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Biotechnological interventions for crop improvement in the context of food security

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We hereby state, that Dawei Yuan, who majored in Biology at the NEN University, has performed under our direction and supervision, and within the Applied Plant Biotechnology group from the department of "Producció Vegetal i Ciència Forestal", the experimental work entitled "Biotechnological interventions for crop improvement in the context of food security",

That the work accomplishes the adequate conditions in order to be defended before the corresponding Thesis Committee and, if the opportunity arises, to obtain the degree by the Universitat de Lleida,

And we sign the current document that this may be officially recorded, to complete formalities demeed necessary,

Lleida, July 2012

Dr.Paul Chirstou Dr.Changfu Zhu Dra.Teresa Capell

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Abbreviations

ADC	Arginine decarboxylase (enzyme)
Adc	Arginine decarboxylase (gene)
AIDS	Acquired immunodeficiency syndrome
Arg	Arginine
BAP	6-Benzylaminopurine, synthetic cytokinin
bp	Base pair(s)
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
CaMV 35S	Cauliflower mosaic virus 35S
cDNA	Complementary DNA
2,4-D	2,4-Dichlorophenoxyacetic acid
dNTP	Deoxynucleoside 5'-triphosphate
EDTA	Ethylenediaminotetraacetic acid
ESTs	Expressed sequence tags
FAO	Food and Agriculture Organization of the United Nations
Fig(s)	Figure(s)
fw	Fresh weight
Gus	β-Glucuronidase
HIV	Human immunodeficiency virus
hpt	Hygromycin phosphotransferase gene
kb	Kilobase pair(s)
kDa	Kilodalton(s)
LB	Luria Burtoni medium
MA	Mugineic acid
Mbp	Megabase pair(s)
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
MS	Murashige and Skoog
ODC	Ornithine decarboxylase (enzyme)
Odc	Ornithine decarboxylase (gene)
ORF	Open reading frame
Orn	Ornithine
Os	*Oryza sativa* (rice)
PAO	Polyamine oxidase
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAM	S-adenosylmethionine
SAMDC (or *Samdc*)	S-adenosylmethionine decarboxylase (enzyme)
Samdc	S-adenosylmethionine decarboxylase (gene)
SDS	Sodium dodecylsulfate
SPDS	Spermidine synthase
SPMS	Spermine synthase
SSC	Standard saline citrate
T0	Primary transformants
T1	First transgenic generation
T2	Second transgenic generation
TBE	Tris-borate-EDTA buffer

Ubi-1	Ubiquitin-1
uORF	Upstream open reading frame
UTR	Untranslated region
UN	United Nations
UNICEF	The United Nations Children's Fund
UV	Ultraviolet
Ψ_w	Water potential
v/v	Volume to volume ratio
w/v	Weight to volume ratio
Zm	*Zea mays* (maize)

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ABSTRACT

Crop productivity is limited by a number of important constraints that need to be addressed urgently in order to avoid an imminent humanitarian crisis. Food security, as articulated in the eight Millennium Development Goals (MDGs), depends on technological interventions to address the key objectives, i.e. increasing yield in a sustainable manner, improving the nutritional value of staple food crops and endowing crops with the ability to withstand major abiotic stresses such as drought.

My thesis provides three diverse yet converging examples of biotechnological solutions that can deliver fundamental knowledge, tools and potential products in the form of improved/enhanced crop plants. I report the cloning and characterization of two promoters from rice *arginine decarboxylase* (*ADC*) genes, which play a pivotal role in the polyamine biosynthesis pathway. Polyamines protect plants against abiotic stresses such as salinity and drought. We therefore urgently need a better understanding of the molecular and physiological mechanisms though which polyamines confer stress tolerance in plants. My results indicate that the promoters of *ADC* genes in rice become more active under drought stress as measured by reporter gene expression, and therefore indicate a potential underlying mechanism for stress-induced polyamine biosynthesis.

Carotenoids are important molecules with many nutritional and health benefits. In a wider program to understand the mechanism of carotenoid biosynthesis in plants I focused on *Gentiana lutea zeaxanthin epoxidase* (*GlZEP*) as this is the key enzyme responsible for the accumulation of antheraxanthin and violaxanthin in *G. lutea* petals (which contain large amounts of lutein, violaxanthin, antheraxanthin and *β*-carotene). The overall aim was to understand the link between carotenoid synthesis and chromoplast differentiation. This is relevant because *ZEP* expression is intimately linked with chromoplast development. I evaluated the ability of the *GlZEP* promoter to drive reporter gene expression in transgenic tomato plants. My results suggest an evolutionarily-conserved link between *ZEP* and the differentiation of organelles that store carotenoid pigments in plants.

Enhanced photosynthesis is a key objective in rice improvement programs and the focus of intense international research aiming to increase the capacity of crops to utilize fixed carbon during photosynthesis. To that effect I introduced five chloroplast-targeted bacterial genes to reconstitute the *Escherichia coli* glycolate catabolic pathway in rice, aiming to reduce the loss of fixed carbon during photorespiration. If successful, this strategy should increase the biomass and yield of the plant. I recovered a large number of transgenic rice plants containing and expressing different combinations of the input transgenes and carried

out detailed molecular characterization. This combinatorial transgenic plant population will facilitate an in-depth analysis of the consequences of expressing these genes simultaneously in rice allowing the development of more refined strategies to achieve the long-term objective of reducing photorespiration.

I conclude my thesis by discussing the potential of biotechnology to address the MDGs. My key conclusion is that although biotechnology can contribute positively and substantially towards many of the MDGs, political expediency and an over-burdening regulatory system threaten to prevent those needing the technology from gaining access, i.e. impoverished subsistence farmers and their families in the developing world.

RESUM

Per evitar una imminent crisi humanitària cal treballar per solucionar els factors que limiten la productivitat dels cultius actuals. La sostenibilitat alimentària, segons els Objectius de Desenvolupament del Mil·leni (ODM) acordats per l'Organització de les Nacions Unides (ONU), depèn de les intervencions tecnològiques que es facin, les quals han d'ajudar a assolir els objectius clau següents: augmentar el rendiment d'una manera sostenible, millorar el valor nutricional dels cultius alimentaris bàsics i dotar-los de capacitat per tolerar els estressos abiòtics com la sequera.

La meva tesi ofereix tres exemples de solucions biotecnològiques, diverses però convergents, que poden contribuir a proporcionar els coneixements fonamentals, les eines i els productes potencials en forma de plantes o cultius millorats genèticament que facilitaran el desenvolupament d'un l'agricultura sostenible.

En el primer capítol presento la clonació i caracterització dels dos promotors dels gens *Arginina descarboxilasa* (*ADC*) d'arròs, els quals participen en la ruta de la biosíntesi de les poliamines. Les poliamines juguen un paper bàsic en la protecció de les plantes davant d'estressos abiòtics com la salinitat i la sequera. Per tant, aprofundir en el coneixement dels mecanismes moleculars i fisiològics pels quals les poliamines confereixen a les plantes la tolerància a diferents estressos, és essencial per tal de trobar solucions biotecnològiques que les protegeixin contra l'estrès. Els meus resultats indiquen que els promotors dels gens d'*ADC* en l'arròs s'activen diferentment en condicions de manca d'aigua, tal com es demostra pels nivells d'expressió del gen marcador i per tant indica l'existència d'una activació transcripcional en resposta a l'estrès que pot ajudar a elucidar el mecanisme de protecció.

Els carotenoides són molècules naturals amb molts beneficis nutricionals i per a la salut. Dins d'un programa més ampli, desenvolupat al laboratori, per comprendre el mecanisme de la biosíntesi de carotenoides en plantes, em vaig centrar en l'estudi de l'enzim zeaxantina epoxidasa de *Gentiana lutea* (GlZEP) amb l'objectiu de conèixer la relació entre la síntesi dels carotenoides i la diferenciació dels cromoplasts. L'expressió del gen *ZEP* està estretament lligada amb el desenvolupament dels cromoplasts, ja que es tracta de l'enzim responsable de l'acumulació d'anteraxantina i violaxantina en els petals (els quals contenen gran quantitat de luteïna, violaxantina, anteraxantina i *β*-caroté). En el segon capítol mostro el clonatge del promotor de *GlZEP* i la seva caracterització molecular. He avaluat la capacitat d'aquest promotor per a induir l'expressió del gen marcador *GUS* en tomateres (*Solanum lycopersicum*) transgèniques. Els meus resultats suggereixen que hi ha hagut una relació

evolutivament conservada entre *ZEP* i la diferenciació dels orgànuls que emmagatzemen els pigments carotenoides en plantes.

L'increment de l'eficiència fotosintètica és un objectiu essencial en els programes de millora de l'arròs i el focus d'una intensa recerca que té per objectiu augmentar la capacitat d'utilitzar el carboni fixat durant la fotosíntesi. En el tercer capítol de la tesi explico com he introduït en arròs cinc gens d'origen bacterià dirigits al cloroplast per a reconstituir la via catabòlica del glicolat d'*Escherichia coli* amb la finalitat de reduir la pèrdua de carboni fixat durant la fotorespiració. Si tingués èxit, donaria com a resultat un increment de la biomassa i del rendiment de la planta. He regenerat un nombre elevat de plantes d'arròs transgèniques que contenen i expressen diferents combinacions dels transgens introduïts i he dut a terme una detallada caracterització molecular. Aquest conjunt de plantes transgèniques seran la base d'un germoplasma amb el qual es podrà estudiar amb més profunditat les conseqüències de l'expressió simultània d'aquests gens en arròs. Aquests resultats facilitaran el disseny d'estratègies més precises per a assolir l'objectiu que a llarg termini ha d'ajudar a reduir la fotorespiració.

En el quart i darrer capítol de la tesi analitzo el potencial de la biotecnologia per a assolir els ODM. La meva conclusió és que, si bé la biotecnologia pot contribuir de manera positiva i substancial a molts dels ODM, la conveniència política i un sistema de regulació que ha imposat una càrrega burocràtica excessiva amenacen d'impedir l'accés de les persones més necessitades a aquestes tecnologies, és a dir, els agricultors pobres que practiquen una agricultura de subsistència i les seves famílies, normalment situades en els països en vies de desenvolupament.

RESUMEN

Los factores que limitan actualmente la productividad de los cultivos están siendo analizados con el objetivo de encontrar soluciones que eviten una posible crisis alimentaría. La sostenibilidad alimentaría, tal y como se describe en los ocho Objetivos de Desarrollo del Milenio (ODM) acordados por la Organización de las Naciones Unidas (ONU), depende de que las intervenciones tecnológicas que se apliquen puedan dar respuesta a los objetivos esenciales planteados, es decir, conseguir aumentar el rendimiento de una manera sostenible, mejorar el valor nutricional de los cultivos alimentarios básicos y dotarlos de nuevas características que les permitan soportar estreses abióticos como la sequía.

Mi tesis presenta tres ejemplos de soluciones biotecnológicas, diversas pero convergentes, que pueden contribuir proporcionando conocimientos fundamentales, herramientas, y productos potenciales en forma de plantas mejoradas y/o cultivos mejorados que faciliten la llegada a una agricultura sostenible.

En el primer capítulo, describo la clonación y caracterización de los dos promotores de los genes *arginina descarboxilasa* (*ADC*) de arroz, los cuales juegan un papel fundamental en la ruta biosintética de las poliaminas. Las poliaminas son importantes para la protección de las plantas frente a estreses abióticos como la salinidad y la sequía. Por lo tanto, profundizar en el conocimiento de los mecanismos moleculares y fisiológicos a través de los cuales confieren tolerancia a las plantas a diferentes estreses, puede proporcionarnos una información importante que nos lleve a encontrar soluciones a estos estreses. Mis resultados sugieren que los promotores de los genes de *ADC* en el arroz se activan diferencialmente en condiciones de falta de agua, tal como demuestran los niveles de expresión del gen marcador y, por tanto indican una activación transcripcional que facilitara la elucidación del mecanismo de protección de la poliaminas a estreses.

Los carotenoides son moléculas naturales importantes porque nos aportan beneficios nutricionales que repercuten en nuestra salud. Dentro del programa más amplio que desarrollamos en el laboratorio, enfocado a comprender el mecanismo de la biosíntesis de carotenoides en plantas, me centré en el estudio de la enzima zeaxantina epoxidasa de *Gentiana lutea* (GlZEP). En el segundo capítulo he presentado el clonaje de su promotor y su caracterización, con el objetivo general de conocer la relación entre la síntesis de los carotenoides y la diferenciación de los cromoplastos. La expresión de *ZEP* está estrechamente ligada con el desarrollo de los cromoplastos, ya que se trata de la enzima clave responsable de la acumulación de anteraxantina y violaxantina en pétalos de *G. lutea* (que contienen grandes cantidades de luteína, violaxantina, anteraxantina y *β*-caroteno). He evaluado la capacidad del

promotor de *ZEP* para conducir la expresión del gen marcador GUS en plantas de tomate transgénicas. Mis resultados sugieren una relación evolutivamente conservada entre ZEP y la diferenciación de los orgánulos que almacenan los pigmentos carotenoides en plantas.

El aumento de la eficiencia fotosintética es un objetivo clave en los programas de mejora genética de arroz y también el foco de una intensa investigación cuyo objetivo es aumentar la capacidad de los cultivos para utilizar el carbono fijado durante la fotosíntesis. Para este estudio en el tercer capítulo, he introducido en arroz cinco genes bacterianos, dirigidos al cloroplasto, para reconstituir la vía catabólica del glicolato de *Escherichia coli,* con el objetivo de reducir la pérdida de carbono fijado durante la fotorrespiración. Si tuviera éxito, esta estrategia debería resultar en un aumento de la biomasa y del rendimiento de la planta. He regenerado bastantes plantas de arroz transgénicas que contienen y expresan diferentes combinaciones de los transgenes introducidos y he llevado a cabo una detallada caracterización molecular. Este conjunto de plantas transgénicas con combinaciones diferentes de genes serán la base de un germoplasma donde se podrá estudiar con más profundidad las consecuencias de la expresión de estos genes de forma simultánea en arroz. Mis resultados ayudaran a diseñar estrategias más refinadas para alcanzar a largo plazo el objetivo de reducir la fotorespiración.

Concluyo mi tesis en el capítulo cuarto, discutiendo el potencial de la biotecnología para alcanzar los ODM. Mi conclusión es que, si bien la biotecnología puede contribuir de manera positiva y sustancialmente a muchos de los ODM, la conveniencia política y un sistema de regulación que ha impuesto una carga burocrática excesiva, amenazan con impedir el acceso de las personas más necesitadas a estas la tecnologías, es decir, agricultores pobres que practican una agricultura de subsistencia y sus familias, normalmente emplazados el países en vías de desarrollo.

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1. Food insecurity and micronutrient malnutrition

The achievement of food security is currently one of the world's greatest challenges, but the situation is projected to deteriorate over the next decade in at least 70 developing countries according to the USDA Economic Research Service and the Food and Agriculture Organization (FAO, 2010). **Table 1.1** provides a glossary of terms associated with food insecurity.

It is estimated that 925 million people currently suffer from chronic hunger and approximately 14,400 children die from hunger-related causes every day. More than one in seven people currently lack sufficient protein and energy in their diet, and even more suffer from some form of micronutrient malnourishment (FAO, 2010). Continuing population growth and rising consumption will mean that the global demand for food will increase for at least another 40 years, and it has been projected that global food production must increase by 70% by 2050 to meet the demand (FAO, 2010). Food insecurity has also increased recently in several regions of the world owing to competing claims for land, water, labor, energy and capital, which generates additional pressure to improve production per unit land (**Table 1.1**; Kajala et al., 2011).

Food insecurity also causes an increase in micronutrient malnutrition, which is known as "hidden hunger" because it is not readily apparent from the clinical signs in most situations (**Table 1.1**). Up to three billion people may suffer from micronutrient deficiency (Graham et al., 2001), which disproportionately affects women and children (Barrett, 2010).

Table 1.1 Glossary of terms associated with food insecurity.

Food security	Exists when all people, at all times, have physical, social and economic access to sufficient amounts of safe and nutritious food to ensure an active and healthy life. The four pillars of food security are availability, access, utilization and stability. The nutritional dimension is integral to the concept of food security.
Food insecurity	Exists when people lack access to sufficient amounts of safe and nutritious food, and are therefore not consuming enough to maintain an active and healthy life. This may reflect a lack of availability or purchasing power, or inappropriate utilization at the household level.
Hidden hunger	Malnutrition, caused by a chronic lack of vitamins and minerals, with ambiguous clinical signs so people who suffer from it may not be aware. Its consequences are nevertheless disastrous: hidden hunger causes stunted mental and physical development, generally poor health and low productivity, and can be fatal. One in three people in the world suffer from hidden hunger. Women and children from the lower income groups in developing countries are often the worst affected.

1.2. **Plant improvement through biotechnology**

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

The application of metabolic engineering in plants will teach us not only how to engineer biochemical changes but also about how the metabolic pathways work. This approach will allow us to identify limitations and rate-determining steps by providing the means to

experimentally define the control points. Transgenic approaches will also allow us to test directly whether a particular gene product is of interest or, more appropriately, important to the plant.

1.2.1. Increasing yield potential

Increasing the maximum yield potential is an important part of any strategy aiming to break through the yield ceiling of crops. The harvest index for many crops is approaching the natural ceiling, so the possibility of genetic intervention to increase photosynthesis and biomass accumulation is being considered (Kebeish et al., 2007).

A successful strategy to enhance photosynthesis is the introduction of C_4-like photosynthesis into C_3 plants. Transgenic rice plants overexpressing the maize phosphoenolpyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPDK) genes, which play key role in organic acid metabolism in the guard cells to regulate stomatal opening, demonstrated 10–30% and 30–35% yield increases, respectively, which was quite unexpected given that only one C_4 enzyme was expressed in each case (Ku et al., 1999). In the PEPC transgenic plants, there was also an unanticipated secondary effect in which RuBisCO showed reduced inhibition by oxygen (Ku et al., 1999).

1.2.1.1. C3 photosynthesis

Approximately 95% of plants, including major crops such as rice, wheat and oat, assimilate CO_2 via the C3 photosynthetic pathway and thus are known as C3 plants (Ku et al., 1996). **Fig. 1.1** shows the CO_2 assimilation pathway in C3 plants, which is known as the Benson Calvin cycle (Calvin, 1989; Kebeish, 2006). One molecule of CO_2 reacts with the five-carbon compound ribulose-1,5-bisphosphate (RuBP) producing an unstable six-carbon intermediate that immediately breaks down into two molecules of the three-carbon compound phosphoglycerate (PGA), hence the name C3 photosynthesis. PGA is converted to glyceraldehyde-3-phosphate during photosynthesis. This triose phosphate is used to form sugars and/or to regenerate the ribulose-1,5-bisphosphate for the next cycle (**Fig. 1.1**; Kebeish, 2006).

