were shorter compared to the wild type lines. This negative effect has not been described in other plants with the same transgene complement; however, one possible explanation might be the site of transgene insertion in the rice genome.

I also observed that Line 1, which expressed the full pathway, had a larger surface area in the last expanded leaf, while Lines 18 and 45, expressing the same gene combination, had significantly less surface area compared to Line 1. The top three leaves of rice plants, especially the flag leaf, contribute to grain yield. Flag leaf plays a major role in biosynthesis and translocation of photoassimilates to the rice seeds, affecting grain yield (Sperotto et al. 2013). Flag leaf is the most metabolically active leaf at grain filling and contributes to 45% of rice grain yield (Al-Tahir 2014). This correlates with Lines 18 and 45, which had a small surface area and at the same time had a very low yield (**Table 3.3**; **Figure 3.4**).

Expression of the full glycolate catabolic pathway enhanced plant growth performance and showed a clear increase in total fresh and dry weight, specifically in Line 3 and Line 61. In the case of Line 1 and Line 68, the results were not as evident, but a similar trend was noted (**Table 3.6**; **Figure 3.7**). The data was thus consistent with published reports in other plants expressing the same combination of genes (Kebeish et al. 2007; Nölke et al. 2014; Dalal et al. 2015).

The expression of the first enzyme of the glycolate catabolic bypass pathway, GDH, only resulted in a significant increase in total biomass in the case of Line 61. Seed number was only increased in Line 68, which expressed the same combination of input transgenes as Line 61. Although the results were not statistically significant, the increase of the number of seeds observed in these lines are in line with the results

reported for other crops where the partial glycolate bypass had been introduced (Kebeish et al. 2007, Nölke et al. 2014; Dalal et al. 2016). Even though in those species the effect of the introduction of GDH alone was more evident, further analyses in subsequent generations need to be carried out.

Lines that expressed the last two genes of the pathway, *gcl* and *tsr*, did not perform any better compared to the other plants. Plant chloroplasts have an oxidation system which is associated with photosynthetic electron transport chain; however, no enzyme responsible for this postulated activity has been isolated (Goyal and Tolbert 1996). It has been hypothesized that there is an enzyme in plant chloroplasts that can oxidize glycolate to glyoxylate. If that were the case, glyoxylate would be consumed by glyoxylate carboligase (*gcl*) in the chloroplast of plants expressing *gcl* and *tsr*. However, if that were the case plants should have exhibited normal or better performance compared to the wild type plants. As this enzyme has not been isolated, it might be that it is not present in rice plants, or it is not functional, and that could be one reason why the plants did not perform any better.

While seed data from *Arabidopsis* are not available (Kebeish et al. 2007), in camelina and potato plants expression of the partial or the full bypass pathway increased seed or tuber yield number per plant. In addition the transgenic plants flowered about a week earlier than wild type plants (Nölke et al. 2014; Dalal et al. 2015). These results are consistent with my results. Transgenic rice plants that expressed the full or the partial bypass pathway (GDH) flowered earlier compared to wild type lines. In the case of lines that expressed only *gcl* and *tsr*, these flowered later compared to the other transgenic lines and in some cases even later than the wild type lines. Further investigation is required to ascertain which aspects of the photorespiratory pathway might be affecting these lines.

I established a correlation between plant height, the surface area of the last expanded leaf and the amount of seeds. Chlorophyll content of the transgenic rice plants was the same as in WT leaves (**Table 3.5**; **Figure 3.6**), similarly to the results obtained in camelina (Dalal et al. 2015).

Leaf size in rice and camelina behaved the same way (Dalal et al. 2015).) To achieve higher yield potential, metabolic activity within the grain must coincide with maximum activity of source leaves which can retain photosynthetic activity well into the grain-

filling period. However, much remains to be investigated on the regulation of photosynthesis during grain-filling in rice plants (Murchie et al. 2002).

Starch and sucrose are the primary products of photosynthesis in leaves of most terrestrial plants (Kötting et al. 2010). In the case of rice starch accumulates in plants as granules in chloroplasts of source organs such as leaves (transitory starch) or in amyloplasts of sink organs such as seeds (Fasahat et al. 2014). Increased photosynthetic CO_2 fixation rates would be expected to increase the amount of starch accumulated in rice and thus I measured total starch. Seeds from transgenic plants which expressed the different combinations of transgenes were used to analyze the starch content. I observed that all lines which expressed the full glycolate catabolic bypass pathway and one of the lines expressing only GDH, accumulated more starch compared to wild type plants; however the differences were not statistically significant.

Interestingly, Line 1 and Line 68, which had a higher number of seeds, were the same lines that also had the highest starch content. More starch is related with the capacity of these plants to have better CO_2 fixation; however, further analysis is needed to confirm if this is indeed the case.

3.6 CONCLUSIONS

I have generated the first population of transgenic rice plants expressing three combinations of genes of the glycolate catabolic pathway. I performed phenotypical analyses of the transgenic plants and I measured a positive effect on the performance of the plants. Line 61 expressing GDH and Lines 1 and 3, expressing the full pathway, had better growth performance compared to the other transgenic lines and the wild type. The yield of Line 1, which expressed the full glycolate catabolic bypass pathway, was higher compared to the control lines. However, analyzing the photosynthetic and the photorespiratory activity of the plants under low CO_2 conditions needs to be performed in order to determine if they are able to use the CO_2 produced during the ligation of two molecules of glycolate.

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CHAPTER 4

Generation and molecular characterization of transgenic rice plants engineered with carbonic anhydrases

CHAPTER 4: GENERATION AND MOLECULAR CHARACTERIZATION OF TRANSGENIC RICE PLANTS ENGINEERED WITH CARBONIC ANHYDRASES

4.1 ABSTRACT

In order to increase the yields of major crops, several strategies have been proposed. One strategy is based on the introduction of the components of the carbon concentrating mechanism (CCM) present in specific microalgae. The main components of this mechanism are the carbonic anhydrases (CA) and bicarbonate transporters. I transformed rice embryos with one or both CA (*Cah1* or *Cah3*). These genes were under the control of two different constitutive promoters, the duplicated CaMV *35S* promoter or the maize *Ubi-1* promoter. Regenerated plants were analyzed at the molecular level to confirm expression of the input transgenes. Transcript abundance was measured to determine the effects of the different promoters. Furthermore, the CA activity was confirmed by enzymatic assays.

4.2 INTRODUCTION

Experimental studies have shown that C_3 plants exposed to high $[CO_2]$ have an increase in yield by suppressing the RubisCO oxygenase reaction and in turn photorespiration (McGrath and Long 2014). One of the potential tools to elevate CO_2 around RubisCO is to engineer a cyanobacterial- or algal carbon-concentrating mechanism (CCM) into C_3 plants. This is a very promising alternative compared to alternative strategies of increasing the concentration of CO_2 around RubisCO, such as introducing C_4 enzymes into C_3 plants. Unlike the later approach a strategy based on CCM does not require anatomical changes in the cell. However, the potential benefits of such an approach remain unexplored (McGrath and Long 2014).

Photosynthetic organisms have evolved in a way to elevate the concentration of CO_2 around RubisCO so they decrease or eliminate photorespiration. In C_4 photosynthesis, the carboxysome and pyrenoid CCMs of single-celled cyanobacteria and algae are examples of mechanisms that allow plants to accumulate CO_2 in the vicinity of RubisCO. Converting C_3 crops into C_4 photosynthetic organisms requires substantial changes in tissue differentiation and enzyme and transporter localization. However, the major constraint to this conversion is that all enzymes and transporters must be localized in the appropriate subcellular location and be biochemically active at the appropriate point in time (Miyao et al. 2011).

CO_2 is the basic substrate of photosynthesis. Most plants can take up CO_2 via stomata; however, under water deficiency conditions, plants close their stomata to minimize water loss but at the same time this limits the entry of CO_2 (Raven 2008). In the case of algae, the problem is completely different. As they live submerged under water the available CO_2 is limited, so they evolved to concentrate inorganic carbon (C_i) and thus increasing the supply of CO_2 in the vicinity of RubisCO (Reinfelder 2011).

The algal CCMs have three major components: (1) Ci uptake systems responsible for accumulating high levels of intracellular C_i; (2) enzymatic systems, including various carbonic anhydrases (CAs), catalyzing rapid inter-conversion between different Ci species and acting coordinated with Ci transporters to facilitate C_i uptake and accumulation; and (3) sequester RubisCO in a specified micro-compartment where CO_2 can be elevated (Wang et al. 2011). One of the most studied algal CCM is that of

Chlamydomonas reinhartii, a unicellular green alga used as a model system to study the eukaryotic CCM for decades due to its well known genetic background (Wang et al. 2011).

Green algae CCMs, like the ones found in *Chlamydomonas reinhartii*, operate into two phases. The first phase involves the acquisition of CO_2 from dissolved C_i sources from the aqueous environment. The C_i species have to cross two barriers, first the plasma membrane and then the chloroplast inner envelope to reach the stroma space (Wang et al. 2011). The main components of this phase are carbonic anhydrases (CAs) in the periplasmic space (CAH1) and a CA in the cytoplasm (CAH9) as well as HCO_3^- transporters and CO_2 channels on the plasma membrane and the chloroplast envelope (Moroney and Ynalvez 2007). HCO_3^- is assumed to be the major Ci accumulated, as it is much less permeable to lipid membranes and the alkaline pH favors its formation (Wang et al. 2011). In the second phase, increased levels of HCO_3^- are generated in the chloroplast stroma through the pH gradient across the thylakoid membrane. Cah6, a plastid CA, has been suggested to be involved in the rapid conversion of CO_2 to HCO_3^- in the stroma (Mitra el al. 2004). As HCO_3^- cannot be directly used by RubisCO, and has to be dehydrated to CO_2, a CA located within the thylakoid lumen (CAH3) catalyzes this critical step and releases CO_2 in the vicinity of RubisCO (Moroney and Ynalvez 2007) (**Figure 4.1**).
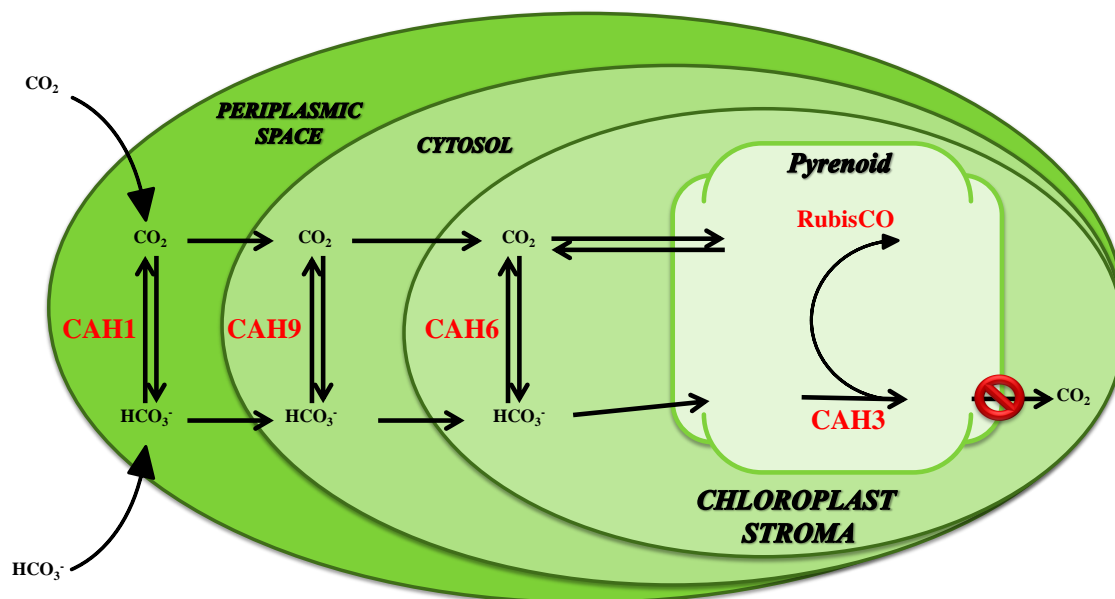
Figure 4.1- Simplified model of the *C. reinhardtii* **CCM.** CO_2 easily penetrates cell membranes and is converted into bicarbonate (HCO_3^-) in the periplasmic space by carbonic anhydrases (Cah1) and thus prevents CO_2 to leak from the plant cell. Bicarbonate is transported to the chloroplast stroma via specific transporter proteins which operate in the cytoplasmic membrane and chloroplast envelope membranes. HCO_3^- diffuses into the chloroplast stroma through pH gradients across the thylakoid membrane. The Cah3 localized in the thylakoid lumen converts HCO_3^- to CO_2 in the vicinity of RubisCO. As CO_2 can easily diffuse from the pyrenoid, a CO_2 recapture system generally prevents leakage of internal CO_2. **RubisCO:** Ribulose-1,5-bisphosphate carboxylase/oxygenase. Adapted from Moroney and Ynalvez (2007).

CAH1 and CAH3 are the main CA found in *C. reinhardtii*. CAH1 facilitates the entrance of CO_2 into the algal cell. This was one of the first CA reported to be responsible for the conversion of HCO_3^- to CO_2 (Moroney and Ynalvez 2007). CAH1 is regulated by environmental changes in CO_2 concentrations and light. Under low CO_2 conditions it is strongly induced and this is when the CCM becomes operational (Fukuzawa et al. 1990). CAH3 is associated with the thylakoid membrane, more specifically inside the pyrenoid tubules (Karlsson et al. 1998; Mitra et al. 2005). Mutants lacking CAH3 cannot grow in ambient levels of CO_2, but they grow normally under high CO_2 levels (Karlsson et al. 1998; Spalding et al. 1983; Moroney et al. 1986). CAH3 mutants accumulate large amounts of C_i but are unable to convert HCO_3^- to CO_2. CAH3 is expressed under low and high CO_2 concentrations, but there is a two-fold increase in message abundance under low CO_2 concentrations (Moroney and Ynalvez 2007).

The *Cauliflower mosaic virus 35S* (CaMV *35S* or *35S*) promoter has been widely used to drive high levels of transgene expression in many plant species. However, gene

expression controlled by the *35S* promoter appears to be specie-dependent (Dutt et al. 2014). Several studies have demonstrated that the expression levels of genes under the control of the CaMV *35S promoter* are usually lower in monocotyledonous (Battaw and Hall, 1990; Benfey et al. 1990) compared to dicotyledonous species (Christensen et al. 1992; Gupta et al. 2001). In my experiments, I used the duplicated CaMV *35S* promoter. This promoter was previously used in tobacco to increase transgene expression (Sack et al. 2007) and I wished to investigate if it had the same capacity to increase transgene expression in rice.

Other constitutive promoters have been used to drive the expression of transgenes in monocot plants, for example the *Z.ea Mays* Ubiquitin 1 promoter (*Ubi-1*) (Christensen et al. 1996) the Actin1 (McElroy et al. 1990), rubi3 (Lu et al. 2008), and OsAct2 (He et al. 2009) from rice. The maize *Ubi-1* promoter is one of the most commonly used promoters in monocots (Bernal et al. 2015). This promoter is active in all cell types and drives strong expression specifically in young roots and leaves (Bernal et al. 2015). The *Ubi-1* promoter has been considered as an ideal candidate due to its ability to drive high gene expression levels in transformed cells (Dutt et al. 2015).

Knowing that the promoter is the most important component regulating the specificity and intensity of expression of transgenes in plants (Zhang et al. 2015), I decided to analyze the effects of both promoters in rice.

4.3 AIMS AND OBJECTIVES

The overall aim of the work described in this chapter was the molecular characterization (at the mRNA level) of transgenic rice plants expressing either one (*Cah1* or *Cah3*) or both CAs (*Cah1* and *Cah3*) under the control of the duplicated CaMV *35S* or the maize *Ubi-1* promoter.

The specific objectives were to:

- Introduce the carbonic anhydrases genes (*Cah1* and *Cah3*) together with a selectable marker gene for hygromycin resistance to generate a population of rice plants containing and expressing these genes.
- Select and regenerate putative transgenic plants.

–   Analyze mRNA expression levels and transcript abundance in transgenic plants.

–   Investigate if the use of the two different promoters had different effects on the levels of expression of the carbonic anhydrase genes.

–   Analyze the enzymatic activity of the carbonic anhydrases in the transgenic rice plants.

4.4 MATERIALS AND METHODS

4.4.1 Transformation vectors

Genes encoding *Chlamydomonas reindhartii* carbonic anhydrase 1 (*Cah1*) and 3 (*Cah3*) were kindly provided by Dr. Stefan Schillberg (Institute for Biology, RWTH-Aachen, Germany). Both genes were independently cloned into the pTRA vector under the control of the duplicated CaMV *35S* promoter and the CaMV *35S* terminator.

Cah1 and *Cah3* were excised from pTRA and transferred into pTRAux which contains the constitutive maize Ubiquitin promoter with its first intron (*Ubi-1*), and the *35S* terminator. The CA genes were inserted into the EcoRI-BamHI sites of pTRAux (**Figure 4.2**).
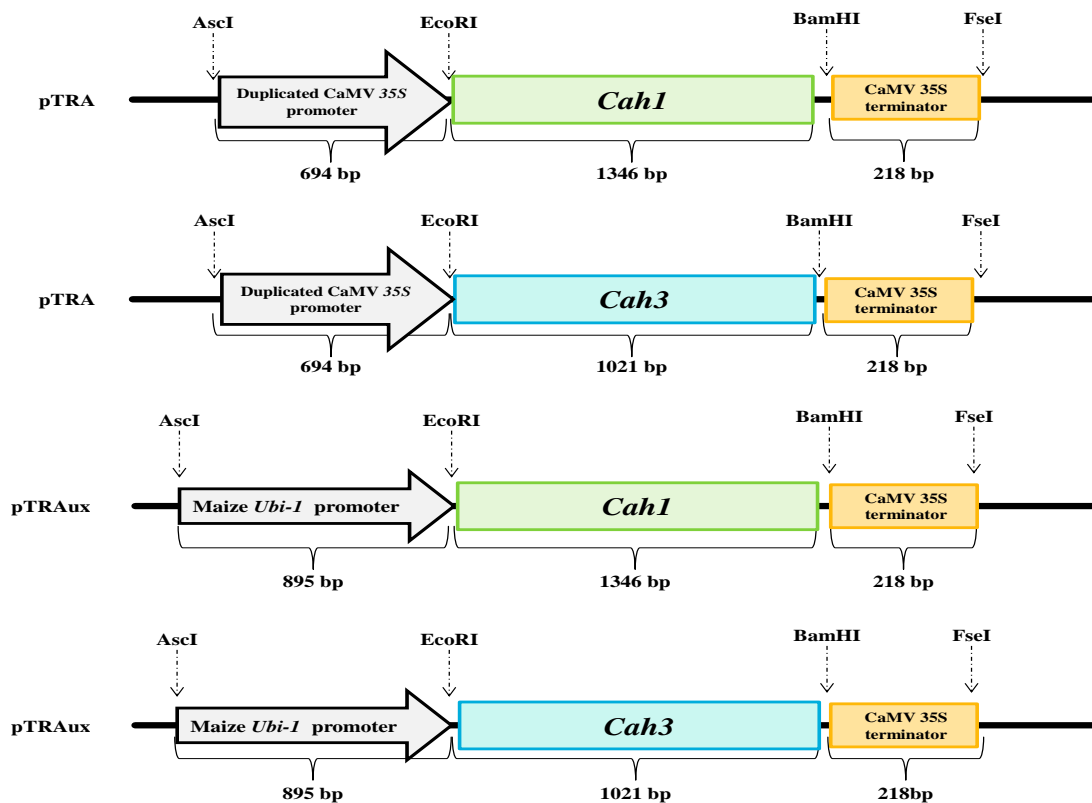
Figure 4.2-Schematic representation of the transgenes used in this chapter.

4.4.2 Rice transformation and plant growth

Mature seed-derived embryos (*Oryza sativa* L. EYI 105) were transformed with the two carbonic anhydrases individually or in combination, and the *hpt* selectable marker as described in Chapter 2, section 2.4.2.

4.4.3 RNA extraction

mRNA extraction in transgenic rice leaves was carried out following the procedure described in Chapter 2, section 2.4.3.

4.4.4 RNA blot analysis

The procedure described in Chapter 2, section 2.4.4 was followed to analyze the expression of the two CA genes.

GENE	PROBE PRIMERS	
	Position	*Sequence*
Cah1	**Forward**	5'- TTTGCCTACTCCACCTCCATT - 3'
	Reverse	5'- ACTGTTGCTTTGGAATCTGGG - 3'
Cah3	**Forward**	5' - CAGTTTCATTTCCACGCACC - 3'
	Reverse	5' - TGGGAGCATAATACCGAGCA - 3'

Table 4.1-Primers used to synthesize DIG-labeled probes for mRNA b4lot analysis.

4.4.5 cDNA synthesis

cDNA synthesis was carried out as described in Chapter 2, section 2.4.5.

4.4.6 Real-time qRT-PCR

Quantitative real-time RT-PCR was carried out to determine the levels of expression of the carbonic anhydrases as described in Chapter 2, section 2.4.6.

GENE	qRT-PCR PRIMERS	
	Position	*Sequence*
*Cah1*_RT	**Forward**	5'- CAGGATTGTTGATGTGCTTGA - 3'
	Reverse	5'- TCCTGCCAAAAGATGTTCACT - 3'
*Cah3*_RT	**Forward**	5' – CGAGGGAGTTGATTGGTTTG - 3'
	Reverse	5' - CCACGAGTCTTGAGTTGAGGA - 3'
*Ubi1*_RT	**Forward**	5' - ACCACTTCGACCGCCACTACT - 3'
	Reverse	5' - ACGCCTAAGCCTGCTGGTT- 3'

Table 4.8-Oligonucleotide sequences of rice ubiquitin and transgenes for Real-Time PCR analysis.

4.4.7 Protein extraction

One hundred µg of young leaves were ground until fine powder and the total soluble proteins were extracted with two volumes of extraction buffer (250 mM sucrose, 100 mM HEPES-KOH pH 7.5, 5% glycerol, 1 mM Na_2MoO_4 x $2H_2O$, 25 mM NaF, 10mM EDTA, 1 mM DTT, 0.5%Triton X-100, protease inhibitor cocktail). The samples were vortexed twice and then centrifuge twice at 13000rpm for 10 min at 4°C to remove plant debris. The proteins were kept on ice or frozen at -20°C until using.

4.4.8 Enzymatic activity of Carbonic anhydrases

Total soluble proteins were extracted from 100 mg ground wild-type and transgenic leaves in 200 µl of extraction buffer (0.2 M Tris-HCl pH 7.5, 5 mM EDTA, and 0.1% Tween-20). One hundred µl of total soluble protein extracts was pippeted into the bottom of a 15ml plastic tube on ice. Carbonic anhydrase from bovine erythrocytes-lyophilized powder (Sigma, St. Louis, USA) was used as a positive control and the extraction buffer as negative control. Six ml of ice cold Trizma buffer (Tris-HCl; pH 8.3) was added and mixed. Four ml of CO_2-saturated water was added and the time required for the pH to drop from 8.3 to 6.3 was recorded. The carbonic anhydrase activity was measured according to the following equation:

Relative activity = $10*(T_{control}/T_{sample} -1)$

$T_{control}$ = time of the uncatalyzed reaction

T_{sample} = time of the enzyme-catalyzed reaction

4.5 RESULTS

4.5.1 Recovery of transgenic rice plants expressing the six different combinations of input transgenes

I used six different combinations of transgenes to co-transform rice mature seed-derived-callus. In three combinations I used the control of the *Cauliflower mosaic virus 35S* (CaMV *35S*) duplicated promoter (Sack et al. 2007) to drive expression of the *Chlamydomonas reinhardtii* carbonic anhydrases (CA). I used two carbonic anhydrases, *Cah1* and *Cah3*, together or individually, simultaneously with the *hpt* gene. In parallel experiments I tested the constitutive maize ubiquitin-1 (*Ubi-1*) promoter. In all six plasmids the CaMV *35S* terminator was used.

A total of 80 hygromycin-resistant putative transgenic plant lines were recovered from the different transgene combinations. Thirty-one independent lines were recovered from experiments where gene expression was under the control of the duplicated CaMV *35S* promoter and 49 independent lines from the experiments where gene expression was under the maize *Ubi-1* promoter. Expression of the input transgenes was analyzed by northern blot and qRT-PCR to confirm expression of the genes.

4.5.2 Expression analysis and molecular characterization of putative transgenic plants

Transgene expression was verified by mRNA blot analysis using leaves from the putative transgenic plantlets. I used gene-specific probes for *Cah1* and *Cah3* to confirm the expression of the input transgenes. As shown in **Figure 4.3**, the mRNA blots confirmed the expression of the carbonic anhydrases (CA) in the regenerated lines. Different combinations of the input transgenes were identified in different plants. In some cases none of the genes of interest were found.
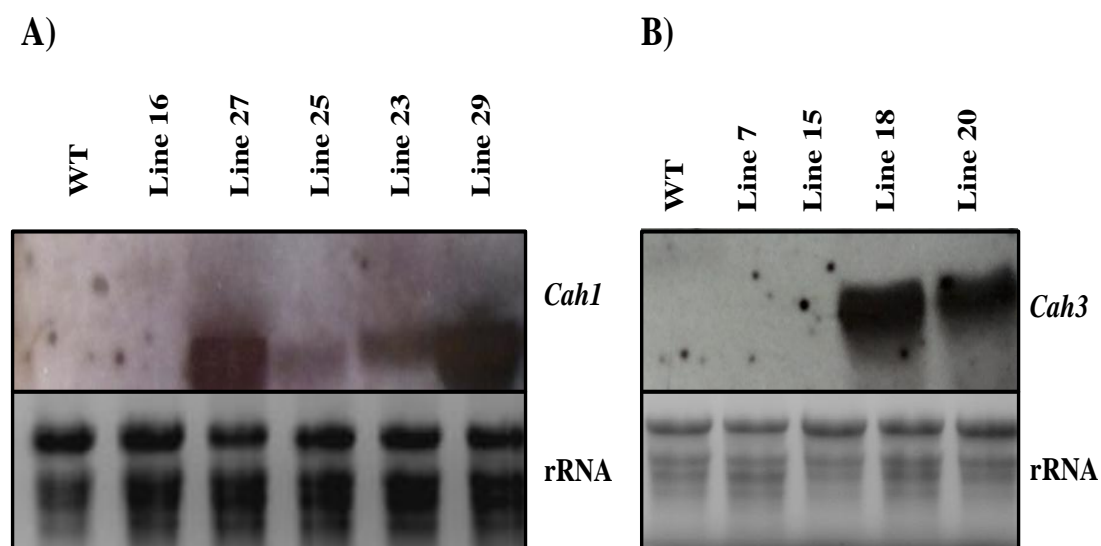
Figure 4.3-mRNA blot analysis showing the expression of *Cah1* and *Cah3* in leaves of T_0 putative transgenic lines. (A) Lines 23, 25, 27 and 29 expressed *Cah1*, whereas line 16 did not express the transgene. (B) Lines 7 and 15 did not express *Cah3*, while lines 18 and 20 expressed the input transgene (C*ah3*).

A total of 80 independent hygromycin-resistant transgenic plant lines were recovered, 31 with the duplicated CaMV *35S* promoter and 49 with the maize *Ubi-1* promoter. Eighteen lines were regenerated from experiments in which the two carbonic anhydrases were co-bombarded under the control of the duplicated CaMV *35S* promoter. Two lines expressed *Cah1* alone, 2 lines expressed only *Cah3* and 11 lines expressed both. I also obtained 3 lines which did not express any of the input genes other than *hpt*. Only 3 lines were regenerated from experiments in which only *Cah1* alone was bombarded under the control of the duplicated CaMV *35S* promoter. Two of those lines expressed *Cah1*, while the other line did not. Ten putative transgenic plants were regenerated containing *Cah3*. I confirmed expression of *Cah3* in 6 lines, whereas 4 lines did not express the transgene.

Nineteen putative transgenic plants were regenerated in which both CA genes were co-bombarded under the control of the maize *Ubi-1* promoter. Two of these lines expressed only *Cah1*. Four lines expressed *Cah3* alone and 5 lines expressed both transgenes. Four lines did not express any of the transgenes. I regenerated 20 lines which were bombarded with *Cah1* under the control of the maize *Ubi-1* promoter; I confirmed the expression of that transgene in 14 lines. Ten lines were recovered from the lines bombarded with *Cah3*. Expression of the transgene was confirmed in 7 of those lines. Regenerated plants were grown to maturity in a growth chamber. **Tables 4.2** and **4.3**

provide a summary of the lines regenerated after bombardment with the different combinations of genes and promoters.

	Lines regenerated	*Cah1* expression	*Cah3* expression	*Cah1 + Cah3* expression	No expression
Duplicated CaMV *35S*: *Cah1*:*Cah3*	18	2	2	11	3
Duplicated CaMV *35S*: *Cah1*	3	2	-	-	1
Duplicated *35S* CaMV: *Cah3*	10	-	6	-	4
TOTAL	31	4	8	11	8

Table 4.2-Lines regenerated with the genes under the control of the duplicated CaMV *35S* promoter.

	Lines regenerated	*Cah1* expression	*Cah3* expression	*Cah1 + Cah3* expression	No expression
Ubi-1*: *Cah1*:*Cah3	19	2	4	9	4
Ubi-1*: *Cah1	20	14	-	-	6
Ubi-1*: *Cah3	10	-	7	-	3
TOTAL	49	16	11	9	13

Table 4.3-Lines regenerated with the genes under the control of the maize *Ubi-1* promoter.

4.5.3 Molecular characterization of putative transgenic plants

I also analyzed the transcript abundance in the regenerated lines to investigate if there were any differences among the transcript abundance of those genes which were under the control of the duplicated CaMV *35S* promoter and the maize *Ubi-1* promoter. qRT-PCR showed that there appeared to be higher transcript abundance in those lines where gene expression was driven by the duplicated CaMV *35S* promoter (**Figure 4.4**, **Figure 4.5**). As the experiments were performed with T_0 plants it was not possible to include biological replicates, as in some cases, only one plant was regenerated. However, it is important to analyze subsequent generations to ascertain stability of transgene expression.
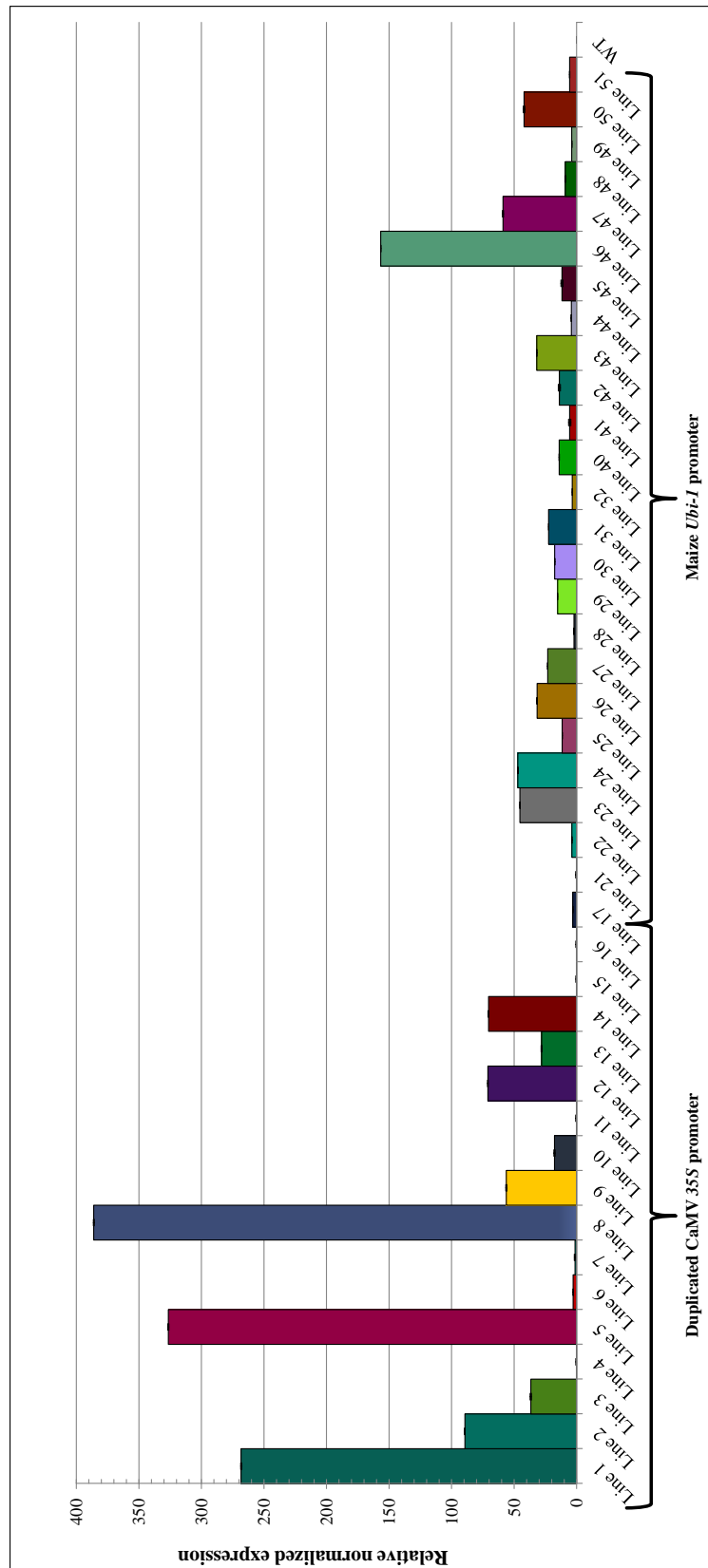
Figure 4.4-*Cah1* transcript accumulation normalized against ubiquitin in wild type (WT) and transgenic lines presented as mean of three technical replicates. Lines 1-17 are different independent lines expressing *Cah1* under the control of the duplicated CaMV *35S* promoter. Lines 21-51 are different independent transgenic plants expressing *Cah1* under the control of the maize *Ubi-1* promoter.
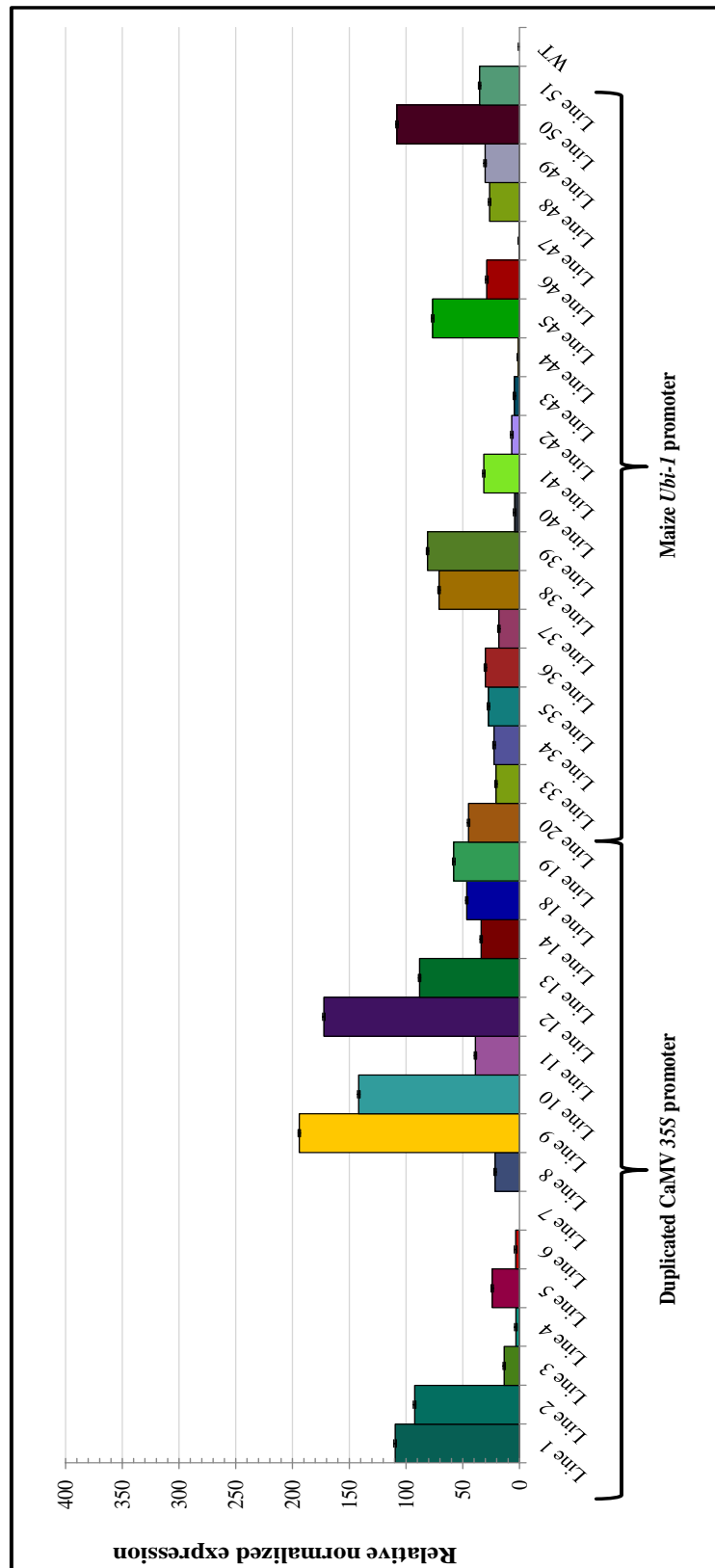
Figure 4.5-*Cah3* transcript accumulation normalized against ubiquitin in wild type (WT) and transgenic lines presented as mean of three technical replicates. Lines 1-14 are different independent lines expressing *Cah3* under the control of the duplicated CaMV *35S* promoter. Lines 18-51 are different independent transgenic plants expressing *Cah3* under the control of the maize *Ubi-1* promoter.