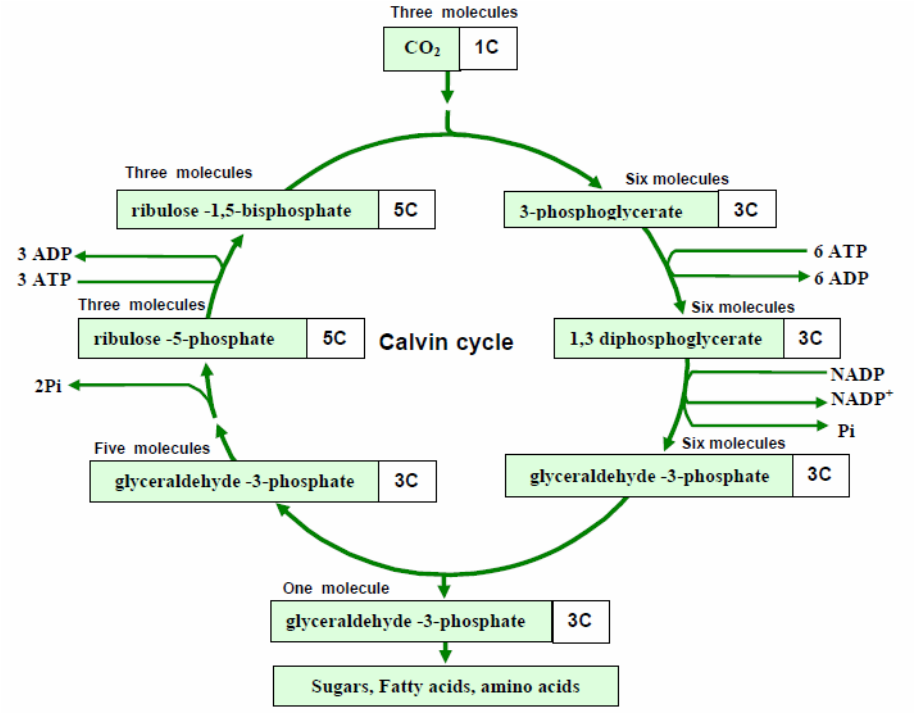

Fig. 1.1 Schematic representation of the Benson Calvin cycle
The carboxylation reaction of RuBisCO yields two molecules of 3-phosphoglycerate (PGA), which is fixed and recycled to ribulose-1,5-bisphosphate (RuBP) in a series of reactions known as the Benson Calvin cycle. The fixation of six molecule of CO_2 requires 12 molecules of NADPH and 18 molecules of ATP. In chloroplasts, CO_2 condenses with RuBP to form two molecules of PGA, which is then reduced to triose phosphate by consuming ATP and NADPH. The triose phosphate is then used to regenerate RuBP and/or to synthesize sugars in the cytosol or starch within chloroplasts (Kebeish, 2006).

1.2.1.2. C4-photosynthesis

About 7500 higher plant species use the C4 photosynthetic pathway **(Fig. 1.2)**, including maize, sugarcane and sorghum. C4 species account of 80% of primary productivity in temperate/tropical grasslands (Sage, 2001). The enhanced photosynthetic performance comes from the ability of C4 plants to concentrate the CO_2 in the vicinity of RuBisCO, and coordinate the activities of two photosynthetic cell types, namely mesophyll cells (MCs) and bundle sheath cells (BSCs).

C4 plants have been divided into three subgroups based on differences in the decarboxylation enzymes in BSCs (Ryuzi Kanai, 1999), i.e. the NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and PEP carboxykinase (PEP-CK) types. However, all C4 plants initially fix CO_2 indirectly as HCO_3^- using phosphoenolpyruvate carboxylase (PEPC) to form oxaloacetate in the MC cytoplasm. **Fig. 1.2** shows the NADP-ME process, where CO_2 enters the MC and is converted to HCO_3^- by carbonic anhydrase (CA; Kebeish, 2006). The HCO_3^- is converted to oxaloacetate (OAA) in the cytosol

by PEPC. OAA then enters the chloroplast and is reduced to malate by malate dehydrogenase (MDH). The malate then diffuses into neighboring BSC chloroplasts through plasmodesmata where it is decarboxylated by malic enzyme (ME). During the decarboxylation of malate, CO_2 is released in the BSC chloroplast. The CO_2 is then fixed by RuBisCO and converted to carbohydrates in the chloroplast. The pyruvate (PYR) produced in the decarboxylation reaction diffuses back to the MC to regenerate phosphoenolpyruvate (Peterhansel et al., 2010).

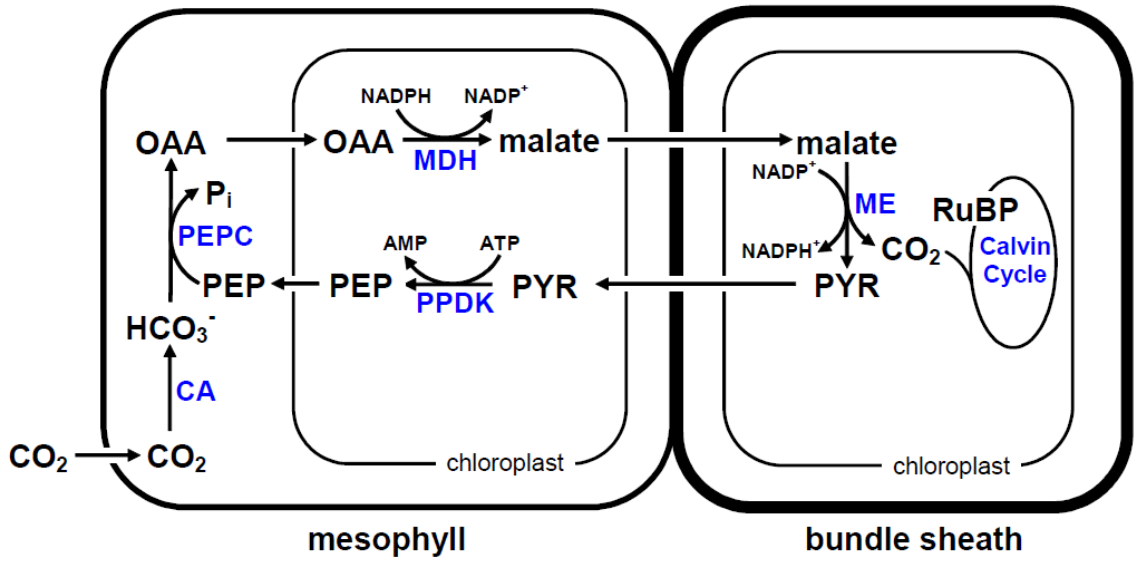

Fig. 1.2 Schematic representation of C4 photosynthesis.
CO_2 enters the MC cytosol where it is converted into HCO_3^-, which reacts with PEP to form oxaloacetate. This diffuses into the MC chloroplast and is converted into malate, which then diffuses into the BSC chloroplast where it is decarboxylated to form pyruvate and CO_2 is released. The pyruvate diffuses back to the MC chloroplast where it is converted into phosphoenolpyruvate and starts a new cycle of CO_2 fixation. The released CO_2 in the BSC chloroplast is used for carbohydrate synthesis via the Benson Calvin cycle. CA: carbonic anhydrase, PEPC: phosphoenolpyruvate carboxylase, OAA: oxaloacetate, MDH: malate dehydrogenase, ME: malic enzyme, PPDK: pyruvate orthophosphate dikinase, MC: mesophyll cell, BSC: bundle sheath cells.

1.2.1.3. Crassulacean acid metabolism (CAM) photosynthesis

Crassulacean acid metabolism (CAM) is another adaptation to increase the efficiency of the Benson Calvin cycle, and is found in species such as pineapple, orchids and cactuses. CAM plants achieve higher water and nitrogen use efficiency than C3 and C4 plants under comparable conditions (Klavsen et al., 2011). However, because their stomata are closed by

day, they are less efficient at CO_2 absorption. This limits the amount of carbon available for growth (Klavsen et al., 2011).

The CAM photosynthetic pathway is divided into two parts. The first part is initiated when CO_2 enters into the cell via the C4 pathway through open stomata when transpirational water losses are low at cooler night temperatures. The CO_2 is converted into malate and stored in vacuoles. The second part is the decarboxylation of malate to generate pyruvate and the CO_2 source that is used in the chloroplast by RuBisCO for carbohydrate biosynthesis during the day (Klavsen et al., 2011).

1.2.2. Abiotic stress tolerance

Abiotic stresses are harmful environmental factors such as drought, salinity and extreme temperatures. Animals are affected by abiotic stress, but plants are much more vulnerable because of their inability to move which means they face constant exposure to detrimental environmental conditions. Environmental stresses reduce crop productivity (Verslues et al., 2006) and increasingly reflect salinization by irrigation and the lower level of rainfall (Vinocur and Altman, 2005). Some land has deteriorated to the extent that it can no longer be used for agriculture, compounding the loss of agricultural land through urbanization. The availability of food is therefore threatened, and the remainig arable land must increase in productivity to maintain food security (Christou and Twyman, 2004). One solution is to create plants that are more tolerant towards abiotic stresses (Takeda and Matsuoka, 2008). The discovery of genes with potential roles in abiotic stress adaptation has accelerated thanks to post-genomics technologies that allow the global analysis of gene and protein functions in plants under stress (Mochida and Shinozaki, 2010).

1.2.2.1. Drought

Drought is the major abiotic stress threatening agricultural productivity. Drought can be defined as a period of below-normal precipitation that limits productivity by introducing a water deficit, and thus a lower water potential in the plant (Yang et al., 2010). About 28% of the world's soil is constitutively affected by drought, and up to 50% is affected periodically due to shallowness, poor water holding capacity and other factors (Salekdeh et al., 2009). Drought is a major contributor to food insecurity and poverty (FAO, http://www.fao.org/nr/water/docs/waterataglance.pdf).

Rice is one of the world's most important food crops, but it is very sensitive to drought stress because of its limited ability to adapt to water-deficit conditions (Yang et al., 2010). In rain-fed ecosystems (approximately one third of all rice crops) drought reduces productivity

by 13–35% (Degenkolbe et al., 2009). Maize is another staple crop that is highly sensitive to water deficit, especially during pollination and embryo development (Yang et al., 2010). The first processes to be affected by drought are cell growth and photosynthesis (Chaves, 2009). Photosynthesis can be affected directly by the reduced availability of CO_2 due to stomatal closure or by oxidative stress.

1.2.2.2. Salinity

Soil salinity is the second most important abiotic stress factor affecting agricultural productivity, particularly in South and South-East Asia and in arid and semi-arid regions with a limited water supply and a hot dry climate (Hakim et al., 2009). Approximately 20% of the world's current farmland and nearly 50% of all irrigated land is affected by salinity. Salinity is expected to increase in the future, resulting in the loss of 30% of arable land by 2025 and 50% by 2050 (Wang et al., 2003). Rice is the most sensitive among the cereals (Munns et al., 2008) although the sensitivity of maize varies according to the developmental stage, and is highest during early vegetative growth (Fortmeier and Schubert, 1995). Many plant species are tolerant towards salt stress, which is expressed as the increase in dry mass at different salt concentrations (Munns et al., 2008). High concentrations of salt in the soil reduce water uptake by the roots, producing similar effects to drought. This is called the osmotic effect and it has an immediate impact on plant growth and development (Munns and Tester, 2008). The osmotic effect of salinity stress reduces photosynthesis and cell growth by the same mechanisms as drought (Chaves et al., 2009). If the stress is prolonged, however, additional stress-response genes are activated, reflecting the combined effects of dehydration, osmotic stress and ion imbalance. Microarray analysis of plants exposed to salt and dehydration has also indicated substantial differences between the gene expression profiles elicited by these stresses (Seki et al., 2002). Strategies to induce salt stress tolerance include the elimination of sodium ions from the cytoplasm and the accumulation of low-molecular-weight protective compounds known as osmolytes or compatible solutes (compatible because they do not inhibit normal metabolic functions). Such molecules include glycine betaine, trehalose, proline, sorbitol, mannitol and ectoine (Hasegawa and Bressan, 2000).

1.2.2.3. Cold stress

Low temperatures also limit plant growth, and this has a major impact on grasses by inducing vernalization and causing low-temperature damage at anthesis (Tester and Bacic, 2005). Cold stress can be divided into chilling and freezing stress depending on the

temperature. Chilling stress occurs at temperatures below the plant's normal growth temperature but not low enough to form ice crystals (Chinnusamy et al., 2007). The primary impact of chilling is to cause membrane leakiness if membranes cannot retain their fluidity at low temperatures (Beck et al., 2004).

In contrast, freezing stress results from the formation of ice crystals in the extracellular space, initially causing dehydration but in many cases also structural damage through expansion. Prolonged cold stress slows down metabolism and leads to the formation of free radicals, which induce oxidative stress. Because cold and drought both induce dehydration as a primary effect, they share more common features than either share with salinity stress (Verslues et al., 2006; Beck et al., 2007).

1.2.2.4. Plant water potential and changes during stress

Water is required by all life forms as a medium for biochemical reactions. In plant cells, water-generated turgor pressure is also a driving force for cell expansion. However, vegetative growth can only occur when the free energy state of water molecules lies within a particular physiological range, and this is expressed as the water potential (ψw). In any plant cell, ψw consists of pressure and osmotic potential. While maintaining a positive turgor pressure, plant cells usually adjust their osmotic potential to balance the water budget and thus meet the requirements of the whole plant (Bernstein, 1961). Substantial changes in the environmental water potential therefore cause osmotic stress, which disrupts normal cellular activities and eventually kills the plant. All abiotic stresses reduce the water potential of plant cells. High salinity and drought are the major causes of osmotic stress in plants (Xiong and Zhu, 2002), but chilling and freezing also cause osmotic stress by reducing water absorption and inducing dehydration (Zhu et al., 1997; Verslues et al., 2006). Exposure to osmotic stress induces a wide range of responses at the molecular, cellular and whole-plant levels (Hasegawa et al., 2000; Xiong and Zhu, 2002).

1.2.2.5. Plant responses to low water potential

Plants respond immediately to declining water potential by closing their stomata, which reduces water loss by transpiration. If the stress is prolonged, plants accumulate solutes to increase their osmotic potential, stiffen their cell walls to counter the loss of turgor pressure (Boyer, 1995), and reduce the rate of shoot and root growth (**Fig. 1.3**). Under severe stress conditions it becomes increasingly difficult to avoid dehydration, and mechanisms that allow the tolerance of reduced water content become important. Most of the dehydration tolerance

mechanisms studied thus far function primarily to protect the cellular structure from the effects of dehydration (Verslues et al., 2006). These mechanisms include the accumulation of osmolytes that protect macromolecular structures from conformational changes (Turner and Jones, 1980), the accumulation of protective proteins and the detoxification of free radicals (Xiong and Zhu, 2002; Degenkolbe et al., 2009).

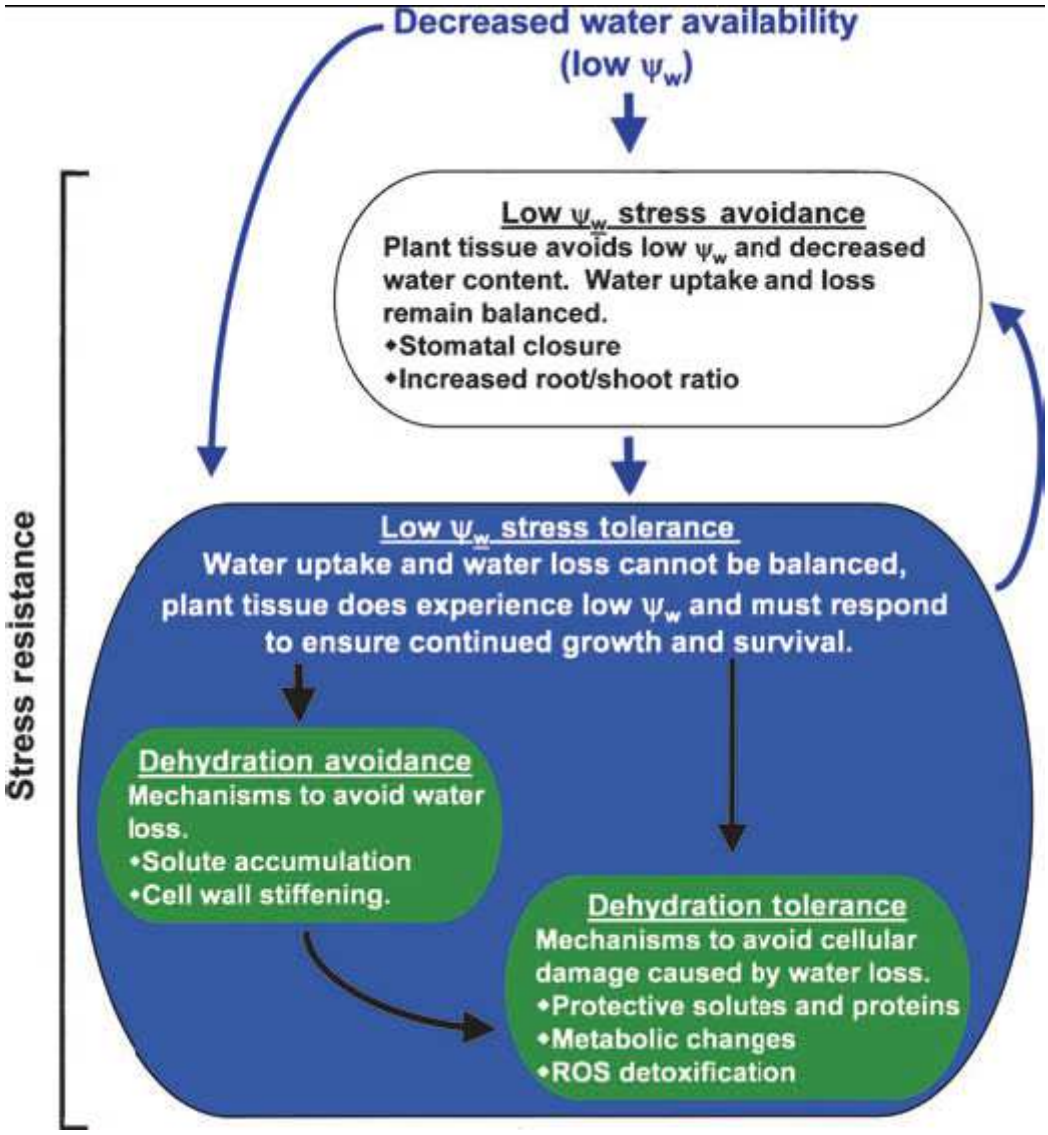

Fig. 1.3 Plant responses to low water potential (Verslues et al., 2006).

1.2.2.6. Importance of stress intensity and duration

Abiotic stresses vary in their intensity and duration, and plants respond in different ways to mild or severe stress and to transient or long-term stress (Degenkolbe et al., 2009).