4.5.4 Enzyme activity assays confirm the functionality of the carbonic anhydrases

Carbonic anhydrases (CA) catalyze the reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. The CA activity in the transgenic rice plants was obtained by the Wilbur Anderson or electrometric method (Wilbur and Anderson 1948). This assay measures the time required for a saturated CO_2 solution to lower the pH from 8.3 to 6.3 at 0°C. The total soluble protein of one T_0 representative line expressing each combination transgene driven by the two different promoters was used to analyze enzymatic activity. As shown in **Figure 4.6**. Transgenic plants had active carbonic anhydrases, as the soluble extracts were able to decrease the pH from 8.3 to 6.3.
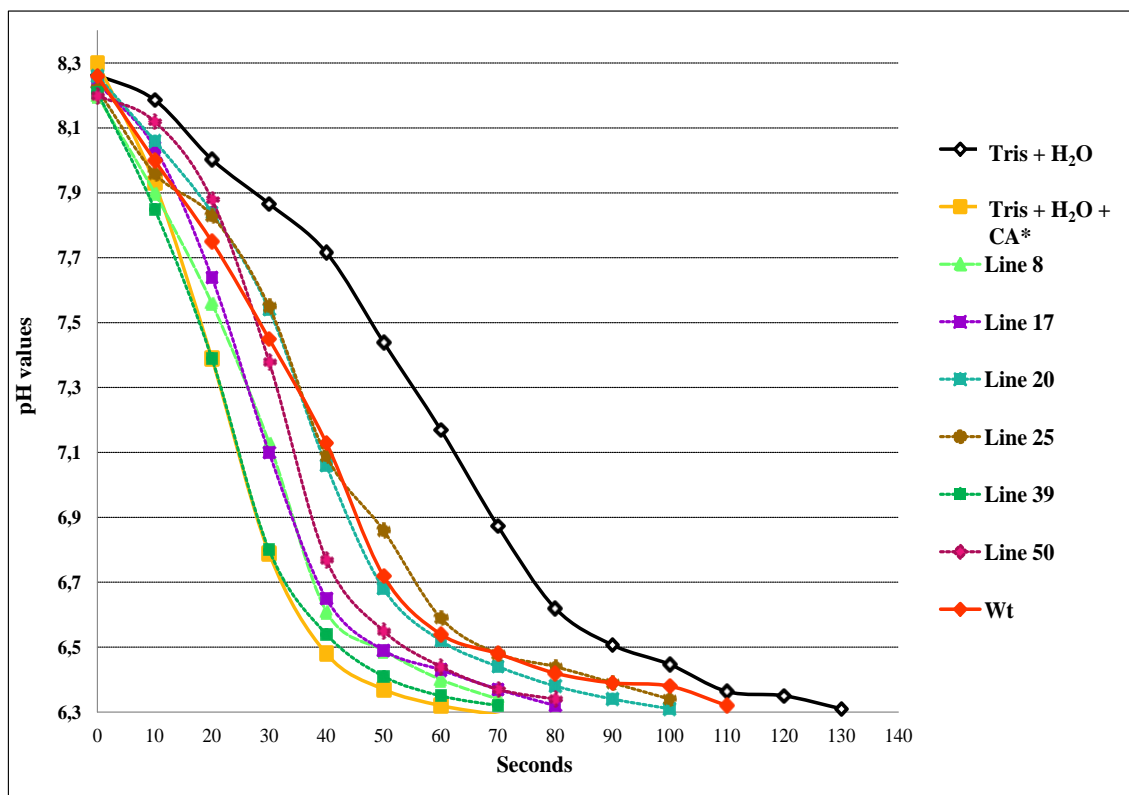
Figure 4.6-CA activity in one representative line of each combination of input transgenes. Line 8 expressed *Cah1* and *Cah3* under the control of the duplicated CaMV *35S* promoter. Line 17 expressed *Cah1* under the control of the duplicated CaMV *35S* promoter. Line 20 expressed *Cah3* under the control of the duplicated CaMV *35S* promoter. Line 25 expressed *Cah1* under the control of the maize *Ubi-1* promoter. Line 39 expressed *Cah3* under the control of the maize *Ubi-1* promoter. Line 50 expressed *Cah1* and *Cah3* under the control of the maize *Ubi-1* promoter.

To analyze the relative activity of the carbonic anhydrases I used the formula: *Relative activity= 10*($T_{control}$/T_{sample} -1)*. Significant results were obtained in Line 50, which had a similar activity as the one obtained from the positive control. On the other hand, lines 20 and 39 had lower activity, similar to the WT plants. Lines 8, 17 and 25 had an

intermediate activity as they were more active than the carbonic anhydrases in line 50 or the positive control, but more active that the WT or lines 8, 17 and 25.

SAMPLE	WILBUR ANDERSON UNITS		
CA$^+$ control	6.87	±	0.45 a
Line 8	6.00	±	0.71 ab
Line 17	4.32	±	0.12 bc
Line 20	4.14	±	0.21 c
Line 25	5.17	±	0.45 bc
Line 39	4.08	±	0.21 c
Line 50	6.05	±	0.91 a
WT	3.30	±	0.33 c

Table 4.4-Enzymatic activity of the carbonic anhydrases in the transgenic plants expressing the different combinations of genes and presented as the mean of three biological replicates. Different letters correspond to statistical significant different groups at p<0.05. Lines not sharing the same letter are significantly different (Tukey HSD; p<0.05).

4.6 DISCUSSION

Improving crop yield is a critical issue in order to meet the predicted food production requirement by 2050 (Long et al. 2015). Major crops such as rice and wheat fix CO_2 by the C_3 photosynthetic pathway. The yield of plants which use the C_3 photosynthetic pathway is lower due to the capacity of RubisCO, the main enzyme the photosynthetic pathway, to fix CO_2 and O_2 (Rolland et al. 2016). Enhancing photosynthetic capacity has been identified as a promising approach to increase crop productivity (Yamori et al. 2016). To circumvent the limited photosynthetic efficiency of C_3 plants, four general strategies describe the main efforts that have been used to increase the yield of photosynthetic CO_2-fixation: (1) improving the catalytic properties of RubisCO; (2) improving the performance of RubisCO through CO_2-concentraing mechanisms (CCMs); (3) engineering synthetic photorespiratory bypasses and (4) engineering synthetic CO_2-fixation pathways (Erb and Zarzycki 2016). It has been recently proposed to use the cyanobacteria or algal CO_2-concentrating mechanisms (CCM) (Rolland et al. 2016). In this chapter I focused on the second strategy, i.e. the improvement of the efficiency of RubisCO through CCMs. Introducing an algal CCM into higher plants is

less technically challenging than engineering C_4 photosynthetic pathways as less genes are required (Price et al. 2013)

During evolution, different CCMs have emerged naturally, for example in C_4 plants, CO_2 is pre-fixed in special compartments into a C_4 acid, malate, which is subsequently transported to the compartment where RubisCO is localized, then this C_4 acid is decarboxylated increasing the local $CO_2:O_2$ ratio in the vicinity of RubisCO (Sage et al 2012). Cyanobacteria and algae, on the other hand, have evolved HCO_3^- transporters and CO_2 uptake systems which enables them to concentrate up to 40mM HCO_3^- intracellularly (Price et al. 2008).

Carbon concentrating mechanisms involve inorganic carbon transporters (C_i) at the plasma membrane and chloroplast envelope and carbonic anhydrases (CA) which act in concert to increase ambient concentrations of CO_2 in the vicinity of RubisCO, usually within a chloroplast microcompartment called pyrenoid in the case of algae and carboxysomes in cyanobacteria (Atkinson et al. 2015). At least 14 genes are considered to be important in order to maintain functional CCM under ambient or below ambient CO_2 concentrations including five C_i transporters, four CAs, two pyrenoid peripheral proteins, a putative methyl transferase and two nuclear transcription regulators (Wang et al. 2015). In this chapter I focused on the introduction of two of these genes, the *Cah1* and *Cah3* carbonic anhydrases.

I regenerated a population of rice plants expressing one or both CA (*Cah1* and/or *Cah3*) from *Chlamydomonas reinhardtii* in order to ascertain the role of these enzymes and their capacity to increase rice yield. Six different combinations of transgenes were used in these experiments. In three combinations I used the duplicated CaMV *35S* promoter to control the expression of one or both carbonic anhydrases (*Cah1* and/or *Cah3*), simultaneously with the hygromycin selectable marker gene (*hpt*). In another series of experiments I used the same combination of transgenes under the control of the maize *Ubi-1* promoter. Eighty independent lines were generated that expressed the different combinations of the input transgenes. Analyses were carried out at the mRNA level, confirming the expression of the transgenes. In addition accumulation of the transcripts was also demonstrated by qRT-PCR.

I also investigated if there were any differences between the duplicated *35S* CaMV promoter and the maize *Ubi-1* promoter in terms of transcript abundance of the transgenes and if that difference was also reflected in the CA enzymatic activity. The most widely used promoter for directing constitutive expression in transgenic plants is the CaMV *35S* promoter, especially in dicots, where it shows a strong and constitutive expression. However, in cereals, this promoter shows a very low activity; therefore the duplicated CaMV *35S* promoter is preferred in monocots (Fischer and Schillberg 2006). On the other hand, the maize *Ubi-1* promoter is preferred for constitutive expression in cereals (Christensen et al. 1996). The maize *Ubi-1* promoter has been widely used in monocots due to its ability to direct high levels of gene expression in all tissues (Park et al. 2010). qRT-PCR analyses showed that in general, those lines whose genes were under the duplicated CaMV *35S* promoter had a higher transcript accumulation compared to the ones where the genes were driven under the maize *Ubi-1* promoter.

The experiments I carried out in this chapter were with T_0 lines, and in some cases it was not possible to analyze biological replicates, which would give a better understanding of the results obtained.

One representative line of each CA combination was used to carry out enzymatic activity assays in order to investigate if the heterologous enzymes were able to convert CO_2 to HCO_3^-. The activities of the CA were measured electrochemically by monitoring the rate of pH change via the Wilbur-Anderson enzymatic assay. Activity was confirmed as the protein extracts were able to decrease the pH from 8.3 to 6.3, as expected. In some cases, the activity was lower than the positive control, but always we found that the activity was higher compared to wild type plants, confirming that the carbonic anhydrases were expressed and were active in the transgenic plants. Future work should consider the introduction of the different C_i transporters, such as LYCA and HLA3 to facilitate the movement of C_i across membranes (Atkinson et al. 2015).

Our attempts to create the first population of transgenic plants expressing the main carbonic anhydrases involved in the microalgal CCM have been successful. Furthermore, the regeneration of these transgenic plants, expressing one or two carbonic anhydrases, allowed me to carry out phenotypic analysis of the plants which is described in detail in Chapter 4.

4.7 CONCLUSIONS

I regenerated a population of transgenic rice plants which expressed the genes of the two carbonic anhydrases from *Chlamydomonas reinhardtii*. qRT-PCR revealed that the transcript accumulation of those genes whose activity was driven by the duplicated 35S CaMV promoter was higher compared to those that were driven under the *Ubi-1* promoter. However, the results presented in this chapter were carried out with T_0 plants and it must be confirmed in subsequent generations. The aim of my experiment was to create a population of transgenic plants which could provide preliminary results and a better understanding of the capacity of the CA to increase the yield of rice. Future studies should be focused on the introduction of the other players in the CCM, such as C_i transporters, which most likely would increase the biomass of rice.

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CHAPTER 5

Phenotypic analysis of transgenic plants engineered with carbonic anhydrases

CHAPTER 5: PHENOTYPIC ANALYSIS OF TRANSGENIC PLANTS ENGINEERED WITH CARBONIC ANHYDRASES

5.1 ABSTRACT

Chlamydomonas reinhardtii is the most studied eukaryotic model of carbon concentrating mechanism (CCMs). The introduction of this mechanism into C_3 plants is expected to increase the concentration of CO_2 in the vicinity of RubisCO and as a consequence, increase the biomass of C_3 plant. One of the components of the CCM is carbonic anhydrases (CA). In this chapter I used transgenic rice plants expressing one (*Cah1* or *Cah3*) or the combination of both CA, under the control of the duplicated CaMV *35S* or the maize *Ubi-1* promoter, to investigate any phenotypic effects that expression of the genes had on transgenic rice plants. Several parameters, such as plant height, number of leave and tillers, surface area of the last expanded leaf and the chlorophyll content; were analyzed. My preliminary data suggest that the introduction of CA into rice chloroplasts could be a useful means to increase the yield and biomass of rice plants.

5.2 INTRODUCTION

Several strategies have been pursued to improve the yield of C_3 crops such as rice. These were disued in detail in Chapter 1. A recent strategy to achieve this objective focused on introducing the bicarbonate transporters and CO_2-uptake complexes of the eukaryotic algal and cyanobacterial systems (Long et al. 2016). These CO_2-concentrating mechanisms (CCMs) are important in terms of overall productivity as mathematical modeling shows that introduction of a CCM can increase the yields of crops by 36-60% in C_3 crops (Long et al. 2016). Additionally, to overcome the limitations that RubisCO has in terms of its ability to fix both CO_2 and O_2, terrestrial plants had to evolve in a way to develop mechanisms to concentrate CO_2. Microalgae and cyanobacteria have evolved highly effective CCMs (Long et al. 2016). The three major components of the CCMs are membrane inorganic carbon transporters, a suite of carbonic anhydrase enzymes in specific subcellular compartments such as the periplasmic space or the pyrenoid, and a micro compartment in which RubisCO is localized, the chloroplast pyrenoid, or the carboxysomes in the case of cyanobacteria (Meyer and Griffiths 2013; Price et al. 2012).

Four steps have been proposed to be followed in order to engineer the cyanobacterial or algal CCMs into C_3 species (Price et al. 2013). The first step is to introduce the bicarbonate transporters into the chloroplast of the host genome to increase the CO_2 levels in the chloroplasts and thus improve the photosynthetic performance of the plant. The second step involves building a functional carboxisome or pyrenoid into the chloroplasts of the host plant, although most of the proteins involved in the organization of these compartments are still unknown (Rae et al.2013). The third step focuses on the elimination of the CA activity from the stroma. If the stromal CA activity is eliminated, the HCO_3^- concentrations will increase up to 2-3 mM, which are more than half the concentration to saturate CO_2 fixation in the newly formed carboxysomes. The last step would require the complex addition of a NDH-1-based (NADPH dehydrogenase type 1) CO_2 pump (Price et al. 2013).

Membrane transporters have been suggested as one of the main components to be introduced into C_3 plants (HLA3, LC11, LCIA or CCP1/2) mainly to reduce the deficit in the steady-state CO_2 concentration between the intracellular space and that in the chloroplast. Increasing the yield of C_3 plants by using the CO_2-fixation approach used in

algae and cyanobacteria is very ambitious. No successful reconstruction of this CO_2-fixation model has been demonstrated in any C_3 plant, most likely because of the complex interplay and interference with the host's native carbon and energy metabolism (Erb and Zarzycki 2016). Further research is needed to develop a definitive molecular and structural basis to the chloroplast pyrenoid as they are essential to ensure that inorganic accumulation is not negated by CO_2 leakage (Price et al. 2011). In Chlamydomonas, the leakage of CO_2 is reduced by the recapturing barrier localized in the pyrenoid matrix. This barrier helps to maintain a high concentration of Ci in the stroma by recapturing the CO_2 leacking from the pyrenoid matrix and transferring to the stromal carbonic anhydrase CAH6. The main proteins involved in this function are LCIB and LCIC (Yamano et al. 2010). The CAs, which catalyze the reversible hydration and dehydration of CO_2 and HCO_3^-, are a key element in the algal and cyanobacterial CCMs. Therefore, it is necessary to determine the mechanisms by which inorganic carbon is delivered to the pyrenoid and the interplay between intra-pyrenoid thylakoid carbonic anhydrases (CA) and the putative inorganic carbon transport (**Figure 5.1**).
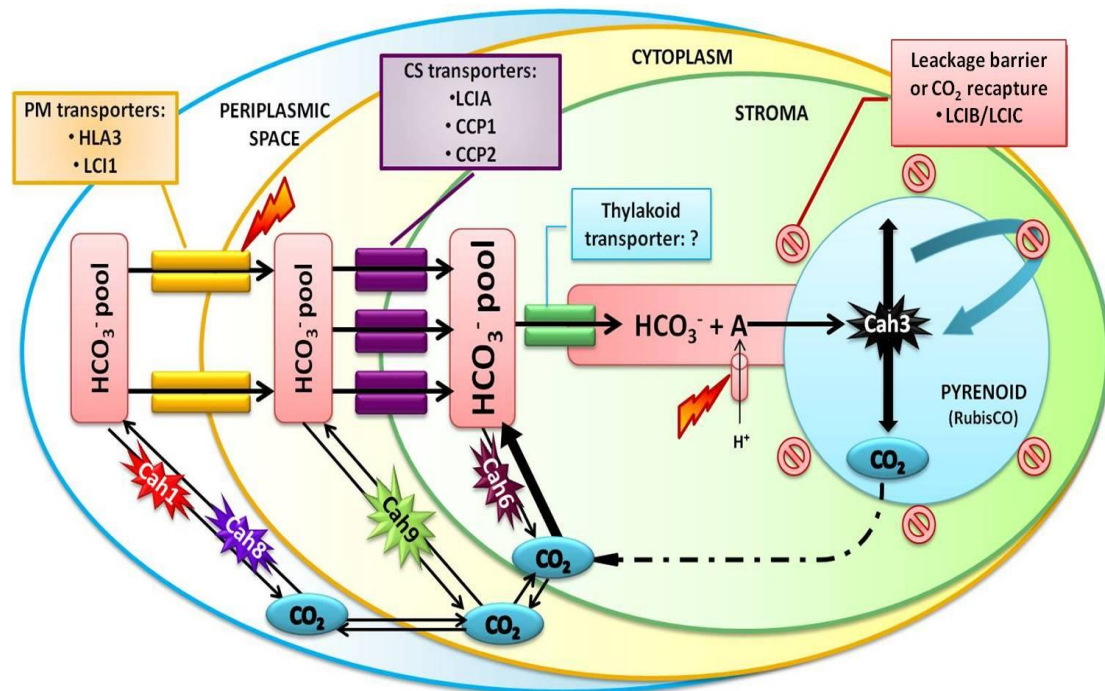
Figure 5.1-Hypothetical model of the *Chlamydomonas reinhardtii* biophysical CCM. The mechanism of CO_2 delivery to RubisCO in *Chlamydomonas* is as follows. First there is an active transport of bicarbonate from the extracellular environment to the cytosol, then to the stroma and finally to the lumen of the pyrenoid. Luminal bicarbonate is converted to CO_2 by a resident CA (CAH3). In the pyrenoid there is a barrier to, or recapture of, CO_2 leaking from the pyrenoid. This barrier is composed by two complexes, namely LCIB/LCIC and a stromal CA (CAH6) and equilibration of charges by proton pumps. In the plasma membrane there are two putative inorganic carbon transporters (ATP-dependent HLA3 and LCI1), and three at the chloroplast membrane (LCIA and two isoforms of the chloroplast carrier protein CCP), however their exact localization is unknown. Adapted from Meyer et al. 2013.

Recently, the expression of a bicarbonate transporter (LCIA or LCIB) or a CA (CAH1 or CAH3), has been reported in tobacco plants (Nölke et al. 2016). Those plants that express one of the components (LCIA, LCIB, CAH1 or CAH3) had a faster growth rate and/or faster vegetative phase, which is the period of growth between germination and flowering. Therefore, tobacco plants expressing one of the CCMs components (LCIA, LCIB, CAH1 or CAH3) flowered earlier and the seeds were mature earlier than the WT plants. In this chapter I focused on the phenotypic analyses of the transgenic plants I described in Chapter 4. These plants expressed one or both CA (*Cah1* and/or *Cah3*) under the control of two different constitutive promoters: the duplicated CaMV *35S* promoter or the maize *Ubi-1* promoter.

5.3 AIMS AND OBJECTIVES

The overall aim of this chapter was to analyze the effects of the introduced CA in rice plants.

The specific objective was to:

- Analyze the phenotypic effects of the introduced CA (*Cah1* and/or *Cah3*) in the transgenic rice plants under the control of two different constitutive promoters (the maize *Ubi-1* or the duplicated CaMV *35S* promoter). I measured the height of the plants, the number of leaves, the number of tillers, the area of the last expanded leaf and the chlorophyll content of the plants expressing one or both CA (*Cah1* and/or *Cah3*).

5.4 MATERIALS AND METHODS

5.4.1 Plant material

Transgenic rice (*Oryza sativa* L. *cv* EYI-105) plant lines expressing one or both carbonic anhydrases (*Cah1* and/or *Cah3*) were generated as described in Chapter 2.

5.4.2 Germination and growth conditions

Seeds from transgenic plants were germinated and grown under the conditions described in Chapter 3, section 3.4.2.

5.4.3 RNA extraction

RNA extraction was carried out following the procedure described in Chapter 2, section 2.4.3.

5.4.4 cDNA synthesis

cDNA synthesis was carried out following the procedure described in Chapter 2, section section 2.4.5.

5.4.5 Real-time qRT-PCR

The procedure described in Chapter 2, section 2.4.6, was followed. The primers used for the qRT-PCR are described in detail in **Table 2.3**, Chapter 2.

5.4.6 Phenotypic analysis

T_1 seeds from different transgenic lines were germinated and analyzed every two weeks to measure different parameters as described below. Plants were maintained as described in Chapter 2 until seed maturity.

5.4.6.1 Measurement of chlorophyll content

The protocol is described in detail Chapter 3, section 3.4.6.1.

5.4.6.2 Area of the last expanded leaf

The protocol is described in detail Chapter 3, section 3.4.6.2

5.4.7 Statistical analysis

A general linear model (GLM) in which genes, promoters and weeks were nested to type (transgenic vs. wild type) was used to analyze the different values measured in the experiment Traits were compared by Tukey's mean separation procedure ($p < 0.05$). All the analyses were performed using the JMP Pro (JMP®, Version 11.0.0. SAS Institute Inc. Cary, NC, 2013).

5.5 RESULTS

5.5.1 Transgene expression

Transgene expression in plants was determined by quantitative real-time RT-PCR. As shown in **Figure 5.2**, all transgenes expressed in the transgenic lines used to carry out further phenotypic analyses.

A)
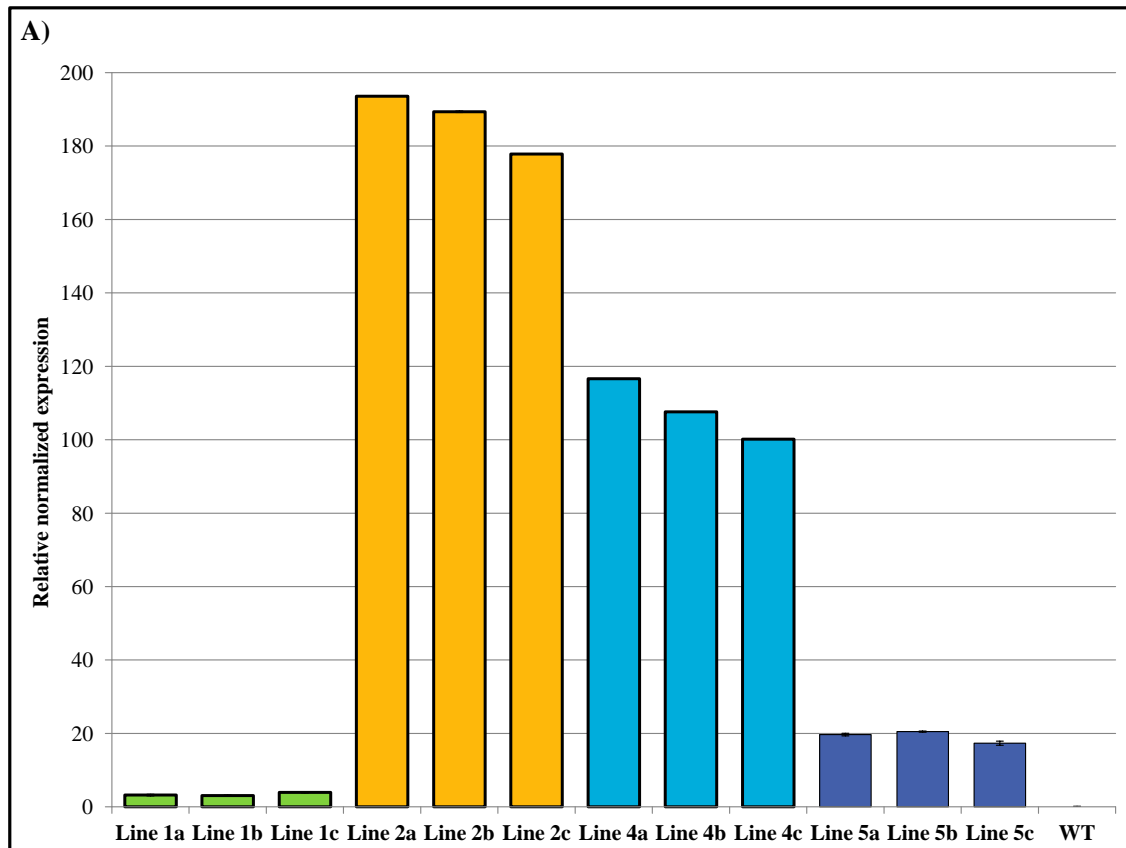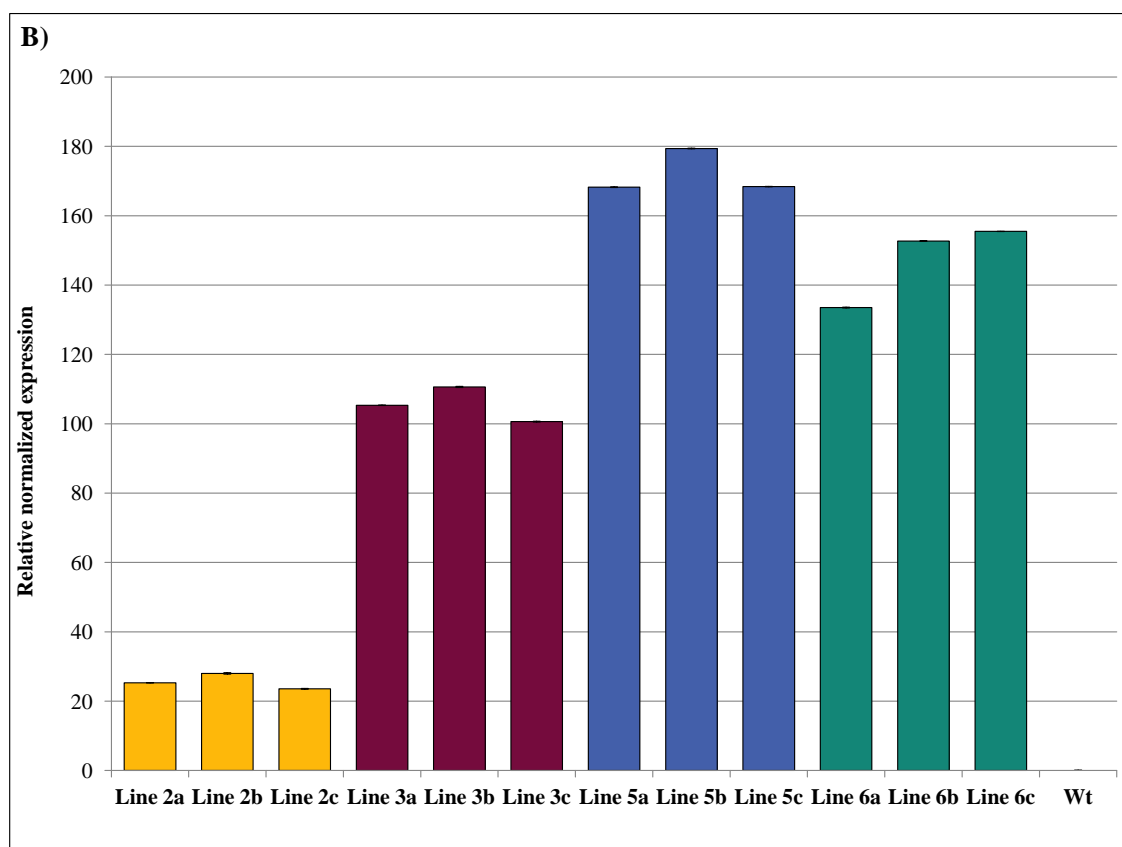
Figure 5.2–Relative mRNA expression of the carbonic anhydrase genes normalized against ubiquitin mRNA and presented as the mean of three replicates ± SE. A) *Cah1* gene expression under the control of the duplicated CaMV *35S* promoter (Lines 1 and 2) and the maize *Ubi-1* promoter (Lines 4 and 5) in T_1 lines. **B)** *Cah3* gene expression under the control of the duplicated CaMV *35S* promoter (Lines 2 and 3) and the maize *Ubi-1* promoter (Lines 5 and L 6) in T_1 lines.

5.5.2 Phenotypic analysis in plants expressing the carbonic anhydrases

Plant height, the total number of leaves and tillers, the surface area of the last expanded leaf and the chlorophyll content in the last expanded leaf, were measured in transgenic rice plants that expressed one or both CA anhydrases (*Cah1* and/or *Cah3*). Three siblings from each independent line were analyzed every two weeks from the date the plants were transferred to soil.

PROMOTER	EXPRESSED TRANSGENES	PLANT LINES
Duplicated CaMV *35S* promoter	*Cah1*	1
	Cah1 + Cah3	2
	Cah3	3
Maize *Ubi-1* promoter	*Cah1*	4
	Cah1 + Cah3	5
	Cah3	6
		WT

Table 5.1-Independent transgenic lines used for phenotype analysis. Three biological replicates were used per line. Transgene expression per biological replicate is shown in Figure 5.1. WT: wild type.

I used a general linear model (GLM) in which genes, promoters and weeks were nested to type (transgenic vs. wild type) to analyze the different values measured in the experiment. The interaction *gene*promoter*week[type]* only resulted in statistically significant differences in chlorophyll content. However, *gene [type]*, *promoter [type]* and *genes * promoter [type]* showed statistically significant differences in most of the parameters measured. The *gene [type]* was significant in all the measurements, therefore the introduction of the CA (*Cah1* and/or *Cah3*) might be influencing the parameters measured (plant height, number of leaves, number of tillers, surface area of the last expanded leaf and chlorophyll content). The *promoter [type]* was statistically significant in plant height, surface area of the last expanded leaf and chlorophyll content of the last expanded leaf. The *gene * promoter [type]* was only statistically significant for plant height, number of leaves and surface area of the last expanded leaf.

The introduction of one or two CA (*Cah1* and/or *Cah3*) under the control of the two different constitutive promoters resulted in a statistically significant change in the height of the transgenic plants compared to wild type. The *gene [type]* and *promoter [type],* as well as their interaction showed that the introduction of these genes might be influencing the height of the plants. Lines 1 and 4, which only expressed *Cah1* under the control of the duplicated CaMV *35S* promoter and the maize *Ubi-1* promoter, respectively, and Line 2, which expressed both CA under the control of the duplicated CaMV *35S* promoter, where the tallest, ca: 56.3 cm, 59 cm and 59.7 cm respectively, compared to the other transgenic lines or the WT, ca: 51cm (**Figure 5.3**).

My preliminary results showed that the total number of leaves was higher at the end of the experiment (week 12th) irrespective of the promoter or transgene used. In the case of the lines where one or both CA expressed, the expression of *Cah1* and *Cah1* together with *Cah3* resulted in ca: 49.6% and ca: 55.17% increase in the number of leaves, respectively. Line 3, which expressed *Cah3* under the duplicated CaMV *35S* promoter, was the line which had a lower increase in the number of leaves, 20.7%, compared to wild type plants. Those lines that expressed one or both CA under the control of the maize *Ubi-1* promoter showed an increase in the number of leaves at the end of the experiment (week 12th). Line 4, which expressed *Cah1* had a 53.2% increased number of leaves, compared to wild type plants. Line 5, which expressed *Cah1* and *Cah3*, had a 33.7% increase in the number of leaves. I would have expected that introduction of both transgenes would result in a more substantial increase in the number of leaves, as was the case for Line 2 which expressed both CA. Line 6, which expressed *Cah3* only, had a 45.8% increase in the number of leaves (**Table 5.3**; **Figure 5.4**). These results suggest that in the general linear model (GLM), as the promoter nested to type is not significantly different, the use of the two different promoters does not affect the number of leaves. Thus the introduced transgenes influence the number of leaves. However, it is necessary to analyze additional independent lines expressing the genes (*Cah1* and/or *Cah3*) to be able to draw a more robust conclusion.

The total number of tillers was also affected by the expression of CA. The lines which expressed *Cah1* alone had a significantly higher number of tillers compared to the wild type plants, independently of the promoter used. Line 1, which expressed *Cah1* alone under the control of the duplicated CaMV *35S* promoter, had a ca: 21.9% increase in the number of tillers. Line 4, which also expressed *Cah1* alone under the maize *Ubi-1* promoter, had a 24.2% increase in the number of tillers at the end of the experiment (week 12th). Interestingly, introduction of *Cah3* under the control of the duplicated CaMV *35S* promoter and introduction of *Cah1* together with *Cah3*, under the control of the maize *Ubi-1* promoter resulted in a decrease in the number of tillers. Even though these lines had less tillers compared to WT plants, the number of leaves was still increased, compared to WT plants (**Table 5.3**; **Figure 5.5**). These results indicated in GLM, as the factor promoter nested to type and the interaction between them was not significantly different, meaning that the use of the two different promoters does not affect the number of leaves. Thus the introduced transgenes influence the number of

leaves. The experiment needs to be repeated with more lines in subsequent generations to ascertain if this trait is constant and if this is also affecting the number of grains in the plants, as it is expected that an increase in the number of tillers will translate to an increase in the number of seeds produced by the plants.

The surface area of the last expanded leaf was only increased in three lines at the end of the experiment (week 12th). Line 2 which expressed *Cah1* and *Cah3;* and Line 3 which expressed *Cah3*, under the control of the duplicated CaMV *35S* promoter showed an 11.2% and 6.52% increase in the area of the last expanded leaf, respectively. On the other hand, there was only an increase (ca: 16.28%) in the area of the last expanded leaf in Line 4, which expressed *Cah1* under the control of the maize *Ubi-1* promoter (**Table 5.3**; **Figure 5.6**). The GLM results showed that genes *[type]* and *promoter [type]* as well as their interaction (*genes*promoter [type]*) were significantly different. In this case the use of the two different promoters affected the surface area of the last expanded leaf, as two of the three lines where the genes were under the control of the maize *Ubi-1* promoter had similar or smaller surface area.

Analyses of more independent lines expressing the CA genes under the control of the two different promoters is necessary to be able to confirm these preliminary findings.

The chlorophyll content was also affected by the expression of the transgenes, but the interaction of both factors (*gene*promoter[type]*) was not statistically significant. The preliminary results showed that there appeared to be a trend in those lines which expressed *Cah1* alone to have higher chlorophyll content, compared to the other lines. Line 1 which expressed *Cah1* alone under the control of the duplicated CaMV *35S* promoter, had a ca: 4.54% more chlorophyll content. Line 4, which expressed *Cah1* alone under the control of the maize *Ubi-1* promoter, had 6.73% more chlorophyll (**Table 5.3**; **Figure 5.7**). Increasing the number of independent lines as well as the lines expressing the different CA (*Cah1* and/or *Cah3*) is necessary to confirm if expression of *Cah1* alone is responsible in increasing the chlorophyll content.

Only one line per combination of CA (*Cah1* and/or *Cah3*) and promoter (duplicated CaMV *35S* promoter or maize *Ubi-1* promoter) were used to carry out phenotypic analyses. This chapter provides preliminary data and it is necessary to carry out experiments through subsequent generations and with a higher number of independent

lines to confirm if the introduction of CA results in an increase in the yield and biomass of rice.