Short periods of severe stress often induce short-term but ultimately unsustainable responses of the “wait and see” variety, whereas long-term stress requires the induction of more extravagant avoidance mechanisms that require significant developmental changes. For example, closing stomata and accumulating osmolytes is a suitable response to transient stress but is difficult to maintain, whereas plants facing long-term dehydration may invest resources to increase the absorption of water by developing deeper roots and increasing the conduction capacity of the root system (Levitt, 1972). Most studies thus far have focused on short-term responses to abiotic stress but there is a need to investigate long-term adaptation strategies as this is similar to field conditions and will be more useful for the development of stress-tolerant crops (Vinocur and Altman, 2005).

1.2.2.7. Recovery from stress

Under natural conditions, plants usually experience cycles of stress and recovery (e.g. dehydration followed by rehydration as part of seasonal weather variations or agricultural practices). The degree of recovery from stress, which also has a molecular basis, is therefore as relevant as the initial stress response (Vinocur and Altman, 2005).

1.2.3. Promoters as key elements for the control of transgene expression

Promoter recognition by RNA polymerase is a crucial step in gene expression and its regulation. The benefits of directing gene expression in specific spatiotemporal or externally-controlled profiles has been understood by researchers studying regulation and development in transgenic organisms for a number of years, as confirmed by the diversity of available promoters.

Eukaryotes have three different RNA polymerases that are responsible for transcribing different subsets of genes: RNA polymerase I transcribes genes encoding ribosomal RNA; RNA-polymerase II transcribes genes encoding mRNA and certain small nuclear RNAs, while RNA polymerase III transcribes genes encoding tRNAs and other small RNAs (Huet et al., 1982; Breant et al., 1983; Allison et al., 1985).

RNA polymerase II (Pol II) promoters typically contain common core-promoter elements that are recognized by general transcription initiation factors, and gene-specific DNA elements that are recognized by regulatory factors, which in turn modulate the function of the general initiation factors (Record et al., 1996).

The transcription of genes by RNA polymerase II is a complex process involving multiple components. One important class of core (or minimal) promoters only consists of a

TATA box, which directs transcriptional initiation at a position about 30 bp downstream of transcriptional start site, and is bound by a subunit known as TBP (the TATA binding protein) (Hernandez, 1993; Burley and Roeder, 1996). The TBP is present in the pre-initiation complexes with all three RNA polymerases (Hernandez, 1993; Burley and Roeder, 1996; Lee and Young, 1998).

Other minimal promoters do not contain a TATA box (and are, therefore, described as TATA-less). In these promoters, the exact position of the transcriptional start site may instead be controlled by another basic element known as the initiator (Inr; Smale, 1994; 1998). Promoters containing only an Inr are typically somewhat weaker than TATA-containing promoters (Smale, 1998). In addition to the two promoter classes mentioned above, there are also promoters which have both TATA and Inr elements, and promoters that have neither (Smale, 1994).

Another promoter element, the downstream promoter element (DPE), which was recently discovered in both *Drosophila melanogaster* and humans, is present in some TATA-less, Inr-containing promoters about 30 bp downstream of the transcriptional start point (**Fig. 1.4**; Butler and Kadonaga, 2012).

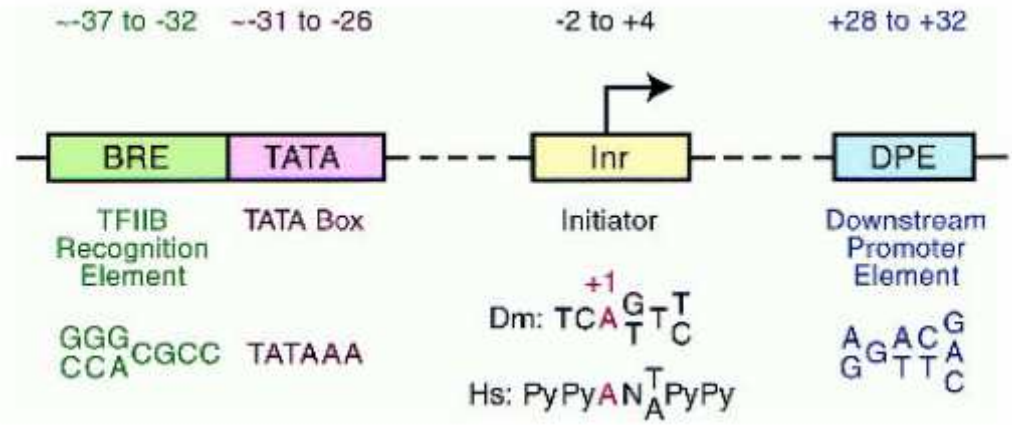

Fig. 1.4 Core promoter elements that can participate in transcription by RNA polymerase II. Each of these elements is found in only a subset of core promoters. Any specific core promoter may contain some, all, or none of these motifs. The BRE is an upstream extension of a subset of TATA boxes. The DPE requires an Inr, and is located precisely at +28 to +32 relative to the A+1 nucleotide in the Inr. The DPE consensus was determined with *Drosophila melanogaster* transcription factors and core promoters. The Inr consensus sequence is shown for both *D. melanogaster* (Dm) and humans (Hs) (Butler and Kadonaga, 2012).

1.2.3.1. Core promoter

The core promoter is the minimal stretch of contiguous DNA sequence sufficient to achieve the accurate initiation of transcription by RNA polymerase II (Struhl, 1987; Weis and Reinberg, 1992; Smale, 1994; 1998; 2001; Burke et al., 1998). Core promoters are much more than simple DNA scaffolds for the basal transcription machinery. Rather, core promoter elements are dynamic and vital participants in the regulation of transcriptional activity. It is important to analyze core promoters because such analysis contributes fundamental insights into the mechanisms by which transcription occurs in eukaryotes and the cascade of events that precedes the activation of transcription must eventually lead to an understanding of the basal transcriptional machinery at the core promoter.

There are several sequence motifs—which include the TATA box, Inr, TFIIB recognition element (BRE), and downstream core promoter element (DPE)—that are commonly found in core promoters (**Fig. 1.4**). These motifs each have specific functions that relate to the transcription process, and it is important to note that each of these core promoter elements is found in some but not all core promoters. It appears that there are no universal core promoter elements (Butler and Kadonaga, 2012).

In addition to the core promoter, other cis-acting DNA sequences that regulate RNA polymerase II transcription include the proximal promoter, enhancers, silencers, and boundary/insulator elements (Blackwood and Kadonaga, 1998; Bulger and Groudine, 1999; West et al., 2002). These elements contain recognition sites for a variety of sequence-specific DNA-binding factors that are involved in transcriptional regulation.

1.2.3.1 Constitutive promoters

Plant viruses have small genes that are easy to define genetically, and small genomes that are easy to manipulate *in vitro*, so many of the earliest constitutive promoters were derived from plant viruses and are still widely used today. The most prevalent of these is the *Cauliflower mosaic virus* 35S promoter (CaMV 35S), which controls the synthesis of the 35S major transcript (Odell et al., 1985; Kay et al., 1987). Although widely used, the CaMV 35S promoter has certain limitations such as its poor performance in monocots, and its suppression by feeding nematodes (Goddijn et al., 1993; Urwin et al., 1997). Alternative virus promoters with similar or improved properties have therefore been sought. Examples include promoters from *Figwort mosaic caulimovirus* (FMV; Bhattacharyya et al., 2002), *Cassava vein mosaic virus* (CsVMV; Verdaguer et al., 1996), *Cestrum yellow leaf curling virus* (CmYLCV; Stavolone et al., 2003), *Mirabilis mosaic virus* (MiMV; Dey and Maiti, 1999) and *Peanut*

chlorotic streak virus (PClSV; Maiti and Shepherd, 1998; Bhattacharyya et al., 2003).

Plant housekeeping genes are another important source of constitutive promoters because housekeeping genes encode proteins that are required by all cells for basic functions such as core metabolism and the maintenance of cell structure and integrity. One of the most commonly used specific promoters is the maize ubiquitin-1 (Ubi-1) promoter, which is about 10 times stronger than the CaMV35S promoter in corn protoplasts when combined with the Ubi-1 first intron (Norris et al., 1993), but 10 times weaker than CaMV 35S in tobacco protoplasts, limiting its potential to cereal monocots (Christensen et al., 1992; Weeks et al., 1993; Gupta et al., 2001).

Constitutive expression can be problematic for several reasons. If a specific transgene is overexpressed at the wrong time in development, in tissues where it is not normally expressed, or at very high levels, it can have unexpected consequences on plant growth and development. For example, the constitutive expression of signal transduction 'master-switches' for pathogen resistance can reduce growth (Bowling et al., 1994, 1997), or increase susceptibility to other pathogens (Stuiver and Custers, 2001; Berrocal-Lobo et al., 2002). Novel strong constitutive promoters need to be identified to expand the choice of regulatory elements beyond the rather limited number of constitutive promoters that exist today (Naqvi et al., 2009).

1.2.3.3. Spatiotemporal promoters

If the constitutive overexpression of transgenes interferes with normal growth and development, then spatiotemporal promoters can be used to restrict and refine the transgene expression profile. The most commonly-available spatiotemporal promoters in plants are those that restrict transgene expression to seeds, and for many reasons the seeds are often a favored target for transgene expression, particularly if the goal of an experiment is to force the accumulation of a heterologous product that might interfere with vegetative growth at high concentrations or to improve the nutritional quality of seeds used as staple foods. Many promoters have been identified that target genes specifically to the seed, or to a particular region of the seed. Genes encoding storage proteins such as corn zein (Schernthaner et al., 1988), rice glutelin (Leisy et al., 1989; Takaiwa et al., 1991; Zheng et al., 1993), barley hordein (Marris et al., 1988), rice prolamin (Qu and Takaiwa, 2004) and wheat glutenin (Colot et al., 1987) have been rich sources of seed-specific promoters, predominantly directing expression to the endosperm (Wobus et al., 1995). Additional promoters have been shown to direct gene expression to the embryo and aleurone (Opsahl-Sorteberg et al., 2004;

Qu and Takaiwa, 2004; Furtado and Henry, 2005).

Many case studies have been published in which multiple transgenes are expressed in specific tissues by combining the use of spatiotemporally-regulated and constitutive promoters, but there have been few examples of studies in which different spatially-restricted promoters have been used in the same plant (Bisht et al., 2004; 2007). One study involved the use of five different promoters to express five transgenes in corn endosperm plus the selectable marker gene under constitutive control (Zhu et al., 2008b). This showed how combinatorial transformation with multiple genes could be used to generate a library of plants with different phenotypes representing carotenoid biosynthesis. The different promoters ensured that, in any combination, it would be possible to isolate plants without multiple copies of the same promoter thus reducing the likelihood of transcriptional silencing (Peremarti et al., 2010; Butler and Kadonaga, 2012).

1.2.3.4. Inducible promoters

Although spatiotemporal promoters are powerful tools for the control of transgene expression, that control is still dependent on the plant and the expression/activity of endogenous trans-activating factors (Peremarti et al., 2010). Inducible promoters are controlled by physical or chemical signals that can be supplied exogenously. These have been combined with both constitutive and spatiotemporal promoters in transgenic plants but are generally not combined with each other. The most widely-used hormone-responsive promoters are those induced by auxins, gibberellins and abscisic acid, although promoters responsive to heterologous hormones (from insects and mammals) are also useful because they do not activate endogenous pathways. For example, Martinez et al. (1999) developed a hybrid system consisting of the tobacco budworm ecdysone receptor ligand-binding domain fused to the mammalian glucocorticoid receptor DNA-binding domain and the VP16 transactivation domain. The receptor responds to tebufenozide (an insecticide better known by its trade name CONFIRM). Similarly, Padidam et al. (2003) have developed a system that is based on the spruce budworm ecdysone receptor ligand-binding domain, and responds to another common insecticide, methoxyfenozide (INTREPID). Another system based on the European corn borer ecdysone receptor also responds to this insecticide (Unger et al., 2002).

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AIMS AND OBJECTIVES

Aims and objectives:

Aims:

The overall aim of my thesis is to explore a number of specific biotechnological interventions involving the creation of plants with enhanced attributes in the context of food security. A further aim is to develop the necessary basic knowledge and unravel the mechanisms that underpin the behavior of these plants. Finally, I will investigate the potential of plant biotechnology to address the Millennium Development Goals.

Specific objectives:

1. To clone and characterize the two promoters of the rice *arginine decarboxylase* (*ADC*) genes by driving the expression of the *gus*A reporter gene in transgenic rice plants, and to try to determine the pivotal role of endogenous polyamine biosynthetic enzymes under drought stress conditions.

2. To clone, isolate and characterize the *Gentiana lutea Zeaxanthin epoxidase* (*GlZEP*) promoter driving the *gus*A reporter gene expression in transgenic tomato plants in order to understand the link between carotenoid synthesis and chromoplast differentiation.

3. To reconstitute the *Escherichia coli* glycolate catabolic pathway in rice in order to explore the potential of this strategy to reduce the loss of fixed carbon during photorespiration, thus enhancing photosynthesis.

CHAPTER I

Cloning and functional characterization of two rice *arginine decarboxylase* gene promoters during abiotic stress

ABSTRACT:

We have cloned and characterized the promoter regions of the rice (*Oryza sativa* L.) paralogs *OsADC1* and *OsADC2* encoding arginine decarboxylase. Sequence analysis showed that both promoters contain a putative TATA box as well as stress-response elements related to drought and methyl jasmonate signaling. We expressed *gus*A under the control of the *OsADC1* and *OsADC2* promoters in transgenic rice plants to investigate the transcriptional regulation of polyamine biosynthesis under normal conditions and during drought stress. Both reporter genes were expressed in leaves, roots, flowers and seeds, and were induced significantly soon after drought stress was applied. Real-time RT-PCR showed that mRNA levels increased 6–12-fold in leaves and 20-fold in roots after 24 h under stress, and returned to normal after a one-week recovery period. The *OsADC2* promoter was more responsive to drought than the *OsADC1* promoter in both leaves and roots. The isolated *OsADC1* and *OsADC2* promoters will be useful for the design and implementation of precise and targeted strategies for the creation of drought-tolerant plants.

INTRODUCTION

Cereals such as rice, wheat, corn and barley are major sources of human food and animal feed, and they also provide the raw material for many industries. Some of the limitations of conventional breeding in cereals can be overcome by applying genetic engineering techniques, especially to improve abiotic stress tolerance (Araus et al., 2002; Shrawat and Lörz, 2006). Drought, salinity and low temperatures are among the most devastating abiotic stresses that affect the growth, development and productivity of agricultural crops worldwide. These stresses induce various biochemical and physiological responses in plants, which must adapt in order to survive. Some genes respond directly to stress at the transcriptional level, and these often encode proteins that (1) act directly to protect the plants against stress, and (2) that regulate stress responses (signaling pathways and gene expression). The first group includes proteins that protect cells from dehydration, such as enzymes required for the biosynthesis of osmoprotectants (compatible solutes), late embryogenensis abundant proteins, antifreeze proteins, chaperones, and detoxification enzymes (Seki et al., 2007). One strategy to improve drought tolerance is to increase the content of compatible solutes by overexpressing genes responsible for the synthesis of amino acids (e.g. proline), quaternary and other amines (e.g. glycine betaine and polyamines) and various sugars and alcohols (Umezawa et al., 2006). The second (regulatory) group includes signal transduction components and transcription factors. These interact directly with the promoters located upstream of genes in the first group, so it is important to study stress-responsive promoters such as those found in polyamine biosynthesis genes.

Polyamines are small, ubiquitous, nitrogenous compounds implicated in a variety of stress responses. In plants and some bacteria, putrescine is synthesized from arginine by arginine decarboxylase (ADC, EC 4.1.1.19) through the intermediate agmatine (Hanfrey et al., 2001). Putrescine is further converted into spermidine and spermine by spermidine synthase (SPDS, EC 2.5.1.16) and spermine synthase (SPMS, EC 2.5.1.22), respectively. These two enzymes add aminopropyl groups generated from *S*-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC, EC 4.1.1.50; Bagni and Tassoni 2001; Bassie et al., 2008). Endogenous putrescine levels increase in response to potassium deficiency in barley (*Hordeum vulgare* L.) through the activation of ADC (Richard and Coleman, 1952). Subsequent studies also confirmed that plants accumulate polyamines when subjected to biotic and abiotic stresses (reviewed in Alcazar et al., 2006; Groppa and Benavides, 2008). Unraveling the stress tolerance mechanisms used by polyamines has been hampered by the

limited availability and understanding of regulatory elements in polyamine biosynthesis gene promoters, which are directly relevant to the control of polyamine metabolism in plants.

Several *ADC* cDNA clones have been isolated from plants and characterized (Primikirios and Roubelakis-Angelakis, 1999 and references therein; Hao et al., 2005 and references therein). Many plants appear to possess a single *ADC* gene (Nam et al., 1997; Perez-Amador et al., 1995; Primikirios and Roubelakis-Angelakis, 1999; Rastogi et al., 1993), but an ancestral *ADC* gene appears to have undergone duplication early in the evolution of the *Brassicaceae* family yielding at least two highly-conserved paralogs in 12 of the 13 taxa surveyed thus far, the exception being the basal genus *Aethionema* (Galloway et al., 1998). For example, three *ADC* cDNAs (*BjADC1*, *BjADC2* and *BjADC3*) have been isolated from the amphidiploid crucifer *Brassica juncea* (Mo and Pua, 1998; 2002), two *ADC* genes (*spe1* and *spe2*) have been identified in *Arabidopsis thaliana* (Watson and Malmberg, 1996; Watson et al., 1997) and several *ADC* sequences have been reported in different tobacco cultivars (Wang et al., 2000; Bortolotti et al., 2004). Although many dicot *ADC* genes are available in the databases, only two monocot representatives have been isolated, from oat (Bell and Malmberg, 1990) and rice (Akiyama and Jin, 2007; Peremarti et al., 2010). The oat *ADC* cDNA encodes a 66-kDa precursor that may be proteolytically processed into 42 kDa and 24 kDa products (Malmberg et al., 1992; Malmberg and Cellino 1994). The rice *ADC* cDNA encodes a 74-kDa protein 702 amino acids in length and no proteolytic processing appears to be required for enzyme activity (Akiyama and Jin, 2007).

Plant *ADC* genes are spatiotemporally regulated, e.g. *ADC* mRNA accumulates predominantly in young tissues in pea (Perez-Amador et al., 1995), varies during tomato fruit ripening (Rastogi et al., 1993) and accumulates to higher levels in the stem and roots compared to leaves in soybean (Nam et al., 1997). In tobacco, increased *ADC* expression and polyamine levels have been associated with vegetative growth abnormalities (Masgrau et al., 1997). The two Arabidopsis *ADC* genes are differentially expressed, with *ADC1* expressed in all tissues and *ADC2* expressed mainly in cauline leaves and siliques, and each induced by different abiotic stresses (Soyka and Heyer, 1999; Perez-Amador et al., 2002; Urano et al., 2003).

In an early study we have shown that paralogous *ADC* genes exist outside the *Brassicaceae*. We have isolated a second *ADC* gene from rice (*ADC2*) and compared its sequence and expression profile with rice *ADC1*, the known oat *ADC* gene, and *ADC* genes from dicots. Whereas rice *ADC1* is expressed predominantly in leaf, root and stem tissues,

ADC2 is restricted to the stem (Peremarti et al., 2010). In this study we report the cloning and spatiotemporal expression of the two *ADC* gene promoters from rice under drought stress conditions. Our data could facilitate new strategies to engineer plants with stress-inducible promoters that could increase stress tolerance without a yield penalty.

MATERIALS AND METHODS

Cloning the *OsADC1* and *OsADC2* promoters

Genomic DNA was extracted from the leaves of two-month-old wild type rice plants (*Oryza sativa* L. subsp Japonica cv. EYI105) as described by Edwards et al. (1991). Four sets of primers were designed to cover 1696-bp and 2273-bp segments of the rice *OsADC1* promoter region (GenBank accession number AY604047), and 1585-bp and 2695-bp segments of the rice *OsADC2* promoter (GenBank accession number AK058573).

The *OsADC1* promoter was amplified using forward primer *OsAdc1P*-1678-*Xho*I-F (5'-ACT CGA GAC GCC ATG ATG TGA CAA TT-3') which annealed 1678 bp upstream of the 5'-UTR, and reverse primer *OsAdc1P-5-Xba*I-R (5'-CTC TAG AGG AGA ACG CTA AAA TCC ACA G-3') which annealed 5 bp downstream of the 5'-UTR, or with forward primer *OsAdc1P*-2278-*Xho*I-F (5'-ACT CGA GGT TTA CAC GTC CTC TCG TTG-3') which annealed 2273 bp upstream of the 5'-UTR, combined with the reverse primer described above. The *OsADC2* promoter was amplified using either forward primer *OsAdc2P*-2686-*Sal*I-F (5'-CGT CGA CGA CTG TTC CAC AGC GTG CCA ATC-3') which annealed 2686 bp upstream of the 5'-UTR, or *OsAdc2P*-1576-*Sal*I-F (5'-AGT CGA CGC GAG AGA ATC TCA GGT TAC TG-3') which annealed 1576 bp upstream of the 5'-UTR, both with the same reverse primer, *OsAdc2P*-9-*Xba*I-R (5'-TTC TAG AGT AGC ATA TGG GAT GTA GAC TGC-3') which annealed 9 bp downstream of the 5'-UTR. All the primers introduced restriction sites for subcloning, which are shown in the above sequences as boxes.

We carried out 35 amplification cycles comprising denaturation (94°C, 45 s), annealing (60°C, 45 s) and extension (72°C, 2 min) using the GoTaq DNA Polymerase Kit (Promega, Madison WI, USA). The PCR products were transferred to the PCR® II TOPO® vector (TA Cloning Kit, Invitrogen, Carlsbad, CA) and sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit on a 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed using DANMA software (UAB, Barcelona, Spain). Ten different clones representing each of the four products were used to confirm nucleotide identity.

Vector construction for plant transformation

The *OsADC* promoter fragments were digested with *Xho*I, *Xba*I and/or *Sal*I (as appropriate), purified by 0.8% TAE agarose gel electrophoresis (Sambrook et al., 1989) and recovered using the Geneclean® II kit (MP Biomedicals, IllKrich, France). The fragments were

introduced into the corresponding restriction sites of a new vector named p25-126, which was assembled by combining a *Bam*HI–*Eco*RI fragment from pAHC25 containing *gus*A and the *nos* terminator (Christensen and Quail, 1996) with a *Bam*HI–*Eco*RI fragment from pTO126 containing the ampicillin resistance gene and a polylinker with *Xho*I, *Xba*I and *Sal*I restriction sites (Okita et al., 1989). This generated four constructs named pl*ADC1:GUS*, ps*ADC1:GUS*, pl*ADC2:GUS* and ps*ADC2:GUS*, which are shown in **Fig. 2.1**. These were introduced into rice explants by particle bombardment along with the plasmid p35S-*HPT* containing the *hpt* selectable marker encoding hygromycin phosphotransferase under the control of the CaMV 35S promoter, which was derived from vector p35S*GUS-HPT* (Sudhakar et al., 1998) by digesting with *Hin*dIII to remove *gus*A and reclosing. All intermediate and final vectors were verified by sequencing.

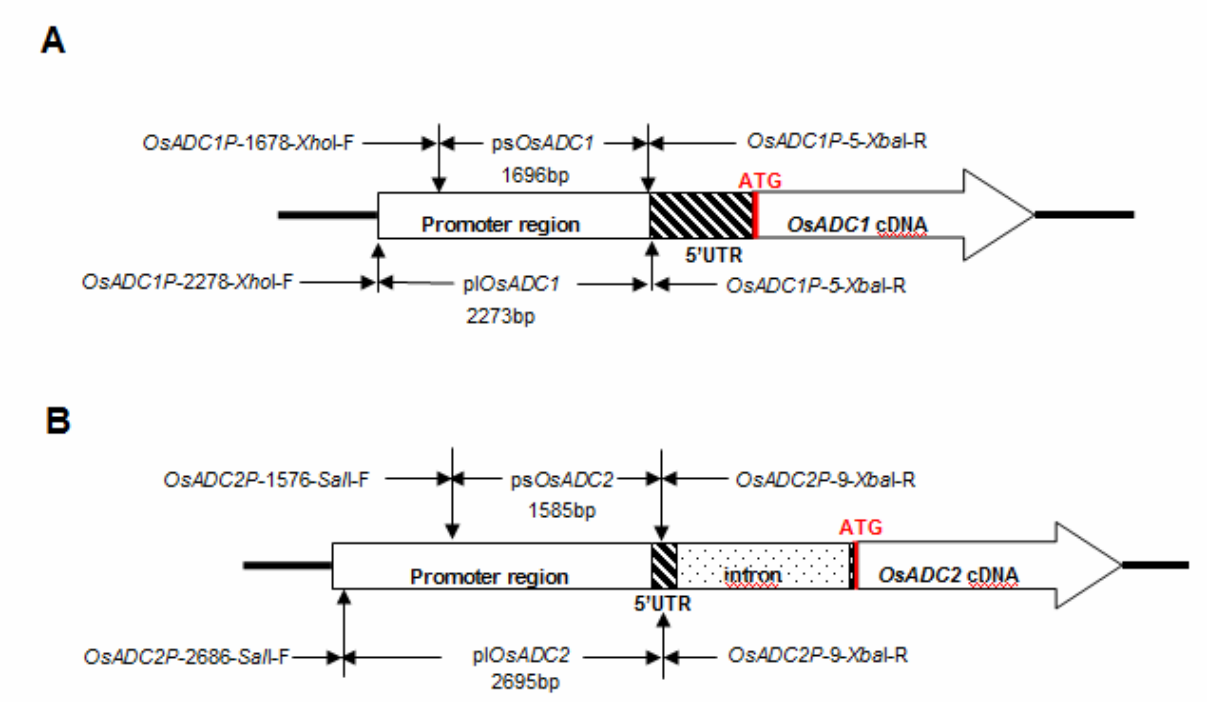


Fig. 2.1 Schematic representation of the rice *arginine decarboxylase* (*OsADC*) promoter regions.

The 5'UTRs are shown as black and white stripes (including the ATG start codon), the intron is shown as a box with black points, primer positions are shown with arrows, and the length of the amplified fragments for cloning the promoters. **A.** *OsADC1*. **B.** *OsADC2*

In silico analysis

The *OsADC1* and *OsADC2* promoter fragments were screened for transcription factor binding sites and other regulatory elements using the PlantCARE portal (http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

Transient expression analysis

Wild type rice plants were grown in the greenhouse at 26 ± 2°C with a 16-h photoperiod (900 µmohn m^{-2} s^{-1} photosynthetically active radiation) and 80% relative humidity. Mature seeds were harvested and embryos were transferred to MS basal medium supplemented with 0.6 M mannitol (osmotic medium) for 4 h before particle bombardment with the promoter constructs as described in Sudhakar et al. (1998) and Valdez et al. (1998). GUS histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Sigma-Aldrich, St Louis, MO) was carried out 48 h after bombardment, as described by Jefferson et al. (1987). The mean number of blue spots from triplicate plates in two different experiments was used to determine the transient expression efficiency.

Transgenic rice plants

The reporter plasmids were mixed with p35S-*HPT* at a 3:1 molar ratio and precipitated onto gold particles (Christou et al., 1991) before bombardment and recovery as previously described (Sudhakar et al., 1998; Valdez et al., 1998). Following regeneration, plantlets were transferred to soil and maintained in the growth chamber as above.

DNA blot analysis

Genomic DNA was extracted from 5 g of frozen leaf tissue as described by Sambrook et al. (1989) and 13-µg aliquots were digested with a panel of enzymes cutting once, twice or not at all in the transgene to determine the transgene integration characteristics and copy number. The fragments were separated by 0.8% agarose gel electrophoresis and blotted onto a positively-charged nylon membrane (Roche, Mannheim, Germany) according to the manufacturer's instructions, and fixed by UV crosslinking. The DNA fragments were hybridized with a digoxigenin-labeled *gus*A-specific probe at 42°C overnight using DIG Easy Hyb buffer (Roche Diagnostics GmbH, Mannheim, Germany) as previously described (Bassie

et al., 2000). The membrane was washed twice for 5 min in 2x SSC, 0.1% SDS at room temperature, twice for 20 min in 0.2x SSC, 0.1% SDS at 68°C, and then twice for 10 min in 0.1x SSC, 0.1% SDS at 68°C. After immunological detection with anti-DIG-AP (Fab-Fragments Diagnostics GmbH, Germany) chemiluminescence generated by the CSPD reagent (Roche, Mannheim, Germany) was detected on Kodak BioMax light film (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The probe was a 512-bp internal *gus*A fragment generated using the PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany) with forward primer 5'-CCT GTA GAA ACC CCA ACC CGT GA-3' and reverse primer 5'-ACG CTG CGA TGG ATT CCG GCA TA-3' and pACH25 as the template (Christensen and Quail, 1996).

RNA blot analysis

Total RNA was isolated from the leaves of six-week-old plants using TRIZOL® Reagent (Invitrogen, Carlsbad, CA), and DNA was removed with DNase I (RNase-Free DNase Set, QIAGEN, Valencia, CA, USA). Denatured RNA (30 µg) was separated by 1.2% agarose-formaldehyde gel electrophoresis using 1x MOPS buffer (Sambrook et al., 1989). The remaining steps were carried out as described above for the DNA blots although hybridization was carried out at 50°C overnight and membranes were exposed to BioMax light film for 2 h at 37°C.

Drought stress experiments

Six-week-old transgenic plants were placed under drought stress by exposure to 20% polyethylene glycol (PEG8000) for 24 h under otherwise standard growth chamber conditions, as previously described (Capell et al., 2004; Peremarti et al., 2009). Leaf and root samples were collected 0, 1, 3, 6 and 24 h after PEG treatment. After 24 h, the PEG solution was replaced with water and the plants were allowed to recover for 1 week before final samples were collected from leaves and roots. The experiment was repeated three times. Samples from six plants were pooled in every repetition. The data were processed by analysis of variance followed by Student's t-test.

Quantitative real-time RT-PCR

Leaf or root tissues from six plants were pooled in one sample and total RNA was extracted as

above and quantified using a NANODROP 1000 spectrophotometer (Thermo Scientific, Vernon Hills, Illinois, USA). We then used 2 µg as the template for first strand cDNA synthesis in a 20-µl reaction volume with Omniscript Reverse Transcriptase, following the manufacturer's recommendations (QIAGEN, Valencia, CA, USA).

Real-time PCR was carried out using a BIO-RAD CFX96^{TM} system. Each 25-µl reaction comprised 10 ng cDNA, 1x iQ SYBR green supermix (BIO-RAD) and 0.2 µM each of the *gus*A-specific forward and reverse primers 5'-CGT GGT GAT GTG GAG TAT TGC-3' and 5'-ATG GTA TCG GTG TGA GCG TC-3' (156-bp product). The rice actin gene *RAc1* was used as an internal control, and was amplified with the forward and reverse primers 5'-GGA AGC TGC GGG TAT CCA TGA G-3' and 5'-CCT GTC AGC AAT GCC AGG GAA C-3' (130-bp product).

To calculate relative expression levels, serial dilutions (0.2–125 ng) were used to produce standard curves for each gene. Each reaction was carried out in triplicate using 96-well optical reaction plates. The reactions comprised a heating step for 3 min at 95°C, followed by 40 cycles of 95°C for 10 s, 58°C for 30 s and 72°C for 20 s. Amplification specificity was confirmed by melt curve analysis in the range 50–90°C with fluorescence acquired after each 0.5°C increment. The fluorescence threshold value and gene expression data were calculated using CFX96^{TM} software.

Western blots

Total protein was extracted from wild type and transgenic rice leaves in an equal volume of buffer (0.2 M Tris-HCl pH7.5, 5 mM EDTA, 0.1% Tween-20). The mixture was agitated for 1 h and centrifuged for 10 min at 13,800 rpm. The protein concentration in the supernatant was determined (Bradford, 1976) using BSA as a standard, and 20-µg aliquots were fractioned by SDS-PAGE (10% acrylamide) according to Laemmli (1970) and transferred to an Immun-Blot PVDF membrane (BioRad, Hercules CA, USA). After blocking overnight with 5% non-fat milk, the membrane was washed three times with PBS containing 0.1% Tween-20 and three times with PBS, each for 15min. GUS was detected for 2 h at room temperature with agitation, using a specific antibody (Sigma, St. Louis, MO) diluted 1:2000 in PBS. The membrane was washed as above and the bound primary antibody was detected for 2 h at room temperature with agitation, using a goat anti-rabbit alkaline phosphatase-conjugated antibody (Sigma, St. Louis, MO) diluted 1:20,000 in TBS. The membrane was washed three times in TBS and the signal was detected with Sigma-Fast

reagent (Sigma) for 4–5 min before the membrane was submerged in water to stop the reaction.

Histochemical GUS assay

Histochemical GUS assays were carried out according to Jefferson et al. (1987) with minor modifications. Spikelets and seeds from T0 plants and one-week-old T1 seedlings were sampled and incubated at 37ºC overnight (12 h) in the dark in 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) in 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% (v/v) Triton X-100 and 20% (v/v) methanol to eliminate endogenous GUS activity (Kosugi et al., 1990). After 12 h staining, tissues were destained in an ethanol series (50, 70, 80 and 95%) to remove chlorophyll, and then stored in 70% (v/v) ethanol, and photographed with a digital camera.

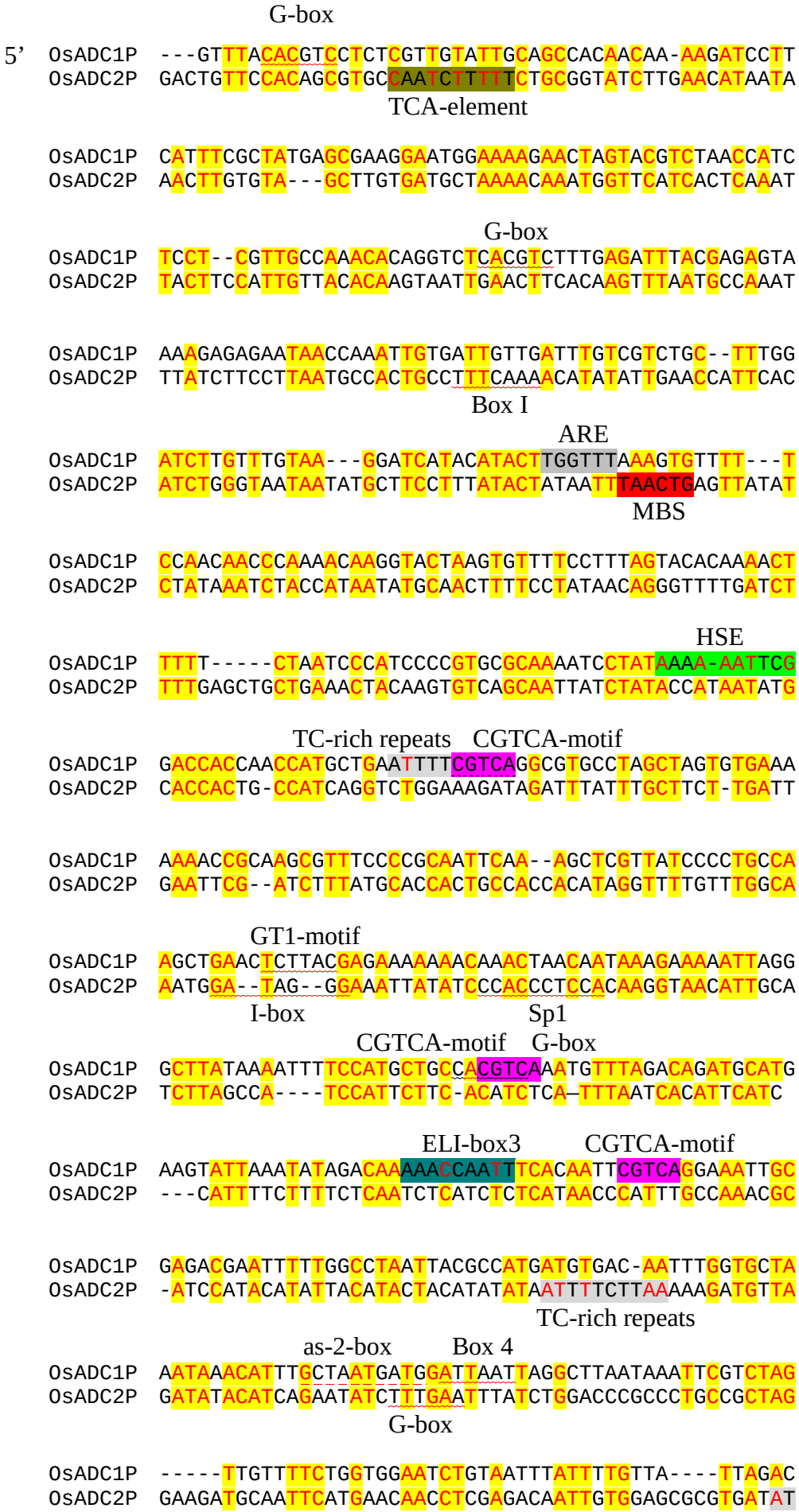
RESULTS

Analysis of *OsADC* promoters and identification of stress response elements

Two rice *ADC* genes and a pseudogene (and their corresponding products) were described by Peremarti et al. (2010a). Thus far, no data have been published concerning regulatory elements located in the promoter regions that might determine the specific expression profiles of each *ADC* gene.