FACTORS	d.f	PLANT HEIGHT (cm)		TOTAL NUMBER OF LEAVES		TOTAL NUMBER OF TILLERS		AREA OF THE LAST EXPANDED LEAF (cm^2)		CHLOROPHYLL CONTENT	
		F value	Prob > F	F value	Prob > F	F value	Prob > F	F value	Prob > F	F value	Prob > F
Week	5	91.8691	<.0001	29.4342	<.0001	28.8747	<.0001	85.0387	<.0001	14.9079	<.0001
Type	1	0.0413	0.8394	27.3107	<.0001	10.2942	0.0019	2.116	0.1495	1.0736	0.3031
Type*Week	5	2.8414	0.0203	4.5472	0.001	1.6451	0.157	1.576	0.1757	5.8516	0.0001
Genes[Type]	2	20.3089	<.0001	13.1343	<.0001	7.2678	0.0012	16.4129	<.0001	28.5586	<.0001
Promoter[Type]	1	14.5889	0.0003	0.2478	0.6199	3.3522	0.0707	30.1661	<.0001	13.313	0.0005
Genes*Promoter[Type]	2	9.594	0.0002	4.5973	0.0127	1.6161	0.2048	10.2621	0.0001	2.9244	0.0592
Genes*Week[Type]	10	2.9236	0.0034	2.0237	0.0406	1.6346	0.1108	2.2263	0.0236	2.1132	0.032
Promoter*Week[Type]	5	0.3081	0.9068	0.6074	0.6944	1.0251	0.4083	1.2366	0.2994	0.4565	0.8075
Genes*Promoter*Week[Type]	10	0.5881	0.8194	0.3865	0.9493	0.2974	0.98	1.1	0.3718	2.2356	0.023

Table 5.2-General linear model (GLM) results. Values under p<0.05 are significantly different.

PROMOTER	EXPRESSED TRANSGENES	PLANT LINE	PARAMETER MEASURED				
			PLANT HEIGHT	NUMBER OF LEAVES	NUMBER OF TILLERS	AREA OF THE LAST EXPANDEND LEAF	CHLOROPHYLL CONTENT
Duplicated CaMV *35S* promoter	*Cah1*	1	9.50%	49.61%	21.87%	-0.81%	4.54%
	Cah1 + Cah3	2	13.60%	55.17%	19.35%	11.21%	-0.77%
	Cah3	3	-11.70%	20.73%	-19.05%	6.52%	-2.76%
Maize *Ubi-1* promoter	*Cah1*	4	14.50%	53.24%	24.24%	16.28%	6.73%
	Cah1 + Cah3	5	-26.60%	33.67%	-4.17%	-20.99%	-22.95%
	Cah3	6	-6.25%	45.83%	21.88%	3.25%	-0.93%

Table 5.3-Increase or decrease in the different parameters measured in independent transgenic plants compared to WT plants at the end of the experiment (week 12th).

Although the GLM showed that the interaction between genes, promoters and weeks, nested to the type of the plants (transgenic vs. WT plants) was only statically significant for the chlorophyll content comparing all the lines together, but not for the other values, I observed that there were interesting results regarding the analyses of the other factors. For example, the GLM showed that the introduction of any of the CA (*Cah1* and/or *Cah3*) was statistically significant in all the parameters analyzed. That is consistent with what I would expect, as it means that introduction of any CA influences all the parameters analyzed. However, in some cases the changes observed in the plants were a decrease, for example, in the number of tillers or the height of the plants. Interestingly, the statistical analysis also revealed that the promoter used was not relevant, as when I analyzed the parameter "promoter" only the height of the plants, the area of the last expanded leaf or the chlorophyll content were statistically significant. Only one line per combination of CA (*Cah1* and/or *Cah3*) with the two different promoters was analyzed. It is necessary to analyze additionally transgenic lines to arrive at a more robust conclusion.

5.6 DISCUSSION

One of the alternative mechanisms that have been proposed to increase the concentration of CO_2 in the vicinity of RubisCO is the introduction of the microalgae carbon concentrating mechanism (CCM) intro the chloroplasts (McGrath and Long 2014). Multiple proteins, such as several carbonic anhydrases (CA) and inorganic carbon (C_i) transporters are implicated in this mechanism. Increasing bicarbonate transporters and/or CA activity is expected to improve RubisCO's CO_2 fixation rate; however the introduction of all the components of the CCMs at the same time is a challenge as at least 14 genes are thought to be important in maintaining a fully functional CCM under ambient or below ambient CO_2 concentrations (Zhou et al. 2016; Atkinson et al. 2016). When multiple transgenes are introduced into plants it is also necessary to ensure that their expression will be stable over many generations (Dietz-Pfeilstetter 2010). By the use of combinatorial genetic transformation it will be possible to create a population of transgenic plants which express the different spectrum of genes involved in the CCM and thus analyze the effect that these genes have on the transgenic plants. Generation of independent transgenic plants expressing the different combinations has to be sufficient, as it depends on several aspects, which include the number of genes likely required to obtain the desired pathway, strong expression of the individual transgenes as well as an optimal balance between expression levels of the transgenes in this pathway (Bock. 2013). The most attractive feature of combinatorial transformation is that there is no theoretical limit of the number of transgene cassettes that can be introduced (Zhu et al. 2008; Bock 2013). Therefore, by using combinatorial transformation it would be possible to analyze the different combinations of genes and identify which combinations resulted in the best performance in terms of biomass and CO_2 fixation. Additionally, a challenging goal to meet when introducing the eukaryotic CCM proteins into crop plants is to ensure that the CCM will perform their intended function in the heterologous host system (Atkinson et al. 2016).

CCMs components, namely LCIA, LCIB, LCIC, LCI1, HLA3, CCP1, CCP2, CAH1, CAH2, CAH3 and CAH6; have been expressed both transiently in tobacco (*Nicotiana tabacum*) and stably (in Arabidopsis) showing appropriate locations and functionality of all components (Atkinson et al. 2015). The results showed that expression of individual C_i transporters did not enhance Arabidopsis growth; therefore it is necessary to stack

additional CCM components to achieve a significant increase in the photosynthetic efficiency in the plants (Atkinson et al. 2015).

The expression of the cyanobacterial bicarbonate transporter BicA in the chloroplasts of tobacco (*Nicotiana tabacum*) via plastome transformation was an important milestone (Long et al. 2016). However, BicA localization was predominantly in the thylakoids, instead of the chloroplast envelope, and lack functionality, confirming that correct targeting and localization of the input transporter is required for correct functionality. Indeed, functionality of bicarbonate transporters in higher plants requires: (i) efficient targeting to the chloroplasts; (ii) insertion in the correct membrane; (iii) an orientation that allows transport of bicarbonate into chloroplasts and (iv) active and regulated transgene expression. It is therefore necessary to understand the mechanisms of activation of the CCM in order to use in the appropriate way the proteins that drive the CCM (Long et al. 2016).

In another study, transient expression of carboxysomal proteins in tobacco chloroplasts showed that these structures were able to assemble in the chloroplast, establishing the feasibility of introducing carboxysomes into chloroplasts for potential compartmentalization of RubisCO or other proteins (Lin et al. 2014a). Theoretical analyses have estimated that engineering a carboxysome into chloroplasts, with the addition of a bicarbonate transporter and removal of stromal carbonic anhydrases could increase crop yield up to 30% (McGrath and Long 2014).

A recent study has been published where introduction of a bicarbonate transporter (LCIA or LCIB) or one carbonic anhydrase (CAH1 or CAH3) resulted in plants growing faster and with a shorter vegetative phase in T_1 generation compared to wild type (Nölke et al. 2016). The increase of each parameter analyzed is described in **Table 5.4**.

PARAMETERS	TRANSGENE EXPRESSED			
	Cah1	*Cah3*	*lcia*	*lcib*
Leaf area	22%	24%	33%	23%
Chlorophyll content	13%	10%	16%	15%
Leaf number	24%	64%	19%	24%
Fresh weight	26%	34%	54%	30%
Dry weight	29%	31%	41%	28%

Table 5.4-Increase of the parameters (leaf area, chlorophyll content, leaf number, fresh and dry weight) measured in tobacco plants expressing a bicarbonate transporter (*lcia or lcib*) or a carbonic anhydrase (*Cah1* or *Cah3*).

In this chapter I focused on the introduction of one or both CA, namely *Cah1* and *Cah3* in rice plants in order to determine if the introduction of these genes had an effect on the yield of the resulting plants.

Introduction of the CA (*Cah1* and/or *Cah3*) resulted in statistically significant differences in the height of the plants. The plants that expressed *Cah1* alone (Lines 1 and 4), independently of the promoter used, were ca: 9.46% and ca 14.52% taller, respectively, than the wild type plants. In the case of Line 2, which expressed both CA under the control of the duplicated CaMV *35S* promoter, the plants were ca 13.56% taller than the wild type plants (**Table 5.3**). This effect could be attributed to the increase of CO_2 in the vicinity of RubisCO. It has been demonstrated in previous studies that when rice plants grow under high CO_2 concentrations; the height of the plants increased (Gória et al. 2013). On the other hand, introduction of bicarbonate transporters (LCIA or LCIB) and CA (CAH1 or CAH3) resulted in an increase in the height of tobacco plants that expressed the introduced transgenes (Nölke et al. 2016). More specifically, when the tobacco plants expressed *Cah1*, the height was increased by ca: 3% and by ca: 46% when the transgene expressed was *Cah3*. The height of the transgenic tobacco plants which expressed LCIA was ca: 68% taller and lines that expressed LCIB were 46% taller.

A)

B)
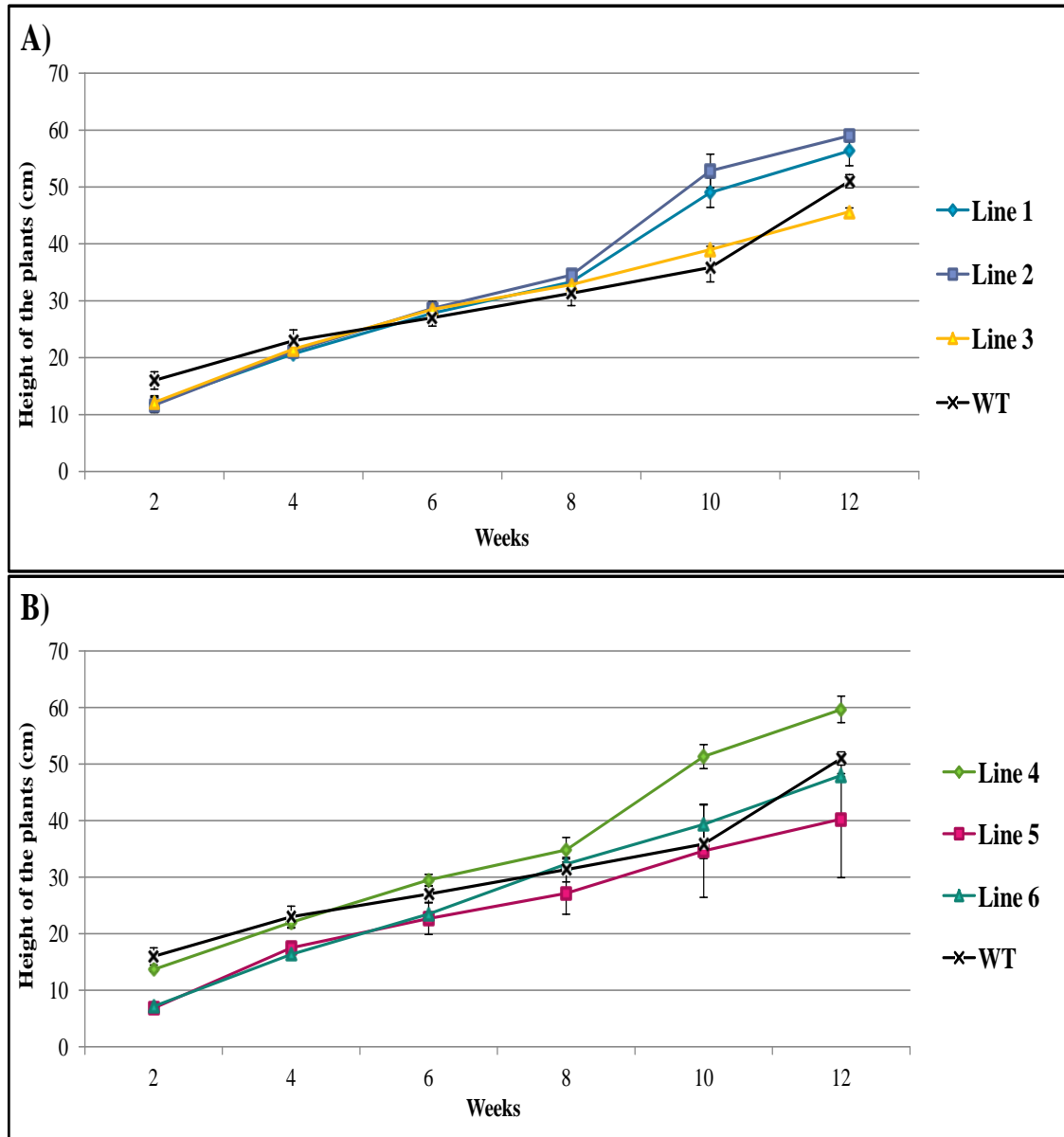
Figure 5.3-**Height of independent transgenic lines compared to wild type (WT) controls. A)** Height of Lines 1, 2 and 3 which expressed *Cah1, Cah1* and *Cah3*, and *Cah3* alone, respectively, compared to the WT. The transgenes were under the control of the duplicated CaMV *35S* promoter. **B)** Height of Lines 4, 5 and 6 which expressed *Cah1, Cah1* and *Cah3*, and *Cah3* alone, respectively; under the control of the maize *Ubi-1* promoter compared to the WT during the experiment.

Plant biomass also increased when tobacco plants were transformed with bicarbonate transporters (LCIA or LCIB) and CA (CAH1 or CAH3). Similarly to tobacco plants, transgenic rice plants which expressed one or both CA had an increase in the number of leaves, irrespectively of the promoter used. Line 1, which expressed *Cah1* under the control of the duplicated CaMV *35S* promoter increased by 49.61% the number of leaves; Line 2, which expressed *Cah1* and *Cah3* under the control of the duplicated

CaMV *35S* promoter increased ca: 55.17% the number of leaves; Line 3, which expressed *Cah3* under the control of the duplicated CaMV *35S* promoter increased ca: 20.73% the number of leaves. Line 4, which expressed *Cah1* under the control of the maize *Ubi-1* promoter increased ca: 53.24% the number of leaves; Line 5, which expressed *Cah1* and *Cah3* under the control of the maize *Ubi-1* promoter increased ca: 33.67% the number of leaves; Line 6, which expressed *Cah3* under the control of the maize *Ubi-1* promoter increased ca 45.83% the number of leaves (**Figure 5.4**). In microalgae, this enzyme catalyzes the formation of CO_2 from HCO_3^- in the lumen of the thylakoids, and as the ambient is acidic, CO_2 diffuses through the thylakoid membrane to the pyrenoid, where it is fixed by RubisCO (Moroney and Ynalvez 2007). Tiller number was also increased in those lines where *Cah1* alone was expressed by ca: 21.87% and ca: 24.24%, when the gene was under the control of the duplicated CaMV *35S* promoter or the maize *Ubi-1*, respectively. When both CA were expressed under the control of the duplicated CaMV *35S* promoter, total number of tillers was increased by 19.35%. The same transgenes expressed under the control of the maize *Ubi-1* promoter has a 4.46% decrease in the number of tillers. Experiments carried out with rice plants grown under high CO_2 atmosphere, resulted in increasing the tiller number up to a 14% and also the height of the plants (+7%) (Wang et al. 2015). It is also reported that under elevated CO_2 conditions, there is a better water use efficiency (WUE) due to a reduction of stomatal conductance. An interesting parameter to be analyzed in the following generations would be the capacity of these lines to have a better WUE.
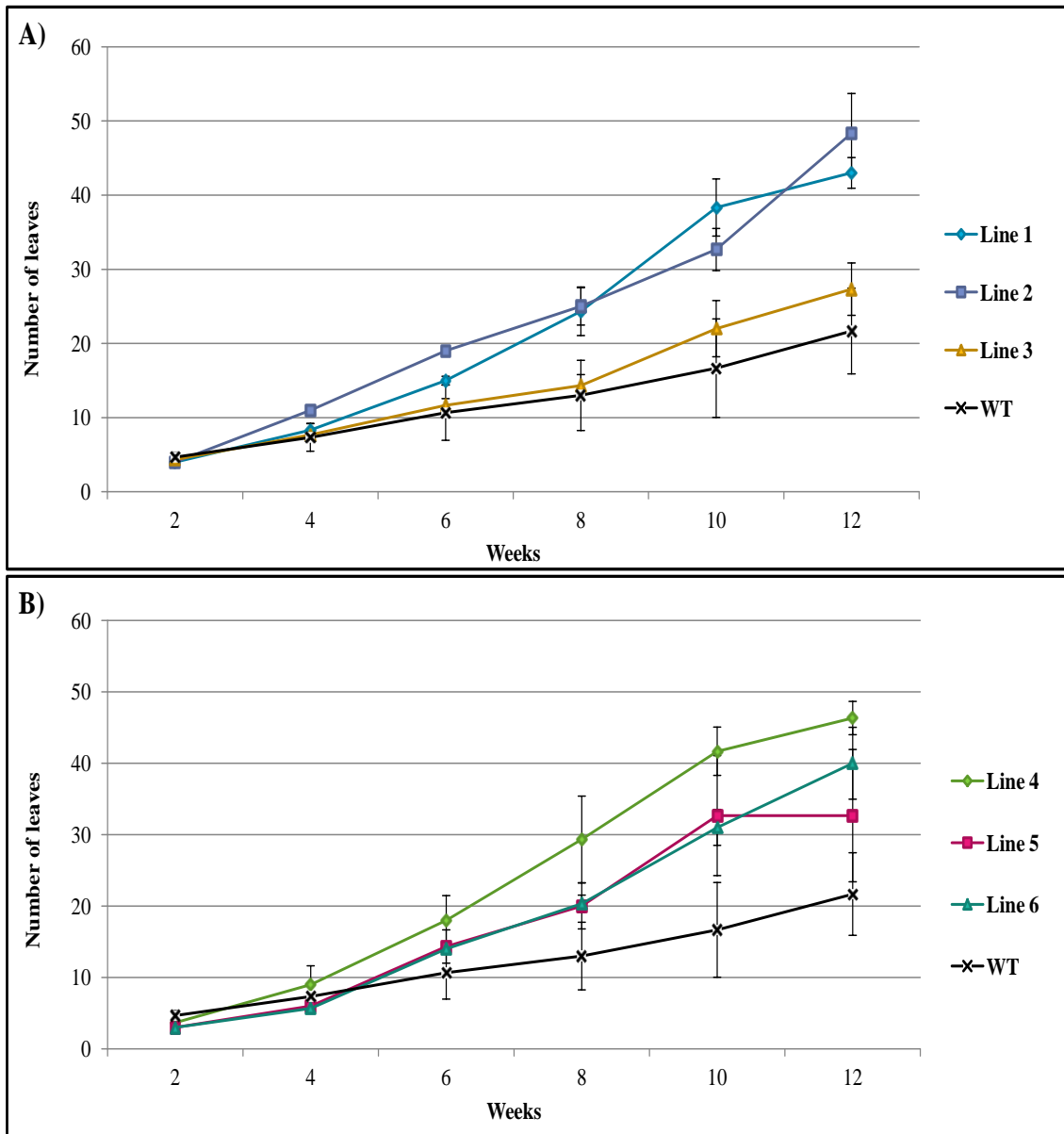
Figure 5.4-Total number of leaves in independent transgenic plants compared to wild type (WT) controls. A) Total number of leaves of Lines 1, 2 and 3 which expressed *Cah1, Cah1* and *Cah3*, or *Cah3* alone, respectively, compared to the WT. The transgenes were under the control of the duplicated CaMV *35S* promoter. **B)** Total number of leaves of Lines 4, 5 and 6 which expressed *Cah1, Cah1* and *Cah3*, and *Cah3* alone, respectively, compared to the WT. The transgenes were under the control of the maize *Ubi-1* promoter.