The short version of the *OsADC1* promoter (1696 bp) spanned nucleotides –1678 bp to +5 bp whereas the long version (2273 bp) spanned nucleotides –2278 bp to +5 bp. Using this nomenclature, +1 is the first nucleotide of the *OsADC1* cDNA (AY604047) and the ATG representing the start of the coding region is found at position +19 (**Fig. 2.1**). The short version of the *OsADC2* promoter (1585 bp) spanned nucleotides –1576 bp to +9 bp whereas the long version (2695 bp) spanned nucleotides –2686 bp to +9 bp. These sequences had a common 3' end because the same reverse primer was used to amplify both products. Using this nomenclature, +1 is the first nucleotide of the *OsADC2* cDNA and the ATG representing the start of the coding region is found at position +82, excluding the sequence of the intron (**Fig. 2.1**). The two *OsADC* promoters show only 41% identity.

Each long promoter sequence was analyzed *in silico* against the PlantCARE database (Lescot et al., 2002) to identify putative *cis*-acting regulatory elements (**Fig. 2.2**). Potential TATA boxes were identified in both promoters, but we wished to investigate the sequences representing potential stress-response elements that would distinguish each promoter. For example, the *OsADC1* promoter contained three copies of the sequences CGTCA and TGACG (the core motifs of the MeJA-response element) as well as a putative heat response element (HSE). The *OsADC2* promoter contained a putative MBS element, which is involved in drought responses. Both promoters also contained putative low-temperature response elements (LTR) involved in cold adaptation, multiple abscisic acid response elements (ABRE), a putative TCA element involved in salicylic acid response, an anaerobic response element (ARE), an elicitor-response element (ELI-box3), and an enhancer-like GC-motif potentially involved in anoxic induction. Multiple light-response elements were also present in both promoters.


G-box
5’ OsADC1P ---GTTTACACGTCCTCTCGTTGTATTGCAGCCACAACAA-AAGATCCTT
OsADC2P GACTGTTCCACAGCGTGCCAATCTTTTCTGCGGTATCTTGAACATAATA
TCA-element
OsADC1P CATTTCGCTATGAGCGAAGGAATGGAAAAGAACTAGTACGTCTAACCATC
OsADC2P AACTTGTGTA---GCTTGTGATGCTAAAACAAATGGTTCATCACTCAAAT
G-box
OsADC1P TCCT--CGTTGCCAAACACAGGTCTCACGTCTTTGAGATTTACGAGAGTA
OsADC2P TACTTCCATTGTTACACAAGTAATTGAACTTCACAAGTTTAATGCCAAAT
OsADC1P AAAGAGAGAATAACCAAATTGTGATTGTTGATTTGTCGTCTGC--TTTGG
OsADC2P TTATCTTCCTTAATGCCACTGCCTTTCAAAACATATATTGAACCATTCAC
Box I
ARE
OsADC1P ATCTTGTTTGTAA---GGATCATACATACTTGGTTTAAAGTGTTTT---T
OsADC2P ATCTGGGTAATAATATGCTTCCTTTATACTATAATTTTAACTGAGTTATAT
MBS
OsADC1P CCAACAACCCAAAACAAGGTACTAAGTGTTTTCCTTTAGTACACAAAACT
OsADC2P CTATAAATCTACCATAATATGCAACTTTTCCTATAACAGGGTTTTGATCT
HSE
OsADC1P TTTT-----CTAATCCCATCCCCGTGCGCAAAATCCTATAAAA-AATTCG
OsADC2P TTTGAGCTGCTGAAACTACAAGTGTCAGCAATTATCTATACCATAATATG
TC-rich repeats
CGTCA-motif
OsADC1P GACCACCAACCATGCTGAATTTTCGTCAGGCGTGCCTAGCTAGTGTGAAA
OsADC2P CACCACTG-CCATCAGGTCTGGAAAGATAGATTTATTTGCTTCT-TGATT
OsADC1P AAAACCGCAAGCGTTTCCCCGCAATTCAA--AGCTCGTTATCCCCTGCCA
OsADC2P GAATTCG--ATCTTTATGCACCACTGCCACCACATAGGTTTTGTTTGGCA
GT1-motif
OsADC1P AGCTGAACTCTTACGAGAAAAAAACAACTAACAATAAAGAAAAATTAGG
OsADC2P AATGGA--TAG--GGAATTTATATCCCACCCTCCACAAGGTAACATTGCA
I-box
Sp1
CGTCA-motif
G-box
OsADC1P GCTTATAAAATTTTCCATGCTGCCACGTCAAATGTTTAGACAGATGCATG
OsADC2P TCTTAGCCA----TCCATTCTTC-ACATCTCA—TTTAATCACATTCATC
ELI-box3
CGTCA-motif
OsADC1P AAGTATTAAATATAGACAAAACCAATTTCACAATTCGTCAGGAATTGC
OsADC2P ---CATTTTCTTTTCTCAATCTCATCTCTCATAACCCATTTGCCAAACGC
OsADC1P GAGACGAATTTTTGGCCTAATTACGCCATGATGTGAC-AATTTGGTGCTA
OsADC2P -ATCCATACACATATTACATACTACATATATAATTTTCTTAAAAAGATGTTA
TC-rich repeats
as-2-box
Box 4
OsADC1P AATAAACATTTGCTAATGATGGATTAATTAGGCTTAATAAATTCGTCTAG
OsADC2P GATATACATCAGAATATCTTTGAATTTATCTGGACCCGCCCTGCCGCTAG
G-box
OsADC1P -----TTGTTTTCTGGTGGAATCTGTAATTTATTTTGTTA----TTAGAC
OsADC2P GAAGATGCAATTCATGAACAACCTCGAGACAATTGTGGAGCGCGTGATAT


OsADC1P TACGTTTAATACTTTAAATATGTGTTCGTATATCTGATGTGATATGTAGG
OsADC2P TTTCTTTAATTCTTTTTCTTTG-GAACTTA-ATAAAAAGGTAGAAGAAGT
TC-rich repeats
OsADC1P G--GTAAATTTTTTTTTGCCAACTAAGCATGCCCTTAATCCAGCAACCG
OsADC2P CAAGTACTGTTGGGGATAAGAGAATAAGAAT-CTCTCAAGCGATGTTGTT
OsADC1P GCAAAATCCAGAA--TTAACCGGAGGTTCAAATCTCCAGTCGTGTTCGGC
OsADC2P GAAGAAGACGCCAAGTTACCAAGATGGTCCT-TCAGTACTTACGGTTCAC
Sp1
OsADC1P CGATCGAGCAGTCGACCGCGACGCCCCCCACA-CGCATGCAGCAGCAGCG
OsADC2P AAATTAATCAATACATGTAGGATCTTATTATAGCACATACATTGATTTCA
Box 4
OsADC1P GCAGCGGCAGCGGCAG-CAGATCGGTCGACCACCTGCTGCAGCGCAGCG-
OsADC2P TCATACAATTAATCATACAGTTTAGCCAACCA--TGCTACAGTAAAGGGT
Box 4
Sp1
OsADC1P -AATCCC------------ACATCCCTATTATTATCTCCCACCCGCGTAT
OsADC2P GAATTAGGGCAGTGTGGTAACATCA-TATAATAAT-TACAACGTGATTTG
Sp1
OsADC1P AAACTGCATCCAAAACCTCCCACAAGTCCGATCTCGGACGCTCCCGCCG
OsADC2P CATTTGGATT-AAAGTGTGATAGTAGTGTGATGTTAAGCTCAACGTACTG
OsADC1P CCGGATCGTGAACGAACGGCCCAGATCGTAC------CGTGG--------
OsADC2P TATAAGTGGGTACGTG-GACACAAATGCTACGTTTCTCGAGGGCGAGAGA
ABRE G-box ACE
OsADC1P ----CTGGCTGCTACCACCACCATC-----ATCATCCATCGCCGCTAGCT
OsADC2P ATCTCAGGTTACTGTGAACATTATCCTACGATAATACATCATTGCTAAAC
Sp1
OsADC1P GCAGCAGCAGCCAGCCAGCCAACCGCCCCGCGCAACAGAACAAGAGAGGG
OsADC2P AAATATGCATATTTGCCCCGTATTACTAGGACGCACACTATCTTATATAA
GC-motif Sp1
OsADC1P ATCAAAGAACGTGACCCCCCCGTGAGGGAGGAAGCAGAGAGAGAGAGGA
OsADC2P TTCGAC-AGTGCTATATATTATGATCCTGGTTTCGTGGCAAATTAAAACT
CATT-motif
OsADC1P GGATTGAAGCATTCAACCACCGTGGGGTGATG-GAGAATTGGAGAGACGA
OsADC2P TTTTTAAGGCGTCTCTCTCCAATAGTGCTCTATGAGAATTATTTGTCAGA
OsADC1P TGACCAATGTTGCCGAGATGAGTAGGCATGTGAA---GTGAGGCGAGAGA
OsADC2P GGA----TTTAACCATCCGGATTAAGCATGAGGATCCGTCTGGTACGACT
Sp1
OsADC1P GAGAGCCAAGCAGATGTGCAGCGATTACCGGGCGCTGTCCGCGACCCCAA
OsADC2P TTAACTCTAGCTCGACTTCTTCTGT-AGCTAAAGTTCAACTAAACAATTA
TCA-element
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OsADC2P TTTGCTCTATCCGAAAAGAGA---GTGGAGCTGGCTGA-AGTGCTCTTAC

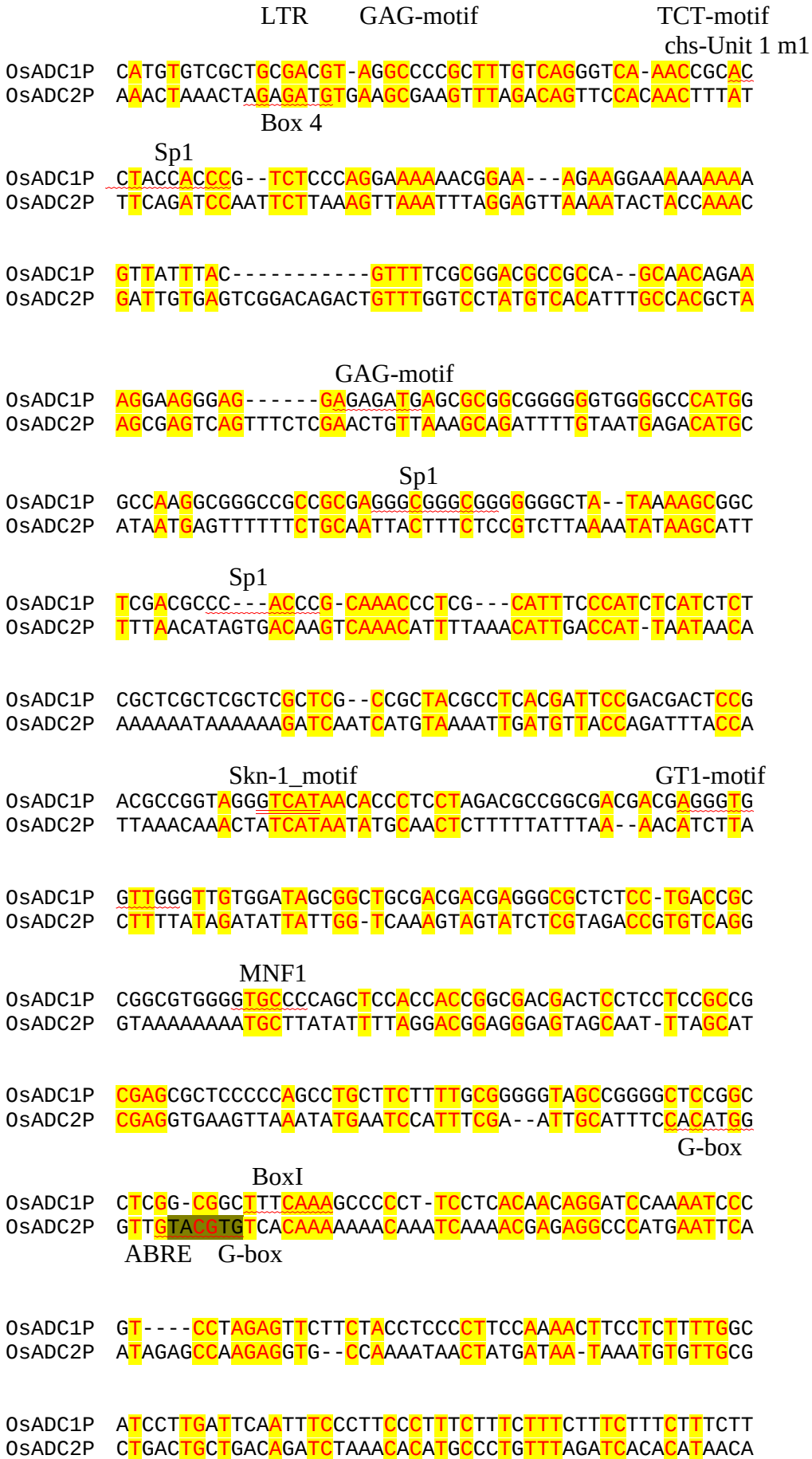
LTR
GAG-motif
TCT-motif
chs-Unit 1 m1
OsADC1P CATGTGTCGCTGCGACGT-AGGCCCCGCTTTGTCAGGGTCA-AACCGCAC
OsADC2P AAACTAAACTAGAGATGTGAAGCGAAGTTTAGACAGTTCCACAACTTTAT
Box 4
Sp1
OsADC1P CTACCACCCG--TCTCCCAGGAAAAAACGGAA---AGAAGGAAAAAAAAA
OsADC2P TTCAGATCCAATTCTTAAAGTTAAATTTAGGAGTTAAAATACTACCAAAC
OsADC1P GTTATTTAC----------GTTTTCGCGGACGCCGCCA--GCAACAGAA
OsADC2P GATTGTGAGTCGGACAGACTGTTTGGTCCTATGTCACATTTGCCACGCTA
GAG-motif
OsADC1P AGGAAGGGAG------GAGAGATGAGCGCGGCGGGGGGTGGGGCCCATGG
OsADC2P AGCGAGTCAGTTTCTCGAACTGTTAAAGCAGATTTTGTAATGAGACATGC
Sp1
OsADC1P GCCAAGGCGGGCCGCCGCGAGGGCGGGCGGGGGGGGCTA--TAAAAGCGGC
OsADC2P ATAATGAGTTTTTTCTGCAATTACTTTCTCCGTCTTAAAATATAAGCATT
Sp1
OsADC1P TCGACGCCC---ACCCG-CAAACCCTCG---CATTTCCCATCTCATCTCT
OsADC2P TTTAACATAGTGACAAGTCAAACATTTTAAACATTGACCAT-TAATAACA
OsADC1P CGCTCGCTCGCTCGCTCG--CCGCTACGCCTCACGATTCCGACGACTCCG
OsADC2P AAAAAATAAAAAAGATCAATCATGTAAAATTGATGTTACCAGATTTACCA
Skn-1_motif
GT1-motif
OsADC1P ACGCCGGTAGGGTCATAACACCCTCCTAGACGCCGGCGACGACGAGGGTG
OsADC2P TTAAACAAACTATCATAATATGCAACTCTTTTTATTTAA--AACATCTTA
OsADC1P GTTGGGTTGTGGATAGCGGCTGCGACGACGAGGGCGCTCTCC-TGACCGC
OsADC2P CTTTTATAGATATTATTGG-TCAAAGTAGTATCTCGTAGACCGTGTCAGG
MNF1
OsADC1P CGGCGTGGGGTGCCCCAGCTCCACCACCGGCGACGACTCCTCCTCCGCCG
OsADC2P GTAAAAAAAATGCTTATATTTTAGGACGGAGGAGTAGCAAT-TTAGCAT
OsADC1P CGAGCGCTCCCCCAGCCTGCTTCTTTTGCGGGGGTAGCCGGGGCTCCGGC
OsADC2P CGAGGTGAAGTTAAATATGAATCCATTTCGA--ATTGCATTTCCACACATGG
G-box
BoxI
OsADC1P CTCGG-CGGCTTTCAAAGCCCCCT-TCCTCACAACAGGATCCAAAATCCC
OsADC2P GTTGTACGTGTCACAAAAAAACAAATCAAAACGAGAGGCCCATGAATTCA
ABRE
G-box
OsADC1P GT----CCTAGAGTTCTTCTACCTCCCCTTCCAAAACTTCCTCTTTTGGC
OsADC2P ATAGAGCCAAGAGGGTG--CCAAAATAACTATGATAA-TAAATGTGTTGCG
OsADC1P ATCCTTGATTCAATTTCCCTTCCCTTTCTTTCTTTCTTTCTTTCTTCTT
OsADC2P CTGACTGCTGACAGATCTAAACACATGCCCTGTTTAGATCACACATAACA

Fig.2.2 Alignment of the pl*OsADC1* and pl*OsADC2* promoters

Gaps were inserted for optimal alignment. The first nucleotides of the two *OsADC* cDNAs are

indicated by +1. The 5′ UTR regions are shown in italics. The *OsADC2* intron is not included in the alignments. ATG initiation codons and putative TATA boxes are indicated in bold and are boxed. Relevant motifs are highlighted. Abbreviations: ABRE, abscisic acid response element; ARE, anaerobic response element; CGTCA/TGACG, MeJA-response elements; ELI-box3, elicitor-response element; GC-motif, enhancer-like anoxic response element; HSE, heat stress response element; LTR, low-temperature response element; MBS, binding site involved in drought response; TCA-element, salicylic acid response element; TC-rich repeats, defense/stress response elements.

The long promoter performs better in transient expression experiments

Transient expression assays in rice embryos were used to confirm that the expression constructs were functional and to compare the activities of the *OsADC1* and *OsADC2* promoter fragments to Ubi-1, which was used as a positive control (Sudhakar et al., 1998). Rice mature embryos were therefore bombarded with the four reporter constructs or the positive control vector pAHC25 (Christensen and Quail, 1996), and the number of blue foci was counted after histochemical staining for GUS activity. Three assays were carried out for each plasmid and in each case 25 embryos were bombarded. The average number of spots per shot was 22 for pl*ADC1:GUS*, 9 for ps*ADC1:GUS*, 8 for pl*ADC2:GUS* and 6 for ps*ADC2:GUS*, compared to 100 for pAHC25 (**Fig. 2.3**). These values confirmed the activity of the vectors and their suitability for expression in stable transgenic plants. We selected the short promoter variants for because transient expression levels were detected in both cases, meaning that they retained promoter activity and are sufficient to confer gene expression.