Figure 5.5-Total number of tillers in the independent transgenic plants compared to wild type (WT) controls. A) Total number of tillers of Lines 1, 2 and 3 which expressed *Cah1, Cah1* and *Cah3*, or *Cah3* alone, respectively, compared to the wt. The transgenes were under the control of the duplicated CaMV *35S* promoter. **B)** Total number of tillers of Lines 4, 5 and 6 which expressed *Cah1, Cah1* and *Cah3*, and *Cah3* alone, respectively, compared to the WT. The transgenes were under the control of the maize *Ubi-1* promoter.

In rice, the flag leaf is the leaf that contributes the most to grain yield, greater carbohydrate translocation from vegetative plant parts to the spikelet and a larger leaf area index during grain filling (Davood et al. 2009). Flag leaf morphology affects significantly yield, grain quality, maturity, pest preference and absorption of plant growth regulators (Fan et al. 2007). Introduction of CA did not result in a significant change in the surface area of the last expanded leaf. However, the surface area of the flag leaf was larger in Lines 1 and 2 (which expressed *Cah1* alone and the combination

of both CA under the control of the duplicated *35S* promoter, respectively) and Line 4 (which expressed *Cah1* under the control of the maize *Ubi-1* promoter) at the 8th week which corresponds to the beginning of grain filling. To achieve a higher yield potential, metabolic activity within the grain has to coincide with maximum activity in source leaves (Murchie et al. 2002). Generally, elevated CO_2 increases photosynthesis, resulting in increased dry matter accumulation, leaf area and plant height, especially in trees and shrubs and to some extent in C_3 plants (Ainsworth and Long 2005). Several studies investigating the effects of elevated CO_2 in crops resulted in contradictory results regarding the effect of leaf size as this trait is affected by several factors such as water, temperature and soil nitrogen (Prior et al. 2004; Ainsworth and Long 2005; Exert 2004). Transgenic tobacco plants expressing one bicarbonate transporter (*lcia* or *lcib*) or one CA (*Cah1* or *Cah3*) had an increase in the leaf area between 22-33%. In the case of the transgenic rice plants I analyzed, the increase of the area of the last expanded leaf was only evident in Line 2 (which expressed *Cah3* under the control of the duplicated *35S* promoter), Line 3 (which expressed *Cah1* and *Cah3* under the control of the duplicated *35S* promoter) and Line 4 (which expressed *Cah1* under the control of the maize *Ubi-1* promoter). If this parameter is affected by several factors it is difficult to conclude that the differences I noted are due to the introduction of the transgenes. Measuring leaf area in subsequent generations is required to determine if the pattern remains the same. Also it could be interesting to carry out the same phenotype experiment under different light and temperature conditions to compare if the changes in these parameters caused any change in the surface area of the leaves.
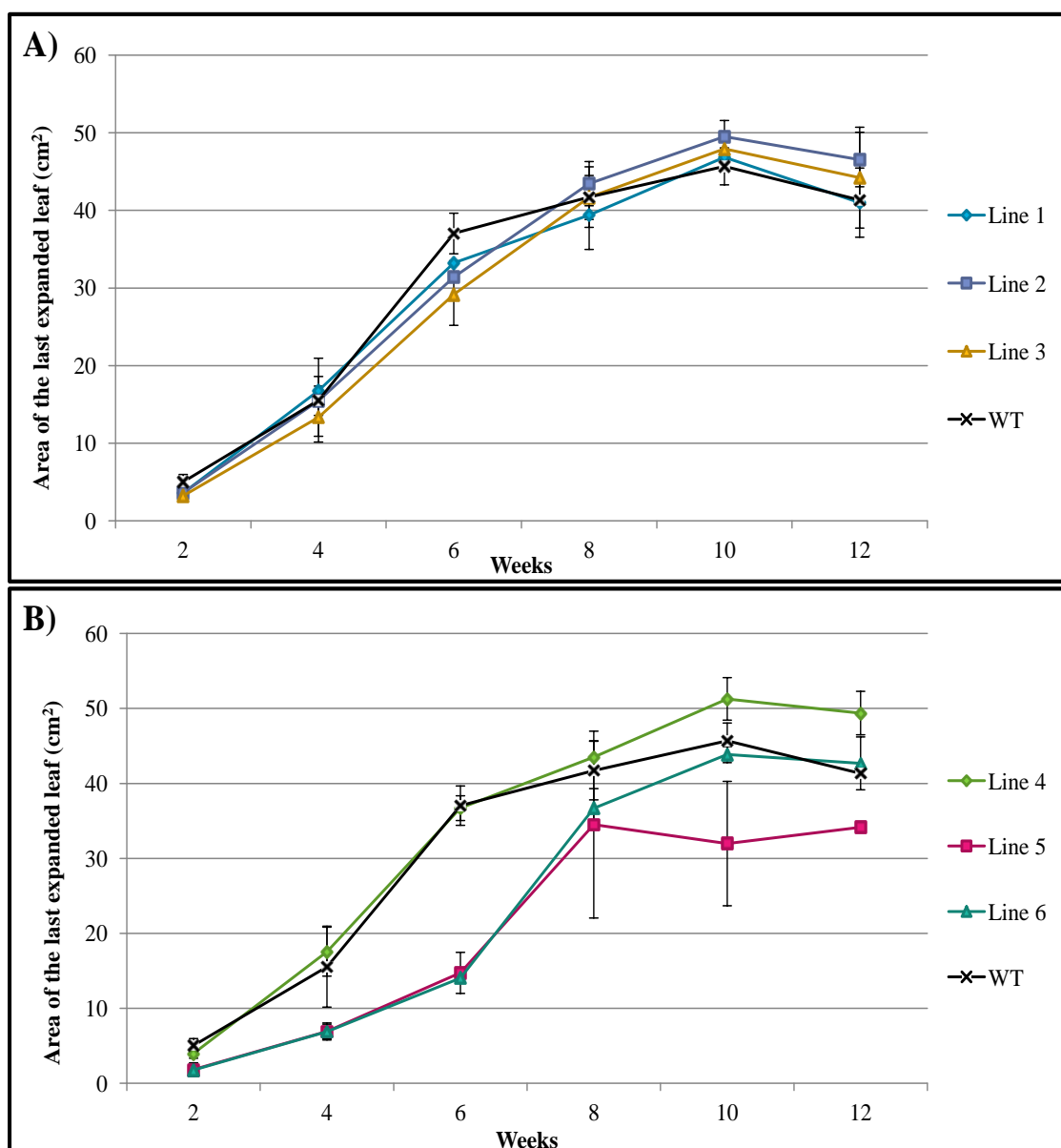
Figure 5.6-**Area of the last expanded leaf in independent transgenic plants compared to wild type (WT) controls. A)** Area of the last expanded leaf of Lines 1, 2 and 3 which expressed *Cah1, Cah1* and *Cah3*, or *Cah3* alone, respectively, compared to the WT. The transgenes were under the control of the duplicated CaMV *35S* promoter. **B)** Area of the last expanded leaf of Lines 4, 5 and 6 which expressed *Cah1, Cah1* and *Cah3*, and *Cah3* alone, respectively, compared to the WT. The transgenes were under the control of the maize *Ubi-1* promoter.

The chlorophyll content in the flag leaf is also an important trait for photosynthetic efficiency (Feng et al. 2014). The three lines that exhibited better performance in terms of number of leaves, tillers and surface area of the flag leaf, were the same lines that had more chlorophyll content compared to the other lines. Increased chlorophyll content is related with increased leaf absorbance and together with greater surface leaf area index it increases light interception. Increasing photosynthetic proteins such as chlorophyll is associated with higher phosphorylation and carbon metabolism, conferring a greater

photosynthetic efficiency in plants (Evans 2013). Photosynthetic rate has been also correlated with increases in chlorophyll content per unit leaf area, and in some cases per unit fresh or dry weight in rice (Takai et al. 2013). The increases in the chlorophyll content observed in Lines 1, 2 and 4, which expressed *Cah1* under the duplicated CaMV *35S* promoter, *Cah1* and *Cah3* under the duplicated CaMV *35S* promoter, and *Cah1* under the control of the maize *Ubi-1* promoter, respectively could mean an increase in the yield of those lines, as well as an increase in the fresh and dry weight of the transgenic plants (**Figure 5.7**). The importance of photosynthesis during the grain-filling period is well documented, and contributes 60-100% of the final grain carbon content (Takai et al. 2005; Panda and Sarkar 2013). It has also been reported that a delay in flag leaf senesce contributes to improved rice grain yield (Horton 2000). A delay in leaf senesce could sustain longer photosynthetic competence during the grain filling stage and maintain and supply assimilates to the grain (Panda and Sarkar 2013).

Due to a limitation of time I was not able to carry out analysis with the seeds produced in the transgenic plants. An in depth analysis in terms of number of grains, weight of the grains and carbohydrate content in the transgenic plants which expressed one or both CA (*Cah1* and/or *Cah3*), should be performed.
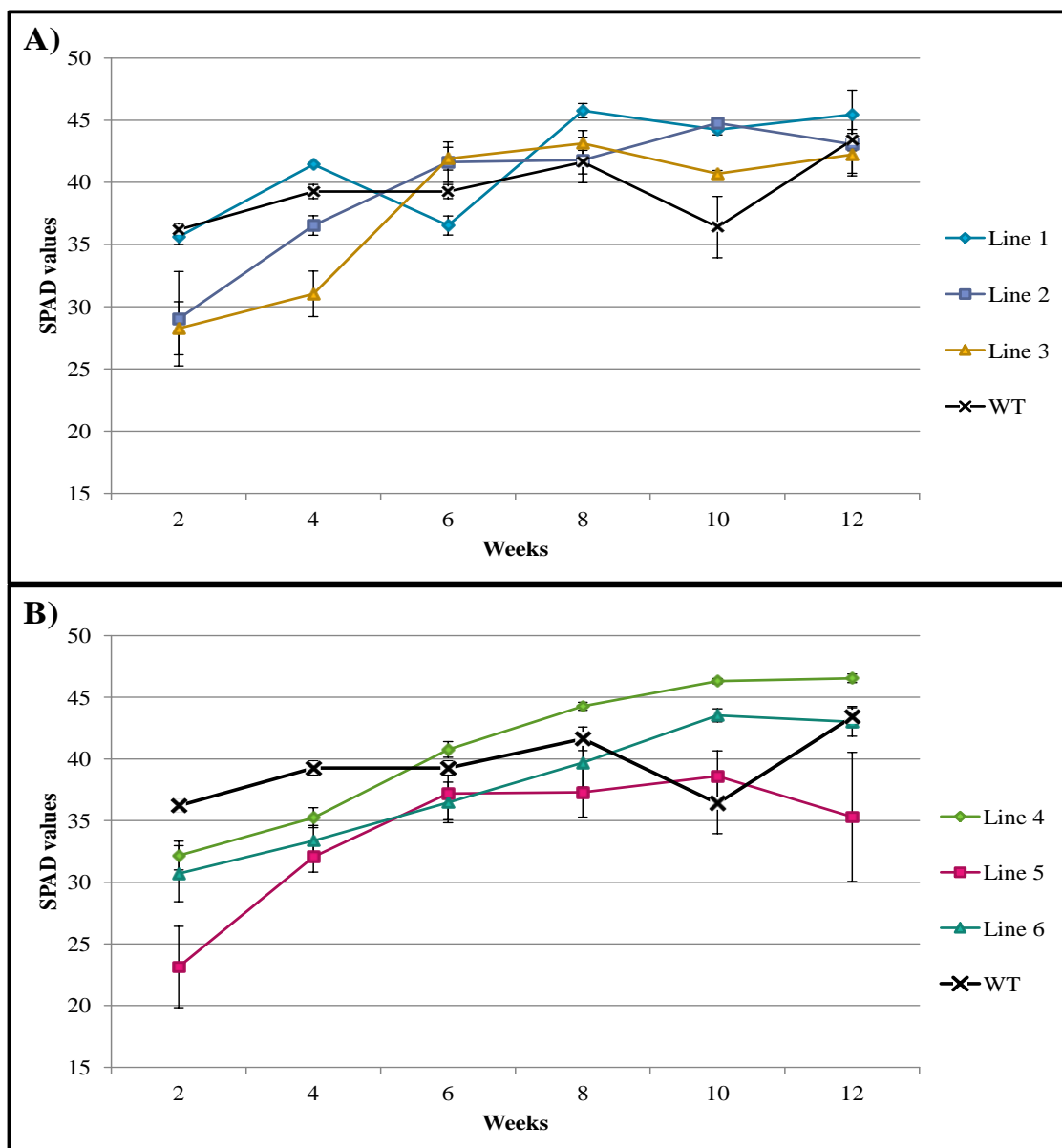
Figure 5.7-Chlorophyll content in independent transgenic plants compared to wild type (WT) controls. A) Chlorophyll content in the last expanded leaf of Lines 1, 2 and 3 which expressed *Cah1, Cah1* and *Cah3*, or *Cah3* alone, respectively, compared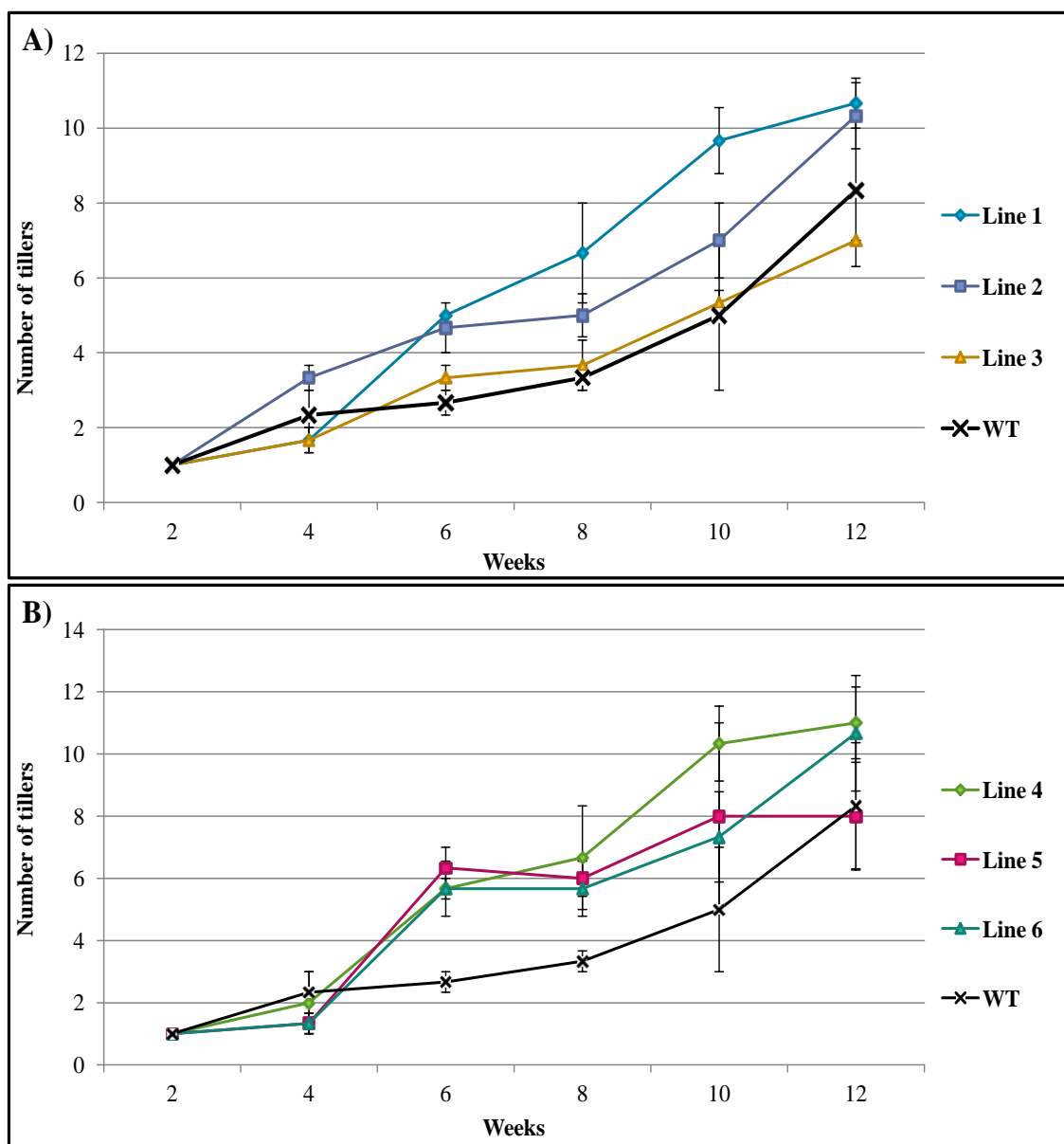 to the WT. The transgenes were under the control of the duplicated CaMV *35S* promoter. **B)** Chlorophyll content in the last expanded leaf of Lines 4, 5 and 6 which expressed *Cah1, Cah1* and *Cah3*, and *Cah3* alone, respectively, compared to the WT. The transgenes were under the control of the maize *Ubi-1* promoter.

5.7 CONCLUSIONS

I have generated a population of transgenic rice plants which expressed one or two carbonic anhydrases, namely *Cah1* and *Cah3*, under the control of two different constitutive promoters, the duplicated CaMV *35S* promoter and the maize *Ubi-1* promoter.

I have performed phenotypical analyses of the transgenic plants by measuring the height of the plants, the total number of leaves and tillers, the surface area of the last expanded leaf and the chlorophyll content of 6 independent lines which expressed one or both CA (*Cah1* and/or *Cah3*) under the control of the two different promoters. I conclude that *Cah1* under the control of both promoters resulted in an increase in the total number of tillers and chlorophyll content. The co-expression of both CA resulted in transgenic plants with a better overall performance when the genes were under the control of the duplicated CaMV *35S* promoter. These experiments were performed in T_1 plants, and analyses of subsequent generations of plants should be carried out to ensure that the measured effects are stable.

Due to a limitation of time I was not able to analyze the number of seeds and the starch content of the transgenic plants.

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GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

1. Twenty four fertile transgenic rice lines with stable expression of transgenes involved in the glycolate catabolic bypass pathway were generated. Four lines expressed the full glycolate catabolic bypass pathway, three lines expressed the glycolate dehydrogenase and six lines expressed the glycolate carboligase and tatronic semialdehyde.

2. Phenotypic analysis of 9 transgenic rice lines which expressed the full glycolate catabolic bypass pathway, GDH or GCL-TSR, resulted in the identification of one line which expressed the full glycolate catabolic bypass pathway and one line with GDH with higher plant biomass compared to wild type plants.

3. Line 1, which expressed the full glycolate catabolic bypass pathway, had higher seed production compared to all other lines.

4. Stable expression of one or both carbonic anhydrases *(Cah1* and/or *Cah3*) from *Chlamydomonas reinhardtii* under the control of two different constitutive promoters was confirmed in transgenic rice plants.

5. Enzymatic activity was confirmed in transgenic plants which expressed one or both CA under the control of the two different promoters.

6. Phenotypic analyses carried out in six independent transgenic lines which expressed one or both CA under the control of the two different promoters, revealed that the number of leaves was increased from 21 to 55% in all transgenic rice plants compared to wild type plants.

7. The co-expression of both carbonic anhydrases (*Cah1* and *Cah3*), resulted in transgenic plants with a better overall performance when the genes were under the control of the duplicated CaMV *35S* promoter. However, due to the limited amount of transgenic lines I cannot reach a conclusion in general terms.

8. Phenotypical analysis carried out in transgenic lines which expressed one or both CA were performed in T_1 plants, and analyses of subsequent generation should be carried out to ensure that the measured effects are stable.

OUTPUTS

OUTPUTS

- **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.

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Note: * Joint first author

ANNEX

Cell
PRESS

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.

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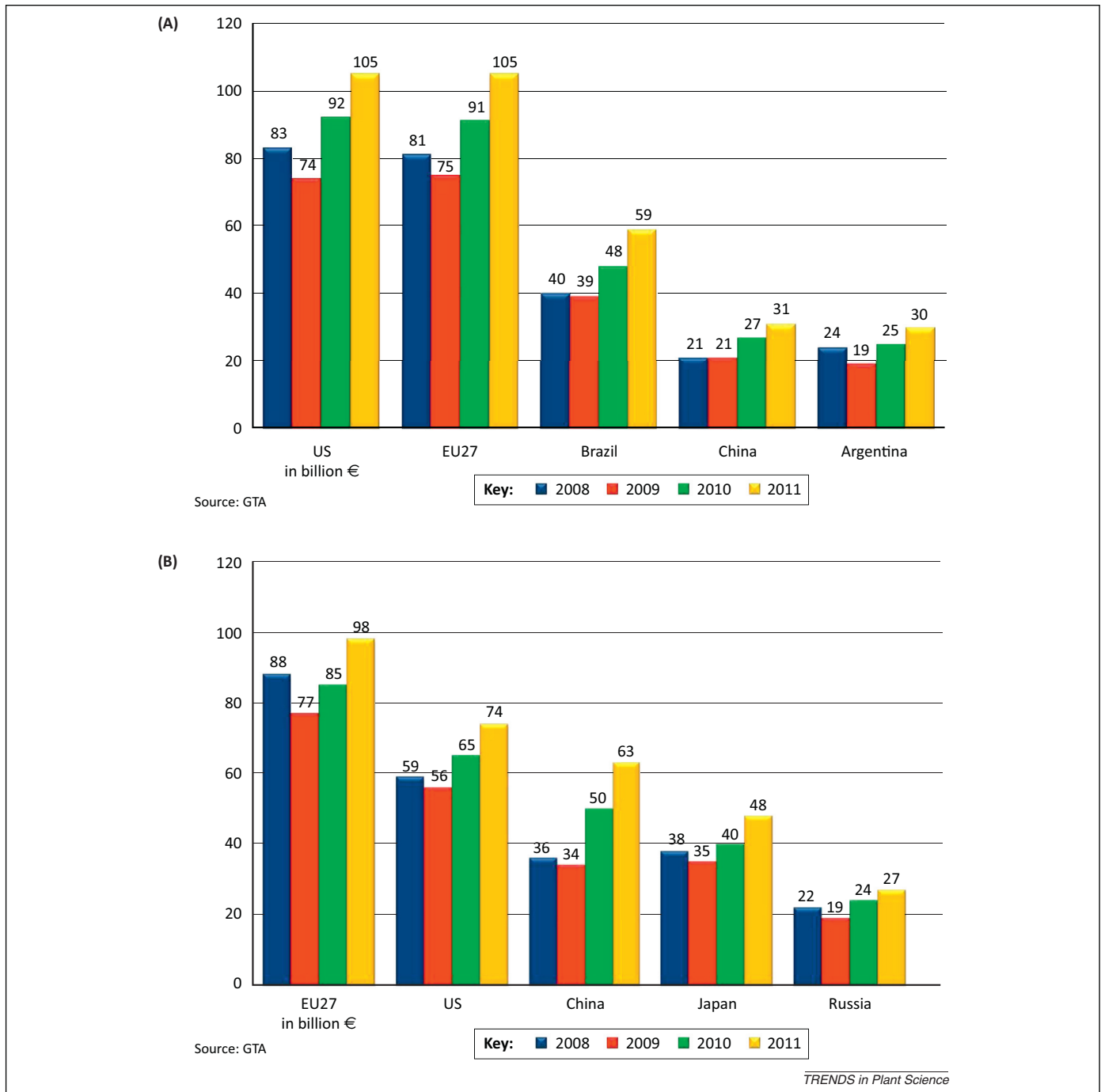
Figure 1. The world's top five **(A)** exporters and **(B)** importers of agricultural products (reproduced from http://ec.europa.eu/agriculture/trade-analysis/map/05-2012_en.pdf). (A) EU exports fell in 2009 but increased in 2010 and 2011 to record levels. (B) The EU is currently the largest importer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

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.

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.

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.

(A)

Total import:
€ 98 billion

Selected products: 42% share

6- Crude palm oil 1.9%

7- Tobacco 1.9%

8- Wine of fresh grapes 1.7%

9- Raw cane sugar 1.6%

10- Maize 1.5%

11- Rape or colza seeds 1.3%

12- Food preparations, n.e.s. 1.2%

13- Fresh grapes 1%

14- Poultry meat 1%

15- Bovine meat, boneless <1%

5- Bananas 2.9%

4- Cocoa beans 3.7%

3- Soybeans 4.7%

2- Soybean meal 6.2%

1- Coffee 8.4%

Source: EUROSTAT-COMEXT

(B)

Total export:
€ 105 billion

Selected products: 40% share

9- Cigarettes 2.3%

10- Spirits 2.1%

11- Food prep for infant use 1.7%

12- Prep for animal feeding 1.6%

13- Solid milk and cream 1.8%

14- Sparkling Wine 1.6%

15- Chocolate 1.5%

8- Cheese 2.3%

7- Beer 2.2%

6- Frozen pigmeat 2.9%

5- Wheat 3.5%

4- Odoriferous subst 3.7%

3- Whiskies 3.8%

2- Food prep 3.9%

1- Wine 6.1%

Source: EUROSTAT-COMEXT

TRENDS in Plant Science

(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).
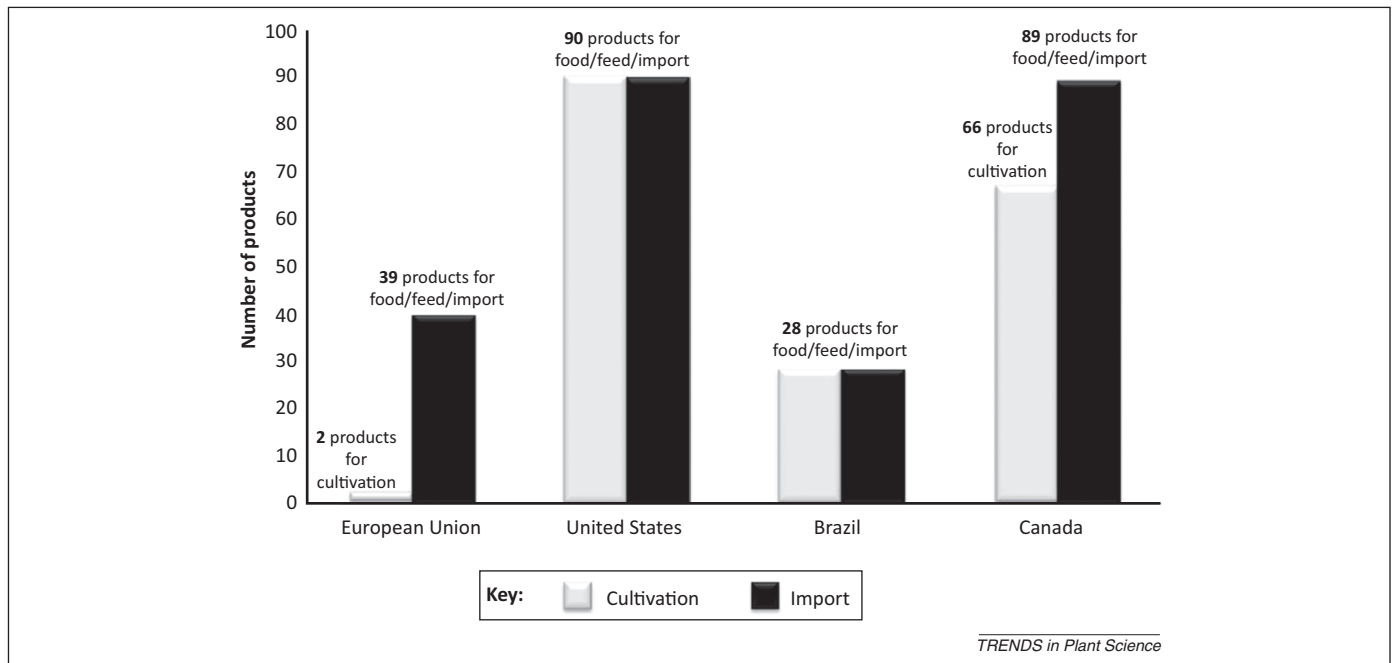
Figure 3. Number of approved genetically engineered (GE) products in the EU, USA, Brazil, and Canada (http://www.europabio.org/sites/default/files/report/approvals_of_gmos_in_eu_europabio_report.pdf). The EU has the lowest number of approved products for cultivation, MON810 and the Amflora potato. However, 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 Europe-an 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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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

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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 (cotransformation) 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 contain-

1. Increase enzyme activity

2. Upstream precursors overexpression

3. Blocking by gene silencing

4. Generation of a metabolic sink

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).

ing 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.

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,
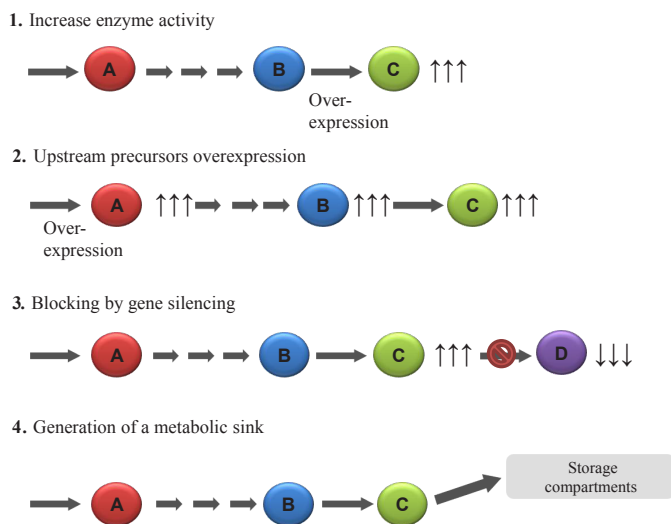
a number of transgenic plants have been generated containing five or more transgenes controlled by the same promoter with no untoward effects (Naqvi *et al.,* 2009). Particle bombardment often generates large, high-copy-number transgenic loci, which are believed to be prone to instability and silencing, 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 frag-

mentation 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
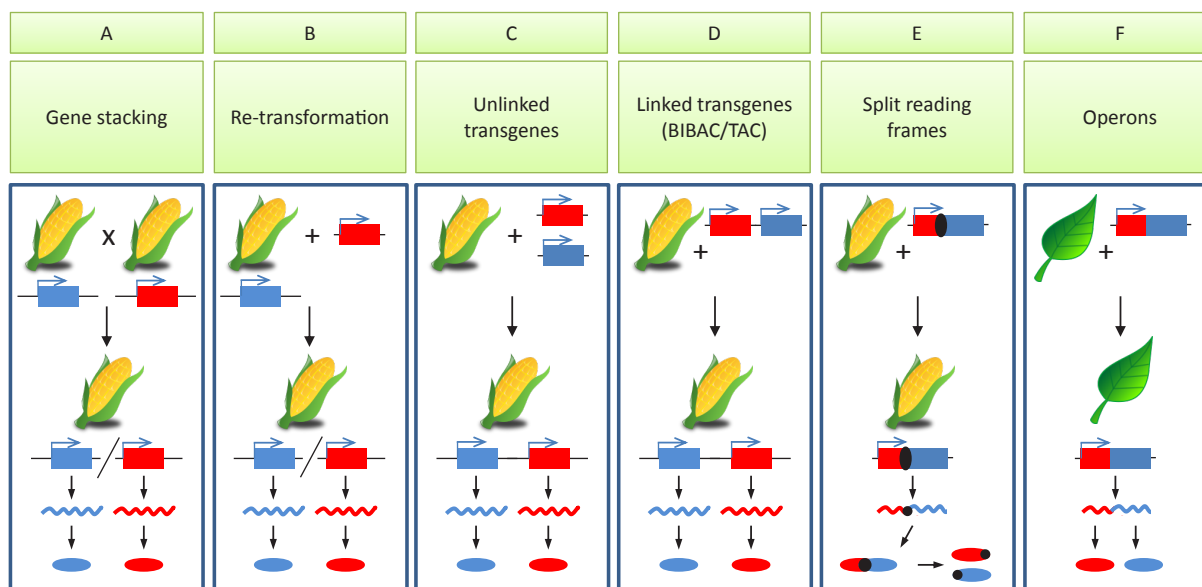