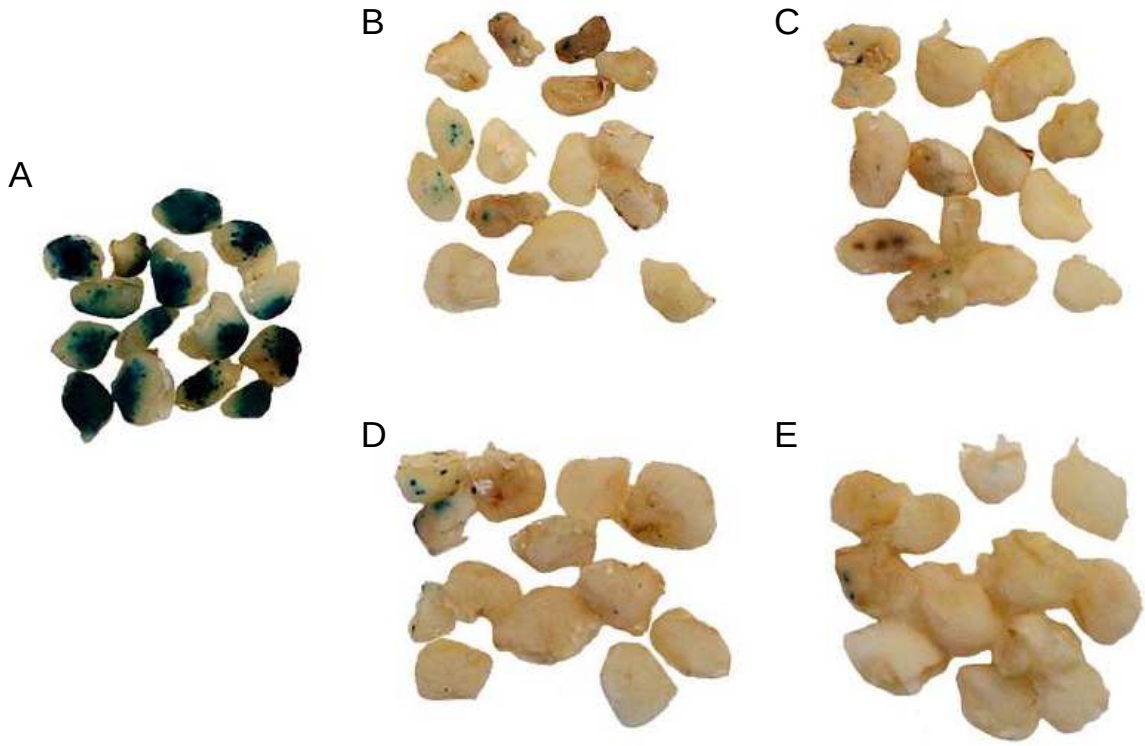

Fig. 2.3 Transient expression analyses

Histochemical GUS staining in embryos after transient expression with *OsADC-gus*A and *Ubi-gus*A constructs. **A**: *Ubi:GUS;* **B**: *plADC1:GUS;* **C**: *psADC1:GUS;* **D**: *plADC2:GUS;* **E**: *psADC2:GUS.*

Characterization of homozygous GUS reporter-transgenic lines

Stable primary transformants of heterozygous rice lines carrying ps*ADC1:GUS* or ps*ADC2:GUS* were used to generate homozygous transgenic plants, and T1 progeny were used for the analysis of reporter gene expression. Twenty independent lines were recovered for each vector and three lines from each were randomly selected for molecular characterization. After self-pollination, we germinated T1 seeds from the six lines and extracted genomic DNA from the leaves. DNA blot analysis was carried out by digesting the genomic DNA with a panel of restriction enzymes according to the construct: *Xmn*I, *Bgl*I, *Xba*I or *Sal*I to cut once in the cassette and determine copy number; and the non-cutter *Hin*dIII to determine the number of transgenic loci. The banding patterns generated by single-cutters were unique in each line (**Fig. 2.4**) confirming they represent independent

events. Only one *Hin*dIII fragment is produced in each line confirming the presence of a single transgenic locus in each case.

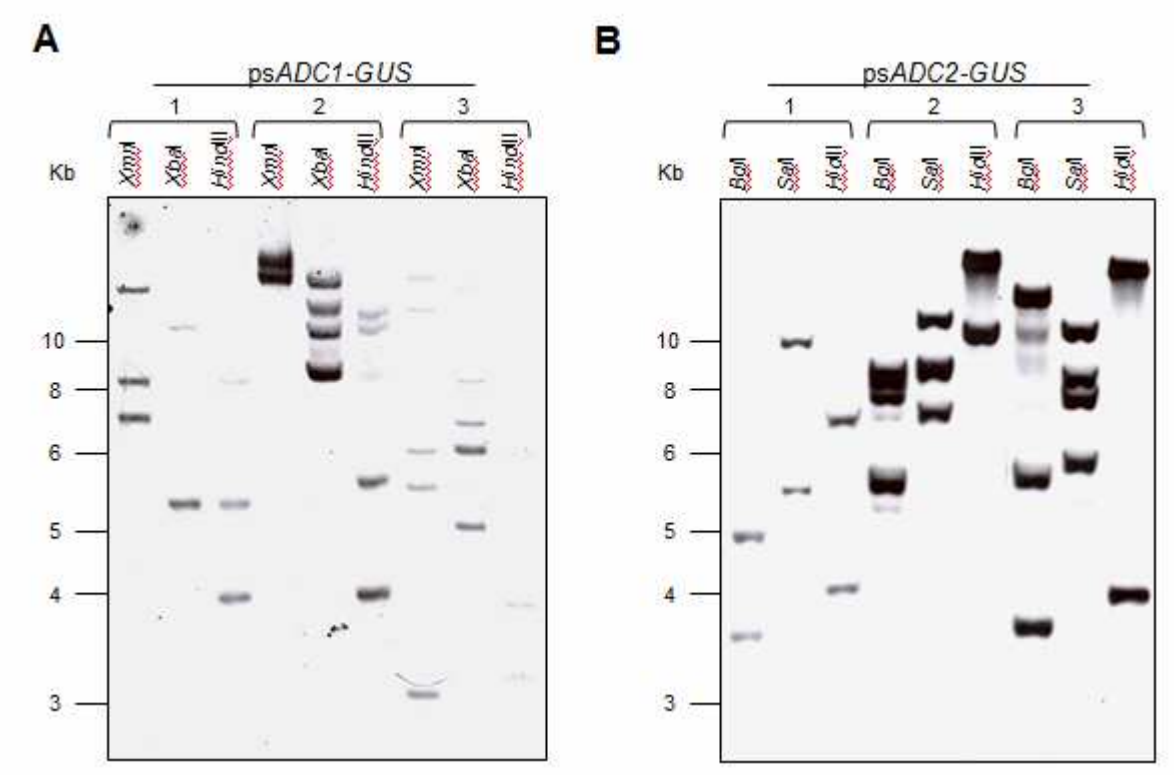

Fig. 2.4 DNA gel blot analysis of transgenic plants containing *psADC1:GUS* and *psADC2:GUS*.

Rice genomic DNA was digested with *Xmn*I, *Bgl*I, *Xba*l, *Sal*I and *Hin*dIII respectively. The digested DNA was separated by gel electrophoresis and blotted onto nylon membranes. Blots were hybridized with the 512-bp *gus*A probe and washed under high stringency conditions and exposed to X-ray film. **A**: three independent lines of *psADC1:GUS*. **B**: three independent lines of *psADC2:GUS.*

GUS histochemical localization

GUS activity was investigated by histochemical staining in seedlings, seeds and flowers from the transgenic plants (**Fig. 2.5**). No GUS activity was detected in wild type tissues, as anticipated (data not shown). We detected GUS activity in all the tissues (leaves and roots from seedlings, spikelets and seeds) from both transgenic lines but staining was generally weaker than that of control plants expressing *gus*A under the control of the Ubi-1 promoter. Seeds were exceptional in that GUS activity in the embryo was higher in the ps*ADC1:GUS*

and ps*ADC2:GUS* transgenic plants than in the positive control (**Fig. 2.5**). In the spikelets, GUS activity was detected in the lemma, palea and stamens.

GUS histochemical localization after drought treatment

T1 seedlings growing for one week under normal conditions were compared with seedlings after 24 h of drought stress. GUS activity increased in all tissues of the ps*ADC1:GUS* and ps*ADC2:GUS* transgenic plants in response to stress, but was clearest in the roots. There was a significant and reproducible difference in activity between the ps*ADC1:GUS* and ps*ADC2:GUS* promoters with the latter yielding consistently higher levels of GUS activity in all tissues (**Fig. 2.5**).

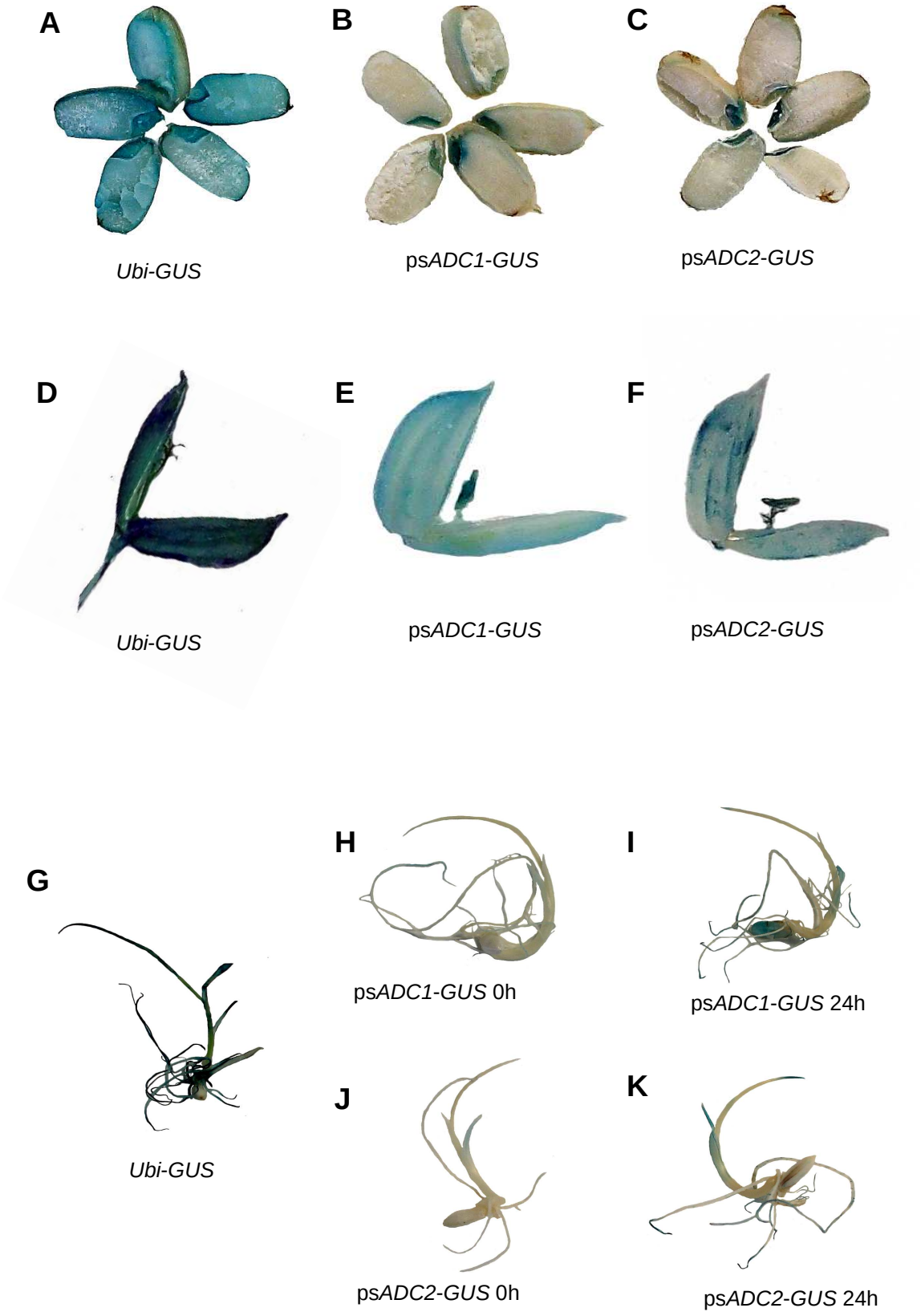

Fig. 2.5 GUS histochemical localization

Histochemical GUS staining in different tissues of T1 ps*OsADC:GUS* and *Ubi-GUS* plants before and after drought stress. **A**: *Ubi–GUS* seeds; **B**: *plADC1:GUS* seeds, **C**: *psADC2:GUS*

seeds; **D**: *Ubi–GUS* spikelet; **E**: *psADC1:GUS* spikelet; **F**: *psADC2:GUS* spikelet; **G**: *Ubi-GUS* seedling; **H**: *psADC1:GUS* seedling before stress. **I**: *psADC1:GUS* seedling after stress. **J**: *psADC2:GUS* seedling before stress. **K** *psADC2:GUS* seedling after stress.

Analysis of *gus*A mRNA and GUS protein levels under normal and stress conditions

The expression of the ps*ADC1-GUS* and ps*ADC2-GUS* reporter genes was confirmed in T0 plants from all six selected lines by RNA blot analysis (**Fig. 2.6**). We then carried out more detailed analysis by real time RT-PCR and western blot in T1 plants under normal and drought stress conditions.

Total RNA was isolated from non-stressed roots and leaves (0 h) and then at time points 1, 3, 6 and 24 h after stress was applied, by exposing the plants to 20% PEG. After 24 h, the plants were placed back into normal conditions and allowed to recover for one week before final RNA samples were taken. These experiments showed that both promoters were more active in leaves than in roots under both normal and drought stress conditions, but the induction ratio under stress conditions was greater in the roots where *gus*A mRNA levels increased 20-fold after 24 h compared to 6–12-fold in the leaves (**Fig. 2.7A**). Induction in the roots was also more rapid, with a 6–8-fold increase in *gus*A mRNA after 1 h of stress, compared to ~2-fold induction in the leaves (**Fig. 2.7B**). It was also apparent from these experiments that the *OsADC2* promoter is more sensitive to drought stress and is induced more efficiently than the *OsADC1* promoter, showing double the activity in leaves and 50% more activity in roots after 24 h (**Fig. 2.7B**).

GUS protein levels as determined by protein blot showed broadly the same profiles as the corresponding mRNAs, accumulating to higher levels in both leaves and roots in response to drought stress (**Fig. 2.6C**). However, because GUS is a stable protein, the levels remained high even after the one-week recovery period whereas the corresponding mRNA levels had returned to normal (**Figs. 2.6 A** and **C**).

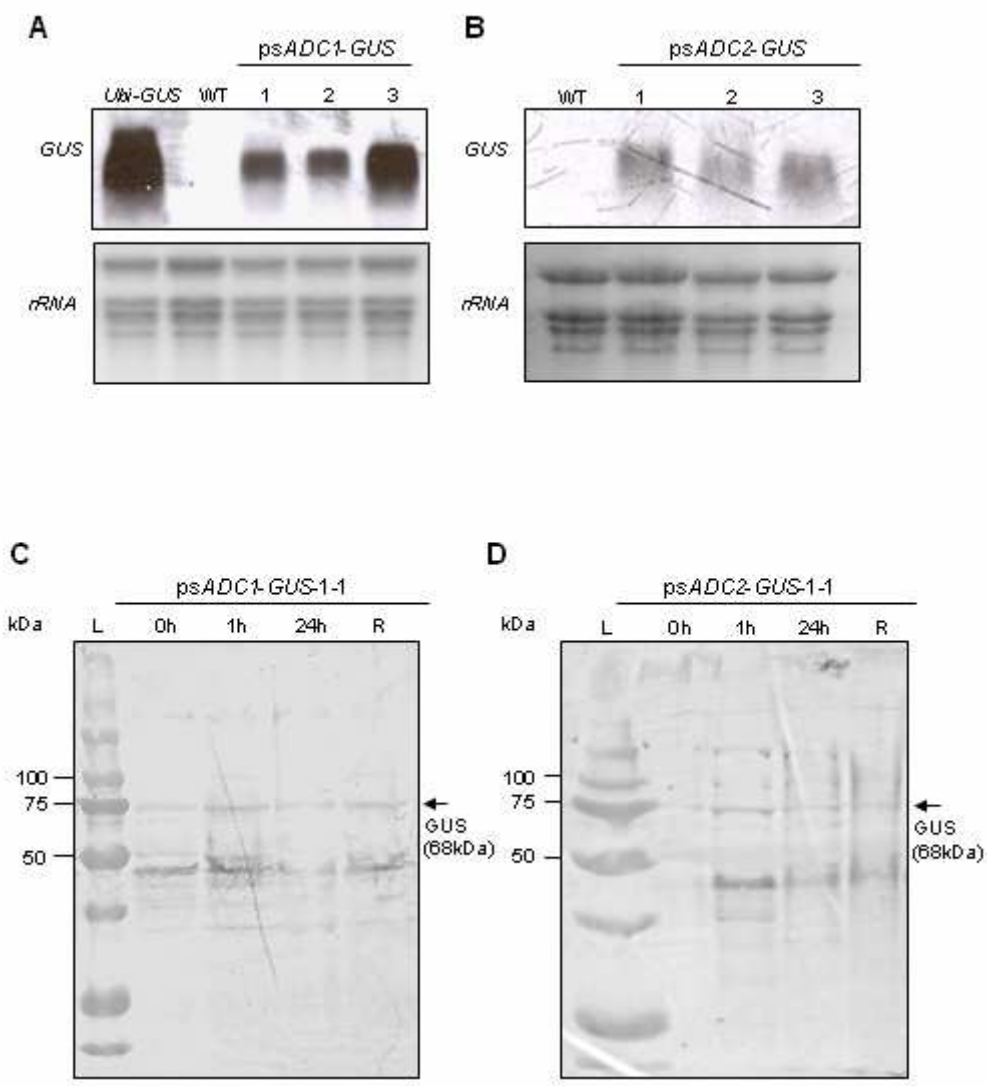

Fig. 2.6 RNA and protein blots of transgenic plants.

A, B: Detection of *gus*A mRNA in plants expressing the ps*ADC1-GUS* and ps*ADC2-GUS* reporter genes, respectively. *Ubi-GUS* was used as positive control. WT, wild type. Lanes 1, 2 and 3 are three independent transgenic T0 lines of *psADC1-GUS* and *psADC2-GUS*. **C, D:** Total leaf protein (20 µg) isolated at different times after the imposition of drought stress were checked for GUS protein accumulation (68 kDa). ps*ADC1-GUS*-1-1 and ps*ADC2-GUS*-1-1 are the T1 plants from line 1 of each group. L = protein size markers.