Fig. 2. Multigene transformation (MGT) methods for metabolic engineering. *Schematic summary of the principles of different methods for multigene transfer. Each panel shows a different method and charts the origin, fate, and activity of two different transgenes (red and blue blocks, with promoters shown 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
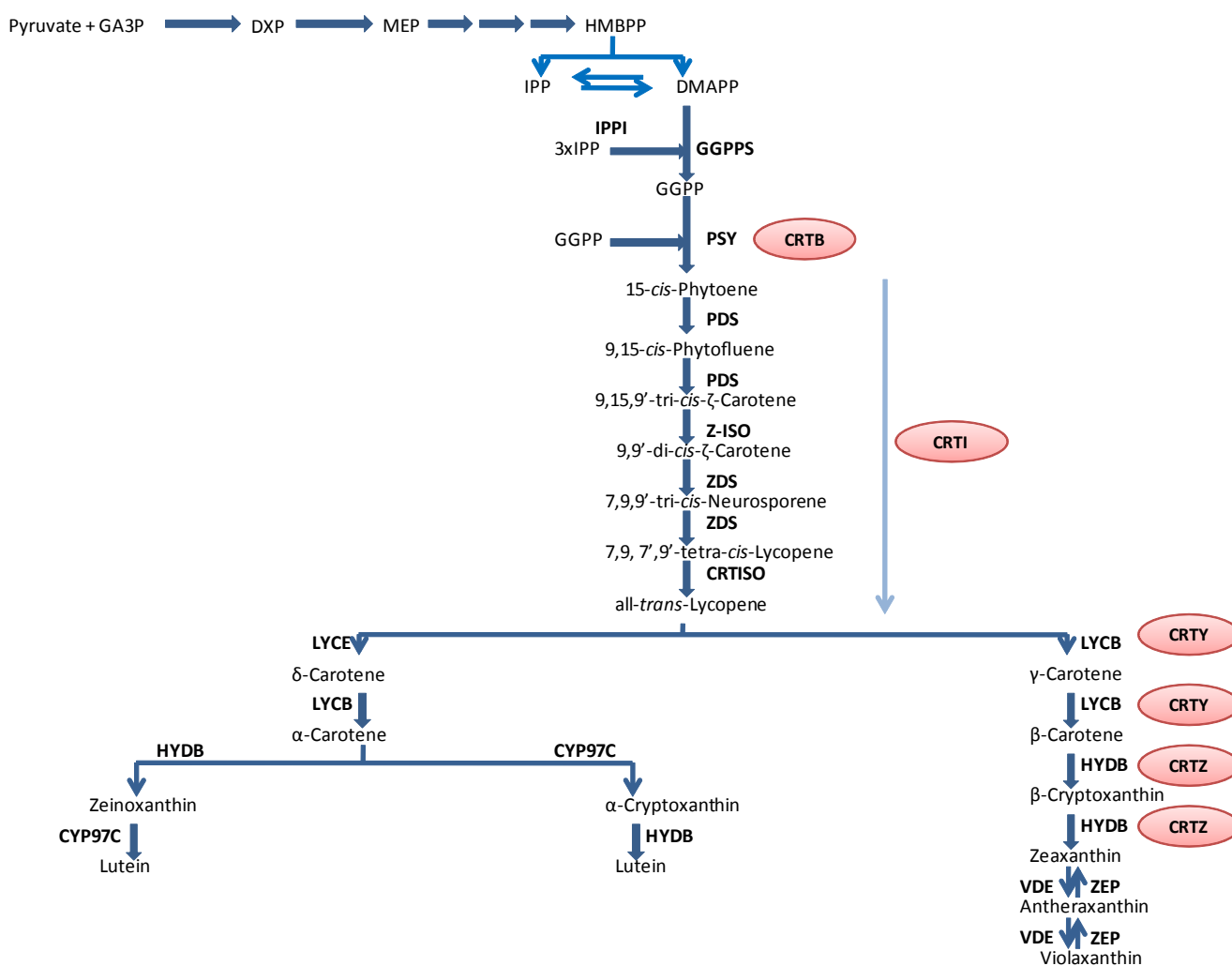
Fig. 3. Carotenoid biosynthetic pathway in plants and equivalent steps in bacteria. *Enzymes in the red ovals are from bacteria. Abbreviations: CRTB, bacterial phytoene synthase; CRTI, bacterial phytoene desaturase, which catalyze all desaturation and isomerization reaction from phytoene to lycopene; 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 hydroxylses (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.*
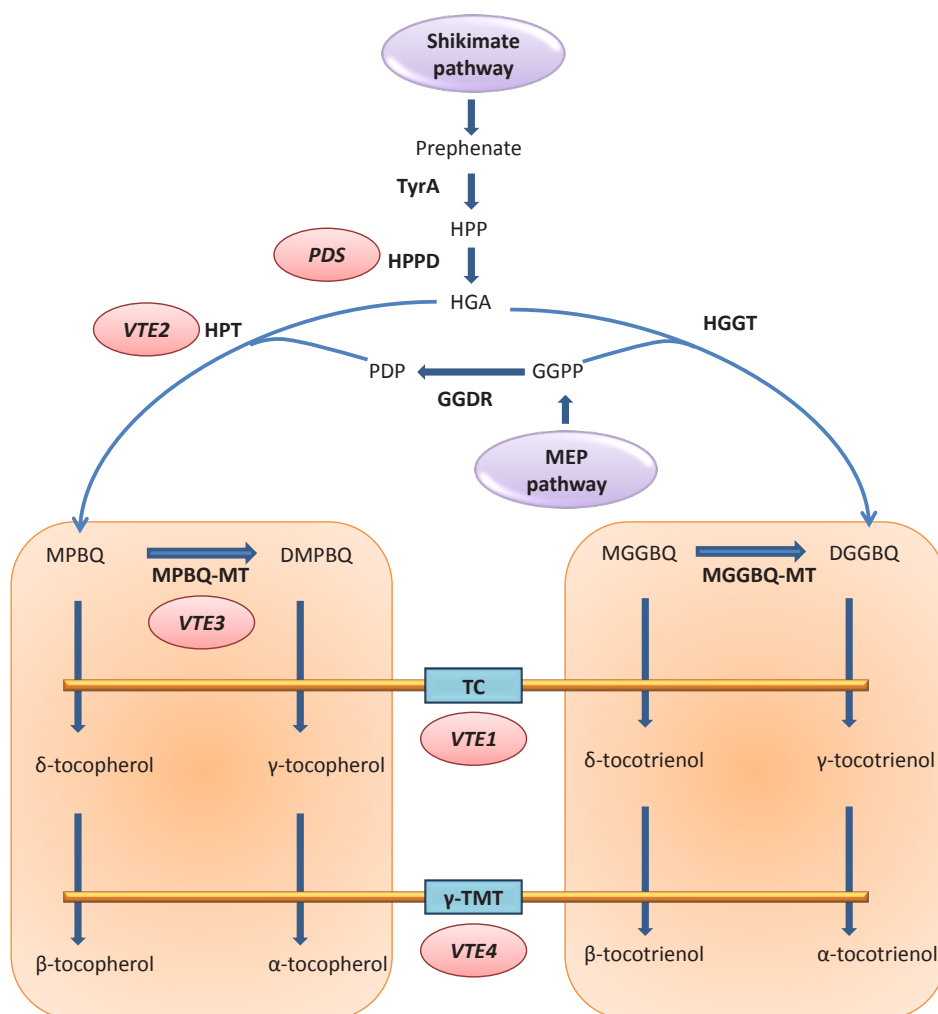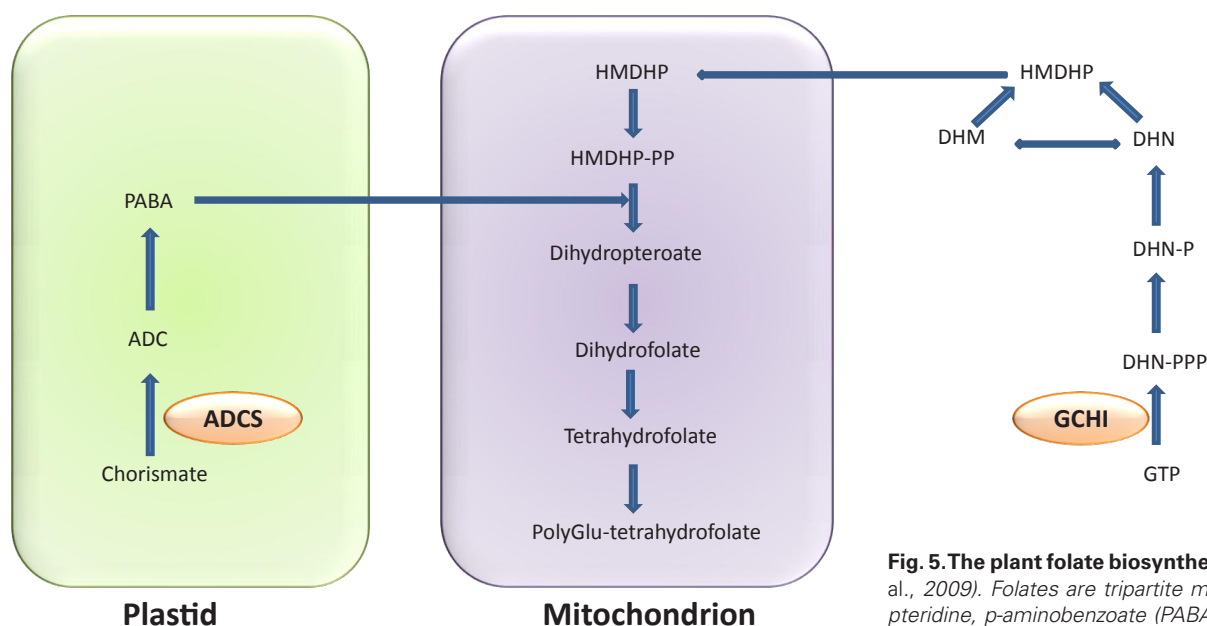
Fig. 5. The plant folate biosynthesis pathway *(Naqvi et al., 2009). Folates are tripartite molecules consisting of pteridine, p-aminobenzoate (PABA), and glutamate moieties, with pteridines synthesized in the cytosol and PABA in the plastids. These moieties are transported to the mitochondria, where 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
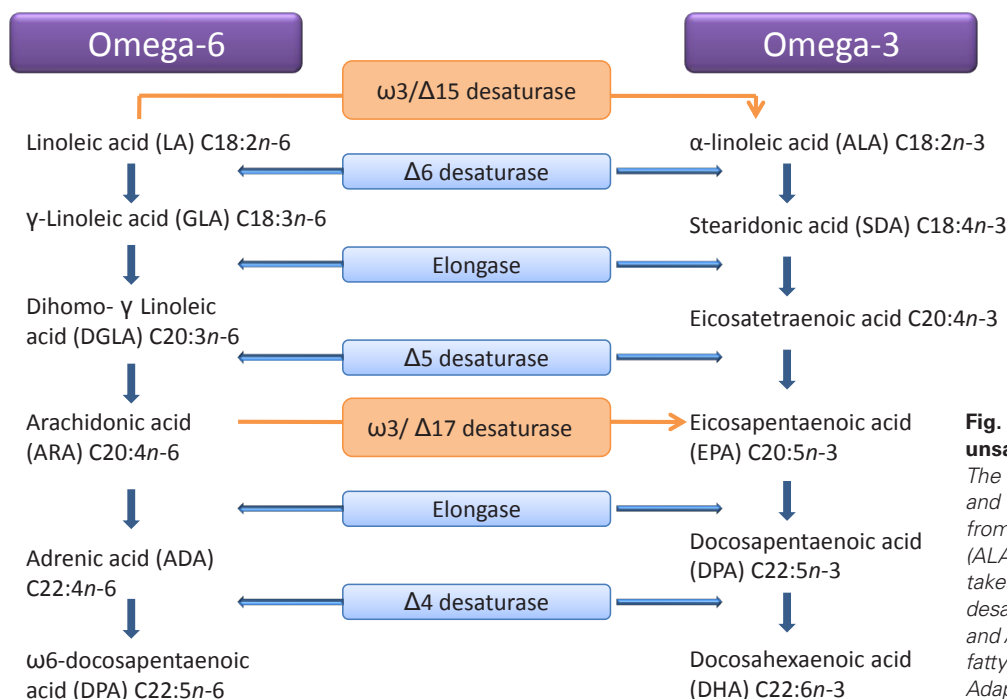
Fig. 6. The biosynthetic pathway of poly-unsaturated fatty acids (PUFAs) in plants. *The conventional Δ6-desaturase/Δ6-elongase and the alternative Δ9-elongase pathways start from linolenic (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3), respectively. PUFAs synthesis takes place in plastids. Since 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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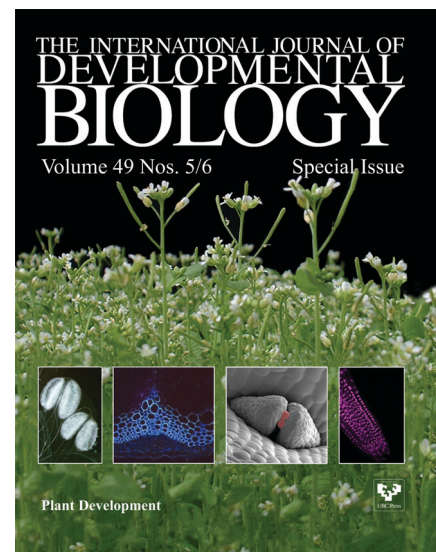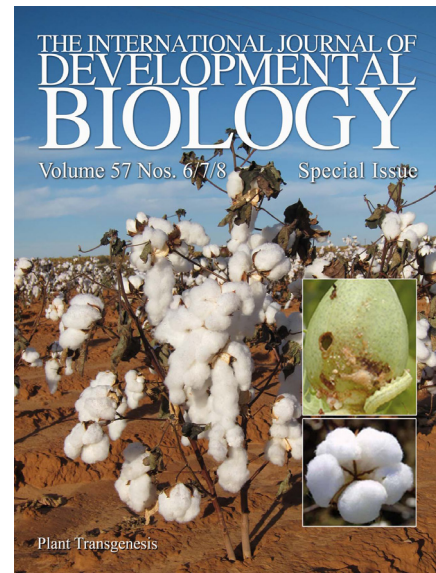Int. J. Dev. Biol. (2012) 56: 761-764

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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).
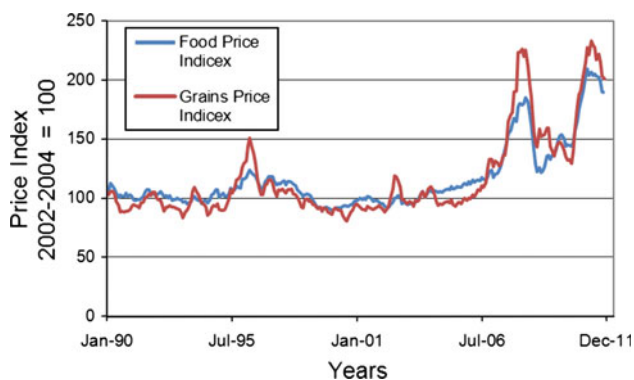

Fig. 1 Global monthly grains price index (*blue*) and total food price index (*red*), January 1990–November 2011. The values for 2002 to 2004 are set at 100. *Source*: FAO (2012b)
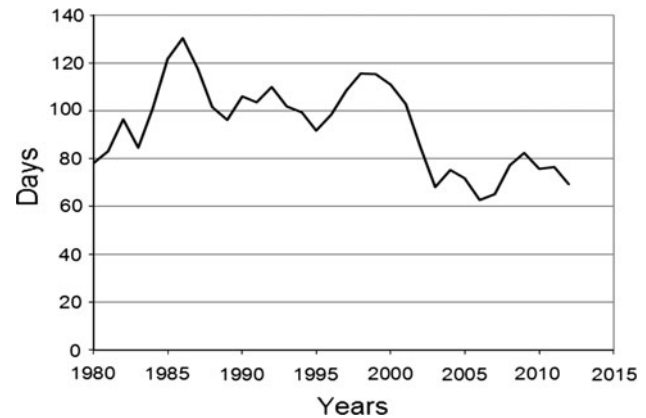
Fig. 2 World grain stocks shown as days of consumption between 1980 and 2012. This shows strong decline since 2000 reflecting policy shifts and greater dependence on trade. *Source*: EPI from USDA (2012a)
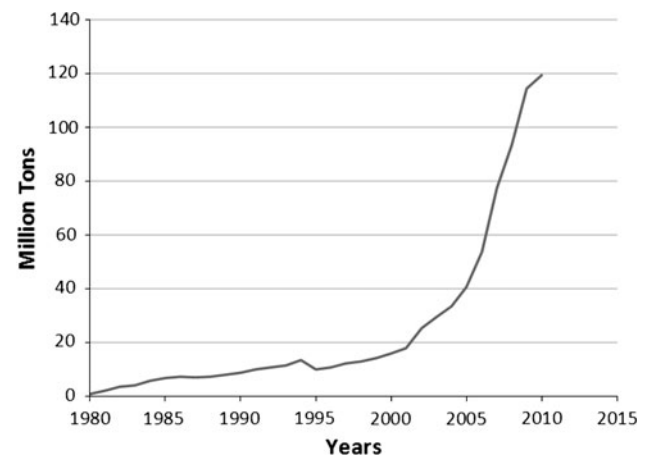
Fig. 3 Corn used for fuel ethanol in the US between 1980 and 2010 in millions of tons. The corn used for fuel is derived from the previous year's harvest, thus the 119 million tons of corn used for fuel ethanol in 2010 represents 28.7 % of the 2009 grain crop (416 million tons). *Source*: EPI from USDA (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)
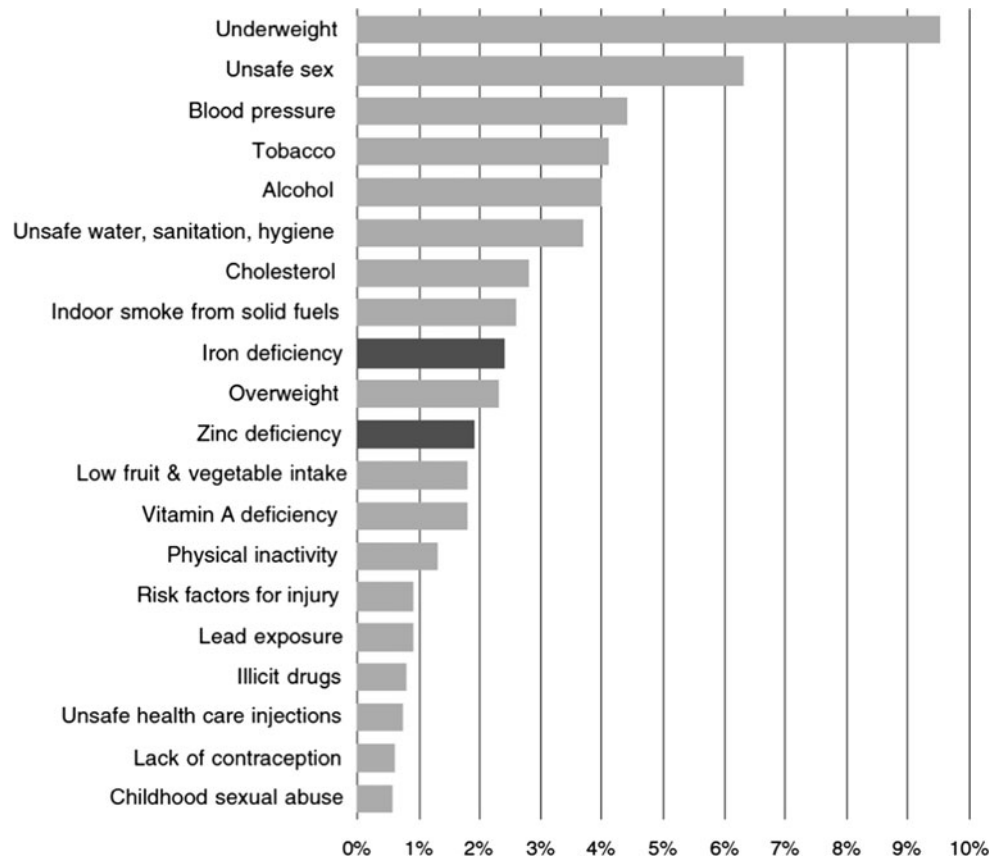
reduced by 1.5 % for every 1 % loss of hemoglobin (Dickinson et al. 2009).

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).
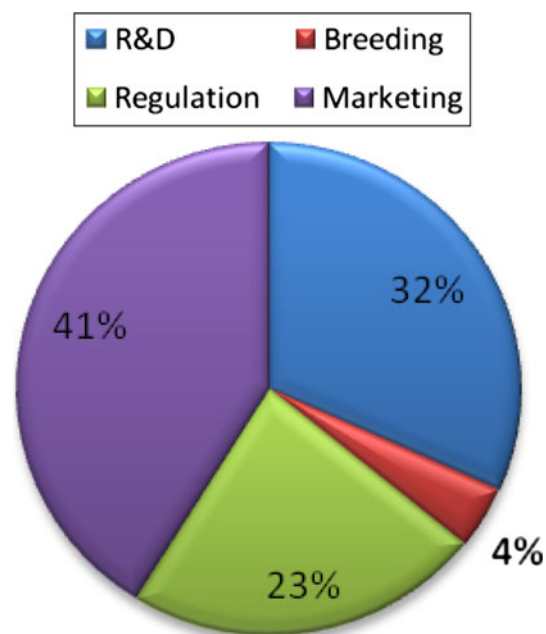

Fig. 5 The proportion of R&D, breeding, regulation and marketing costs required for the development of a genetically engineered crop (adapted from Stein et al. 2006)

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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