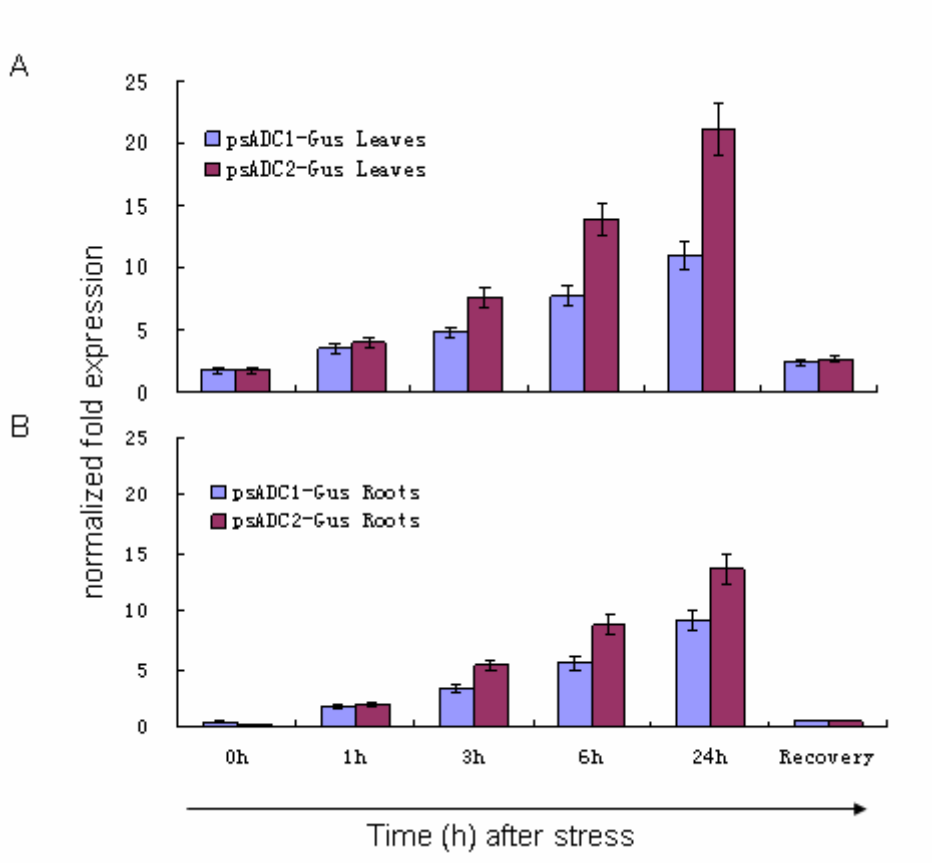

Fig. 2.7 Real-time PCR of leaf and roots tissues after drought treatment.

Expression of *gus*A gene in leaf (**A**) and root (**B**) tissues of transgenic rice plants carrying ps*ADC1-GUS* and *psADC2-GUS*. Quantitative real-time PCR was performed with cDNA prepared from leaves and roots. Relative expression was determined in triplicate measurements in four independent biological replicates. Columns represent the relative *gus*A expression levels normalized against the *β*-actin gene with standard errors.

DISCUSSION:

General role of polyamines, role in stress, and the importance of ADC

To determine the roles of individual polyamines in drought stress tolerance we have created a diverse population of transgenic rice lines expressing various genes from the polyamine biosynthesis pathway (Capell et al., 1998; Lepri et al., 2001; Noury et al., 2000; Thu-Hang et al., 2002). From these experiments we have derived a threshold model which shows how polyamine levels can trigger drought stress responses, and the basis of this model is the abundance of putrescine, which is directly regulated by the enzyme ADC (Capell et al., 2004). Many primary metabolic enzymes in plants and animals exist as differentially-expressed isoenzymes with specific roles, and ADC is present as multiple isoenzymes in some plant species. In order to investigate the function of ADC isoenzymes in plants, particularly their role in drought stress adaptation, we decided to search for additional ADC enzymes in our favored model, rice. A rice *ADC* cDNA was isolated by Akiyama and Jin (2007) but the only other known cereal-derived *ADC* gene was isolated from oat two decades ago (Bell and Malmberg, 1990). After searching through the published rice genome sequence, we isolated two *ADC* genes, one corresponding to the known cDNA (now named *ADC1*) and the other to a novel sequence (*ADC2*). We also identified a third *ADC* sequence, a pseudogene related to *ADC1*, which was truncated and non-functional (Peremarti et al., 2010a). The *ADC1* gene sequence contains a long 5'-UTR that was not present in the cDNA sequence described by Akiyama and Jin (2007) and reveals the presence of an additional transcriptional initiation site. Furthermore, although the deduced amino acid sequences of *Arabidopsis thaliana* (*At*) *ADC1* and *AtADC2* are highly similar, their expression profiles are distinct. *AtADC2* is induced by dehydration, NaCl and ABA whereas *AtADC1* is expressed constitutively under stress (Urano et al., 2003). To understand how the regulatory network influences rice ADC expression, we have cloned the *OsADC* promoters and evaluated the expression of a reporter gene in response to drought stress, reflecting the interaction between transcription factors present in different tissues and *cis*-acting response elements in the promoters.

Analysis of the *OsADC* promoters

The presence of duplicated *ADC* genes in Arabidopsis and in other species could reflect functional diversification based on differential responsiveness. Soyka and Heyer (1999) demonstrated the specific involvement of *AtADC2* in hyperosmotic stress induced by the application of 0.6 M sorbitol, whereas *AtADC1* transcripts were unaffected. JA and ABA also

induced *AtADC2* expression but not *AtADC1* (Perez Amador et al., 2002; Urano et al., 2003). Yamaguchi-Shinozaki and Shinozaki (2005) analyzed the *AtADC2* promoter sequence for known drought-response sequences and identified two ABA-response elements, but no dehydration-response element (DRE)/C-repeat (CRT) motifs, which respond to dehydration in an ABA-independent manner. Furthermore, Hummel et al. (2004) identified a DRE sequence only in the *AtADC1* promoter whereas a stress-response element (STRE) was found in both promoters. Using the public database PlantCARE, we tried to identify putative cis-acting regulatory elements that differed between p*ADC1* and p*ADC2*. We identified a putative drought stress-inducible element (MSB) in the *OsADC2* promoter and ABA response elements in both promoters (**Fig. 2.2**).

OsADC promoter spatiotemporal activities

The expression of *ADC* genes is spatiotemporally regulated in the *Brassicaceae* family (Hummel et al., 2004a; 2004b). In our earlier studies, we found that *OsADC1* and *OsADC2* are also spatiotemporally regulated. *OsADC1* was more abundant in leaves and roots and *OsADC2* was more abundant in stems (Peremarti et al., 2010a). However, the *gus*A reporter experiments showed common promoter activity in the different tissues we analyzed. Under normal conditions, real-time RT-PCR results showed no differences in expression between ps*ADC1*:GUS and ps*ADC2*:GUS (**Fig. 2.7)**. Both constructs were expressed in seeds, specifically in the embryo and also in the spikelet, including the lemma, palea and also the stamens.

OsADC promoter responses to drought stress

We were previously unable to detect changes in the steady-state *OsADC1* mRNA level even after 6 days under drought stress (Capell et al., 2004). Furthermore, the transcript was less abundant in transgenic rice plants overexpressing *DsSAMDC*, inducing phenotypic signs of drought stress (Peremarti et al., 2009). However, Akiyama and Jin, (2007) detected a slight increase in *OsADC1* expression in response to drought stress. To unravel the role of the *OsADC* promoters under drought stress, we tested seedlings and T1 plants carrying the short promoter construct under drought stress conditions. Both promoters were active in leaves and roots, and real-time RT-PCR showed that both *OsADC* promoters responded rapidly to stress and took a long time to return to normal activity, with the roots responding more rapidly and more potently probably reflecting their front-line position in exposure to drought.

Modulated activity of the *OsADC* promoters

The transcription of a gene is not only induced or repressed by the binding of transcription factors to *cis*-acting elements, but may be modulated by other factors such as the presence of transposable elements in the promoter, 5'UTR or introns, or by epigenetic modifications (Samadder et al., 2008). A transposable element was found in the *AtADC1* promoter by El Amrani et al. (2002) which contained several *cis*-acting elements recognized by functionally characterized Arabidopsis transcription factors. This association between the transposable element and the *ADC1* promoter would confer a distinct pattern of activity compared to *ADC2*. Several tourist-like MITE elements have been identified in the promoter regions of rice *ADC1* and *ADC2* but there is no evidence thus far that they regulate gene expression.

The accumulation of putrescine (Capell et al., 2004; Peremarti et al., 2009; Peremarti et al., 2010b) in response to drought stress is the sum of several different mechanisms involving transcriptional, posttranscriptional, translational and post-translational modifications that are not well understood. The post-transcriptional and post-translational regulation of ADC accumulation and activity described in many reports could be responsible for the striking differences between ADC stress responses and the *ADC* stress motifs found in the promoter sequences. Further studies are required to dissect the specific contribution of the intron present in the 5'UTR of *OsADC2* and the transposable elements in other parts of the gene.

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CHAPTER II

Functional characterization of the *Gentiana lutea zeaxanthin epoxidase* (*GlZEP*) promoter in transgenic tomato plants

ABSTRACT

The accumulation of carotenoids in plants depends critically on the spatiotemporal expression profiles of the genes encoding enzymes in the carotenogenic pathway. We cloned and characterized the *Gentiana lutea zeaxanthin epoxidase* (*GlZEP*) promoter to determine its role in the regulation of carotenogenesis, because the native gene is expressed at high levels in petals, which contain abundant chromoplasts. We transformed tomato (*Solanum lycopersicum* cv. Micro-Tom) plants with the *gus*A gene encoding GUS under the control of the *GlZEP* promoter, and investigated the reporter expression profile at the mRNA and protein levels. We detected high levels of *gus*A expression and GUS activity in chromoplast-containing flowers and fruits, but minimal levels in immature fruits containing green chloroplasts, in sepals, leaves, stems and roots. *GlZEP-gus*A expression was strictly associated with fruit development and chromoplast differentiation, suggesting an evolutionarily-conserved link between ZEP and the differentiation of organelles that store carotenoid pigments. The impact of our results on current models for the regulation of carotenogenesis in plants is discussed.

INTRODUCTION

Carotenoids are abundant isoprenoid pigments produced by all photosynthetic organisms as well as certain non-photosynthetic bacteria and fungi (Goodwin, 1980). In chloroplasts, carotenoids are accessory light-harvesting pigments that protect the photosynthetic apparatus from photo-oxidation (Frank and Cogdell 1996; Demmig-Adams and Adams 2002). They also act as precursors for the plant hormones ABA (Creelman and Zeevart, 1984) and strigolactone (Gomez-Roldan et al., 2008; Umehara et al., 2008). Chromoplasts are specialized plastids that have adapted to store carotenoids and are found in flowers and fruits. The accumulation of carotenoids confers a range of pigmentation in the yellow-orange-red spectrum that attracts animals and therefore facilitates the dispersal of pollen and seeds (Bartley and Scolnik, 1995).

There is significant interest in the regulation of carotenoid biosynthesis in plants because of their health-promoting antioxidant activity (Kloer and Schulz, 2006) and the specific nutritional importance of pro-vitamin A carotenoids such as *β*-carotene (Von Lintig and Vogt, 2004; Giuliano et al., 2008; Farre et al., 2010; Bai et al., 2011). However, this has shifted attention away from the key roles that carotenoids play in the continuation of the plant life cycle by attracting pollinating insects and herbivores that distribute seeds. Therefore, relatively little is known about the regulation of carotenoid biosynthesis in petals and fruits, and the link between carotenoid synthesis and chromoplast differentiation.

Gentiana lutea flowers contain large amounts of lutein, violaxanthin, antheraxanthin and *β*-carotene (Zhu et al., 2003). The chromoplasts in *G. lutea* petals originate either from pre-existing fully-developed chloroplasts or from immature proplastids (He et al., 2002). There is a strong temporal correlation during flower development between the accumulation of carotenoids and the formation of chromoplasts, which coincides with the induction of carotenogenic gene expression (Zhu et al., 2002; 2003). Zeaxanthin epoxidase (ZEP) catalyzes the conversion of zeaxanthin to violaxanthin via antheraxanthin, and is therefore the key enzyme responsible for the accumulation of antheraxanthin and violaxanthin in *G. lutea* petals (Zhu et al., 2003). ZEP is also the first committed enzyme in the ABA biosynthesis pathway (Marin et al., 1996; Seo and Koshiba, 2002). Expression profiling in *G. lutea* has shown that *GlZEP* mRNA is abundant in fully-developed petals that contain mature chromoplasts but only minimal amounts are present in younger petals that still contain chloroplasts, and in leaves and stems (Zhu et al., 2003). Steady state *GlZEP* mRNA levels increase 1.8-fold between the hard bud stage (S1) and the fully-open flower stage (S5) (Zhu et al., 2003).

To gain insight into the regulation of *GlZEP* during petal development and chromoplast differentiation, we isolated the *GlZEP* promoter and evaluated different constructs for their activity in transgenic tomato plants by fusing them to the *gus*A reporter gene. Histochemical GUS assays revealed that a construct containing 677 bp of the *GlZEP* upstream promoter was sufficient to confer strong GUS activity in chromoplast-rich tissues but not in tissues containing chloroplasts, similar to the expression profile of the native gene in *G. lutea*. These data indicate that the 677-bp *GlZEP* promoter contains evolutionarily-conserved sequences that confer high level expression in chromoplast-rich tissues.

MATERIALS AND METHODS

Plant material

Gentiana lutea leaves, stems and flowers were obtained from the Hokkaido Experimental Institute of Health Science (Japan). The tissues were frozen in liquid nitrogen immediately after harvesting and then stored at –80°C.

Tomato (*Solanum lycopersicum* cv. Micro-Tom) plants were grown in the greenhouse at 25°C with a 16-h photoperiod. The leaves, stems, roots, flowers and fruits of wild-type and T4 homozygous transgenic plants were used for GUS histochemical assays immediately after harvesting, or were frozen in liquid nitrogen and stored at –80°C until required for GUS analysis or *gusA* mRNA profiling. Fruits were harvested at four different stages: immature green (IG), mature green (MG), orange and ripe red.

Isolation of genomic DNA and RNA

Genomic DNA was extracted from 5 g of leaf tissue as described by Sambrook et al. (1989). Briefly, 50–100 mg of fresh leaf tissues was ground to a fine powder using liquid nitrogen. We then added 500 µl of extraction buffer and 500 µl of phenol/$CHCl_3$ (1/1) and mixed gently for 10 minutes on a shaker followed by centrifugation at 15,000 x g for 10 min. The upper phase was transferred to a fresh tube and 0.1 volumes of 3 M sodium acetate (pH 5.2) and two volumes of 96% ethanol were added and mixed well. Genomic DNA was precipitated by incubation for 30 min at 20°C followed by centrifugation (15,000 x g, 4°C, 20 min). The resulting pellet was washed with 700 µl 70% ethanol, dried and resuspended in 100–200 µl of sterile water. Gel electrophoresis was performed according to Sambrook and Russel (2002). DNA fragments (70 bp to 10 kbp) were isolated from agarose gels using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

Total RNA was extracted from 120 mg of ground leaf tissue by incubating with 1.2 ml Trizol® reagent for 5 min at room temperature, mixing with 240 µl of chloroform, and centrifuging at 13,000 rpm for 15 min at 4ºC. The supernatant was transferred to a fresh Eppendorf tube, mixed with 600 µl isopropanol and incubated for 10 min at room temperature. The supernatant was removed after centrifugation at 13,000 rpm for 10 min at 4ºC and 1 ml of 70% ethanol was added to wash the RNA for 1 h. The mixture was centrifuged at 13,000 rpm for 15 min at 4º,Cdissolved in sterile double distilled water containing 1 µl of RNAse inhibitor (Roche Diagnostics GmbH, Mannheim, Germany). RNA quality was verified by

fractionating 2 µl of isolated RNA by 1.2% agarose gel electrophoresis. Total DNA and RNA were quantified using a NANODROP 1000 spectrophotometer (Thermo Scientific, Vernon Hills, Illinois, USA).

Cloning the *GlZEP* promoter

G. lutea genomic DNA (20 µg) was completely digested with *Ban*II and self-ligated using 10 Weiss units of T4 DNA ligase (Invitrogen, Carlsbad, CA, USA) to generate circular molecules. These were used as templates for amplification of the *GlZEP* promoter region by long accurate (LA) PCR with the Takara LA PCR kit (Takara, Shuzo, Japan), using forward primer FP1 (5'-CCC TAA ACC CTT CAA CAT CAC TGG TTT CAA GAT TCC-3', covering positions +311 to +346 where position +1 is the first nucleotide of *ZEP* cDNA) and reverse primer RP1 (5'-GAA TGA GAG CCA ATC CAA GGA CAT GAA GCA GCA CCA-3', covering positions +119 to +154) based on the GenBank *GlZEP* cDNA sequence (accession number EF203254). The product was transferred to vector PCR® II TOPO® (TA Cloning Kit, Invitrogen, Carlsbad, CA, USA) for sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit on a 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Promoter-*gus*A constructs

GlZEP promoter fragments were fused to the *gus*A gene in vector pBI101 (Clontech Laboratories, Mountain View, CA, USA) (Jefferson et al., 1987). The full-length *GlZEP* promoter region was amplified from *G. lutea* genomic DNA using forward primer 5'-GTC GAC CCT TAA TGG CGG TAA TTA TGT TCT GTT ATC-3' (positions –2225 to –2194; *Sal*I restriction site underlined) and reverse primer 5'-GGA TCC TAA TCC AAT TAC AAA AGA GTG AAA AGA-3' (positions –27 to –1; *Bam*HI restriction site underlined). The 2225-bp amplified promoter fragment was transferred to the PCR® II TOPO® vector using the Invitrogen TA Cloning® kit, to generate plasmid pCR-GlZEPPro. Both plasmids (pCR-GlZEPPro and pBI101) were digested with *Sal*I and *Bam*HI, allowing the GlZEPPro fragment to be inserted upstream of *gusA* in the pBI101 vector, yielding the final construct pBI-GlZEPPro-GUS (*Zep-gus*A).

Three 5' deletions of the *GlZEP* promoter region were also created by PCR. Primers 5'-GTC GAC CCT TAA TGG CGG TAA TTA TGT TCT GTT ATC-3' (positions –2225 to

–2195) and reverse primer 5'-GGA TCC TTC TTG CTT CAA TTT AGT TAC AAT TTG CTA G-3') (positions +252 to +283) were used to amplify the full-length *GlZEP* promoter and 5' UTR for construct pBI-GlZEPPro-5UTR-GUS (*Zep5utr-gus*A). Then forward primers D1 (5'-GTC GAC TTA TGA GTA CCG AGG TAT GCC TT-3') (positions –1709 to –1684), D2 (5'-GTC GAC GAG TGC AGG TCT GTT ACA GTC AG-3') (positions –1134 to –1109) and D3 (5'-GTC GAC GAT TCG AAT TGA GCG AAT AGT C-3') (positions –677 to –655) were combined with reverse primer 5'-GGA TCC TAA TCC AAT TAC AAA AGA GTG AAA AGA-3' (positions –27 to –1) to generate the three stepwise deletions, with *Sal*I and *Bam*HI restriction sites underlined. The amplified truncated promoters were transferred to PCR®II TOPO and then pBI101 upstream of *gusA* using the strategy described above. The resulting vectors were named pBI-GlZEPProD1-GUS (*D1709-gus*A), pBI-GlZEPProD2-GUS (*D1134-gus*A) and pBI-GlZEPProD3-GUS (*D677-gus*A). All the intermediate and final constructs were verified by sequencing.

Transient expression of promoter-*gus*A constructs in tomato

Plasmids pBI101, pBI121 (*35s-gus*A), pBI-GlZEPPro-GUS (*Zep-gus*A), pBI-GlZEPPro-5UTR-GUS (*Zep5utr-gus*A), pBI-GlZEPProD1-GUS (*D1709-gus*A), pBI-GlZEPProD2-GUS (*D1134-gus*A) and pBI-GlZEPProD3-GUS (*D677-gus*A) were transferred to *Agrobacterium tumefaciens* strain LBA 4404 by electroporation (Mattanovich et al., 1989). Individual colonies were seeded into 5-ml aliquots of YEM medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM $MgSO_4$, pH 7.2) containing 50 µg/ml kanamycin and 25 µg/ml rifampicin, and were shaken at 300 rpm, 28°C overnight. Each culture was then used to inoculate 50 ml induction medium (YEM medium supplemented with 20 µM acetosyringone, 10 mM MES, pH 5.6) containing 50 µg/ml kanamycin and 25 µg/ml rifampicin, and incubated as described above. Bacteria were recovered by centrifugation (2700 x *g*), resuspended in infiltration medium (10 mm $MgCl_2$, 10 mM MES, 200 µM acetosyringone, pH 5.6) to an OD_{600} of ~1.0, and then incubated at room temperature with gentle agitation (20 rpm) for 3 h. Approximately 600 µl of the infiltration medium was then injected into fruits at the mature green stage (25–30 days after anthesis) through the stylar apex using a 1-ml syringe and needle (Orzaez et al., 2006). Injected fruits were left on the vine for 3 days, and then harvested and sectioned for histochemical staining.

Transgenic tomato plants

Tomato stems from one-month-old sterile plants were transformed using the procedure described by Pfitzner (1998). Briefly, 0.5–1 cm stems from plants growing on sterile germination medium (MS salts with 0.6% agar) were severed, placed on MSOZR medium (MS salts, MS Fe-EDTA, B5 vitamins and 30 g sucrose supplemented with 5 µM acetosyringone, 2 mg/l zeatin riboside and 0.6% agar) and pre-incubated for 24 h in a growth chamber (25°C, 16-h photoperiod). The stems were then dipped into the bacterial suspension in MSO medium, blotted on sterile paper and placed on MSOZR plates. After 2 days in the growth chamber, the stems were transferred to plates containing selective shoot regeneration medium (MSOZR medium supplemented with 50 µg/ml kanamycin, 500 µg/ml carbenicillin and 0.6% agar) and incubated in the growth chamber as above for 2 weeks. The shoots were subcultured on fresh medium for another 2 weeks and then transferred to selective shoot regeneration medium (MSOZR medium supplemented with 50 µg/ml kanamycin, 250 µg/ml carbenicillin and 0.6% agar) to regenerate shoots from proliferating callus. Shoots up to 1 cm in length were excised from callus and transferred to 5 x 10 cm containers with selective root medium (MSO medium supplemented with 1 mg/l zeatin riboside, 50 µg/ml kanamycin, 250 µg/ml carbenicillin and 0.6% agar). Plantlets with roots appeared after 2–3 weeks and were transferred to soil in the greenhouse (25°C, 16-h photoperiod). Transgenic tomato lines were selfed to the T_4 homozygous generation for further analysis.

Histochemical and fluorimetric GUS assays

Histochemical GUS assays were carried out according to Jefferson et al. (1987) with minor modifications. Leaves, flowers, hand-cut stem and root segments, and sectioned fruits at different developmental stages were incubated at 37°C overnight (12 h) in the dark in 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) in 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% (v/v) Triton X-100 and 20% (v/v) methanol to eliminate endogenous GUS activity (Kosugi et al., 1990). After 12 h, tissues were destained in an ethanol series (50%, 70%, 80% and 95%) to remove chlorophyll, and then stored in 70% (v/v) ethanol, and photographed with a digital camera.

Fluorometric GUS assays were carried out as described by Jefferson et al. (1987) with minor modifications. Plant tissues (100 mg) were ground to powder under liquid nitrogen, dispersed in 0.8 ml extraction buffer (50 mM Na_2HPO_4 pH 7.0, 10 mM EDTA, 0.1% (v/v)

sodium dodecanoyl(methyl)aminoacetate, 10 mM 2-mercaptoethanol and 0.1% (v/v) Triton X-100) and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant (250 µl) was mixed with 250 µl 2 mM 4-methylumbelliferyl-*β*-D-glucuronide (MUG) on ice and 200 µl was transferred immediately to a fresh tube containing 2 ml of GUS stop buffer (0.2 M Na_2CO_3) to serve as a control. The GUS assay mixture was incubated at 37°C for 1 h before the reaction was stopped by adding 2 ml of GUS stop buffer. The released fluorescent product, 4-methylumbelliferone (MU), was measured on an FP-750 spectrofluorometer (JASCO, Germany) with excitation at 365 nm and emission at 455 nm. The protein content of extracts was determined as described by Bradford (1976). GUS enzyme activity was expressed in pmoles MU/hr·µg of soluble protein. Each assay was carried out twice.

DNA blot analysis

Leaf genomic DNA (20 µg) was digested with *Eco*RI, fractionated by 0.8% (w/v) agarose gel electrophoresis and transferred to a positively-charged nylon membrane (Roche, Mannheim, Germany) according to the manufacturer's instructions. Nucleic acids were fixed by UV crosslinking and hybridized with a digoxigenin-labeled 512-bp *gusA* probe at 42°C overnight using DIG Easy Hyb buffer (Roche Diagnostics GmbH, Mannheim, Germany). The probe was synthesized by PCR using the PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany), forward primer 5'-CCT GTA GAA ACC CCA ACC CGT GA-3', reverse primer 5'-ACG CTG CGA TGG ATT CCG GCA TA-3' and pBI121 as the template. The membrane was washed twice for 5 min in 2x SSC, 0.1% (w/v) SDS at room temperature, twice for 20 min in 0.2x SSC, 0.1% (w/v) SDS at 68°C, and then twice for 10 min in 0.1x SSC, 0.1% (w/v) SDS at 68°C. After immunological detection with anti-DIG-AP (Fab-Fragments Diagnostics GmbH, Germany) chemiluminescence generated by chloro-5-substituted adamantyl-1,2-dioxetane phosphate (CSPD) (Roche, Mannheim, Germany) was detected on Kodak BioMax light film (Sigma-Aldrich, St. Louis, USA).

Quantitative real-time RT-PCR

First strand cDNA was synthesized from 2 µg total RNA using Ominiscript Reverse Transcriptase in a 20-µl total reaction volume following the manufacturer's recommendations (QIAGEN, Valencia, CA, USA). Quantitative real-time RT-PCR was performed on a BIO-RAD CFX96^{TM} system using a 25-µl mixture containing 10 ng cDNA, 1x iQ SYBR Green Supermix (BIO-RAD) and 0.2 µM of each primer. For the amplification of *gusA*

(GenBank accession no. U12639; Jefferson et al., 1987) we used forward primer 5'-CGT GGT GAT GTG GAG TAT TGC-3' and reverse primer 5'-ATG GTA TCG GTG TGA GCG TC-3'. For the internal tomato β-actin control (GenBank accession no. U60482; Agarwal et al., 2009) we used forward primer 5'-GCT GGA TTT GCT GGA GAT GAT GC-3' and reverse primer 5'-TCC ATG TCA TCC CAA TTG CTA AC-3'. To calculate relative expression levels, serial dilutions (0.2–125 ng) were used to produce standard curves for each gene. PCRs were performed in triplicate using 96-well optical reaction plates, comprising a heating step for 3 min at 95°C followed by 40 cycles of 95°C for 10 s, 57°C for 30 s and 72°C for 20 s. Amplification specificity was confirmed by melt curve analysis of the final PCR products in the temperature range 50–90°C with fluorescence acquired after each 0.5°C increment. The fluorescence threshold value and gene expression data were calculated using the CFX96^{TM} system software.

RESULTS

Cloning the *GlZEP* promoter

The *GlZEP* promoter was cloned by inverse PCR using cleaved and circularized *G. lutea* genomic DNA as the template and outward-facing primers based on the *GlZEP* cDNA sequence (GenBank accession number EF203254). After sequencing the resulting product, a 2637-bp fragment was isolated directly from genomic DNA using gene-specific primers based on a new template with restriction sites to facilitate further subcloning. This fragment (Genbank accession number: EF203262) comprised 2225 bp of the upstream promoter and 412 bp of the *GlZEP* cDNA. The 2225 bp promoter fragment was designated the full-length *GlZEP* promoter, and position +1 was assigned to the first nucleotide of the *GlZEP* cDNA (Zhu et al., 2003). The PlantCARE database (Lescot et al., 2002, http://www.dna.affr c.go.jp/PLACE/signalscan.html) was used to identify putative *cis*-acting regulatory elements, revealing two potential TATA boxes at positions -72 and -84 as well as six CAAT boxes, which are known to play an important general role in eukaryotic promoter efficiency (**Table 3.1**). We identified several elements that respond to light, including a GT1 motif, two box I motifs, three G-boxes, a GAG motif, four box 4 motifs (which form part of a conserved light responsive DNA module) and a chs-CMA2a motif (**Table 3.1**). The multitude of light-response elements is likely to regulate *GlZEP* expression according to day length and other cues involved in the control of flower development. We also identified several hormone/stress response elements including one ERE (ethylene response element), two CGTCA motifs (methyl jasmonate sensitive), two MYB binding sites involved in drought stress, a heat shock element, three Box-W1 motifs that respond to fungal elicitors, and a circadian control element (**Table 3.1**).

Table 3.1 List of putative *cis*-acting regulatory elements identified in the 2225-bp *GlZEP* promoter region using the PlantCARE database (Lescot et al., 2002)

Function	Name	Sequence	Position in cDNA	Source
Common *cis*-acting element in promoter and enhancer regions	CAAT-box	CAAT	–1957, –349,–221	*Hordeum vulgare*
		CAAAT	–2157, –1369	*Brassica rapa*
		CAATT	–1496	*Glycine max*

Light-response elements	Box I	TTTCA AA	–2160 –275	*Pisum sativum*
	Box 4	ATTAAT	–599,–262 , –237,–215	*Petroselinum crispum*
	chs-CMA2a	TCACT TGA	–1567	*P. crispum*
	GAG-motif	AGAGAGT	–1068	*Arabidopsis thaliana*
	G-box	CACATGG	–1613	*Solanum tuberosum*
		CACGTC	–1585, –1355	*Zea mays*
	GT-1-motif	GCGGTA ATT	–2217	*Oryza sativa*
Ethylene-response element	ERE	ATTTCAAA	–276	*Dianthus caryophyllus*
MeJA-response element	CGTCA-motif	CGTCA	–2064, –1527	*H. vulgare*
MYB biding site (drought-inducibile)	MBS	CAACTG	–1191, –997	*A. thaliana*
Heat stress response element	HSE	CNNGAANNTT CNNG	–2123	*Lycopersicon esculentum*
Fungal elicitor response element	Box-W1	TTGACC	–2082, –746, –100	*P. crispum*
Circadian control element	circadian	CAANNNNNAT C	–822	*L.esculentum*

Transient expression of *GlZEP* promoter-*gusA* fusions in tomato fruits

Tomato fruits at the mature green stage were injected with bacterial cultures carrying the vectors pBI101 (promoterless *gusA*), pBI121 (*35s-gusA*) and *Zep-gusA*. Fruits were harvested 3 days later and transverse sections were stained for GUS activity. As expected, fruits expressing *gusA* controlled by the CaMV35S promoter (*35s-gusA*) or the full-length *GlZEP* promoter (*Zep-gusA*) exhibited high GUS activity (**Fig. 3.1**) whereas no GUS activity was detected in fruits containing the promoterless control vector pBI101(**Fig. 3.1**).

The promoter was characterized in more detail by generating a series of 5' stepwise truncations containing 1709, 1134 and 677 bp of upstream sequence, respectively, and

inserting these fragments upstream of the *gus*A gene in vector pBI101. We also created a construct containing the full-length *GlZEP* promoter plus the 5'-UTR (*Zep5utr-gus*A). We evaluated the four new constructs by transient expression in tomato fruits as above, using the *35s-gus*A vector as a control. Histochemical GUS assay showed that all four constructs exhibited a similar expression pattern to the full-length *GlZEP* promoter (**Fig. 3.1**). *Zep5utr-gus*A was slightly more active (histochemical GUS assay) than the full-length *GlZEP* promoter (*Zep-gus*A construct) indicating that the 5' UTR contains sequences necessary for high-level expression. The GUS activities of the *D-1709-gus*A and *D1134-gus*A constructs were comparable to the full-length *GlZEP* promoter (*Zep-gus*A). The *D667-gusA* construct was slightly less active than the full-length *GlZEP* promoter, *D-1709-gus*A and *D1134-gus*A (**Fig. 3.1**). Nevertheless the histochemical GUS pattern of the *D667-gus*A construct was very similar to that of the full-length promoter suggesting that all *cis*-acting elements necessary to confer high-level *gus*A expression in tomato fruits are contained within the proximal 677 bp of the *GlZEP* promoter sequence.

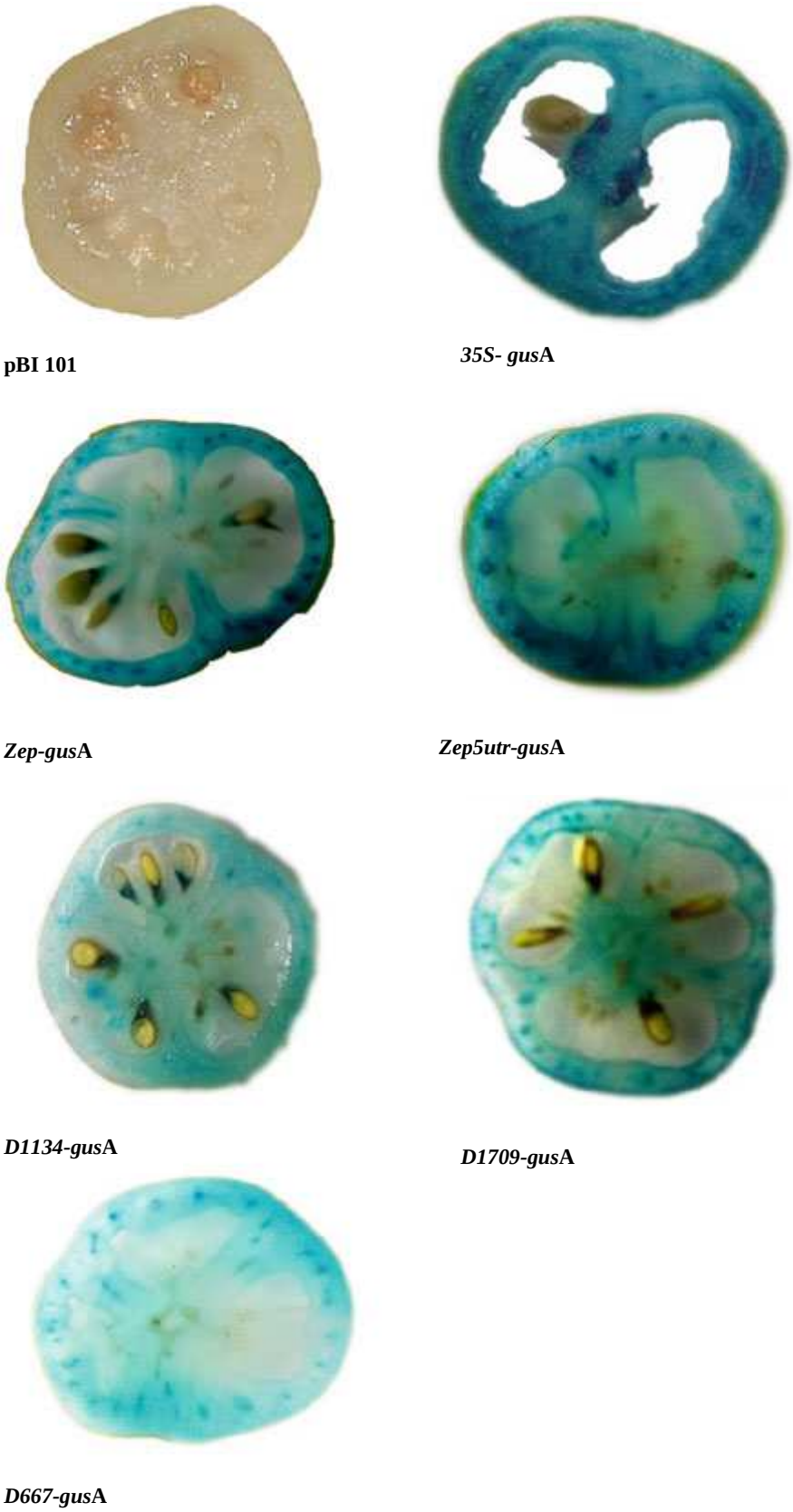

Fig. 3.1 GUS activity in tomato fruits transiently expressing promoterless-*gus*A (pBI101), 35s-*gus*A, Zep-*gus*A, Zep5utr-*gus*A, D1709-*gus*A, D1134-*gus*A and D667-*gus*A, respectively

Histochemical analysis of GUS activity in stably-transformed tomato plants

Tomato plants were stably transformed with two of the constructs described above: the full-length *GlZEP* promoter-*gus*A fusion (*Zep-gus*A) and the positive control pBI121 (*35s-gus*A). Histochemical GUS assays were carried out on 12 primary transformants expressing *Zep-gus*A and eight expressing *35s-gus*A. In the *Zep-gus*A plants, GUS activity was detected in fruits from the mature green stage onwards, but not in leaves, sepals, petals or immature green fruits. The distribution of GUS activity was the same in all 12 independent lines, although the intensity differed significantly. In contrast, GUS activity was detected in all the tissues of all eight *35s-gus*A plants.

We carried out DNA blots on four representative independent *Zep-gus*A lines showing 3:1 segregation of the *gus*A gene and containing one or two transgene copies with little variation in GUS activity, using *gus*A as the probe (**Fig. 3.2**). One *Zep-gus*A line (lane. 4) and one *35S-gus*A line also exhibiting 3:1 segregation were selected to produce T4 homozygous lines for further promoter analysis. Plants from these lines were grown to maturity and GUS activity was analyzed in different tissues.

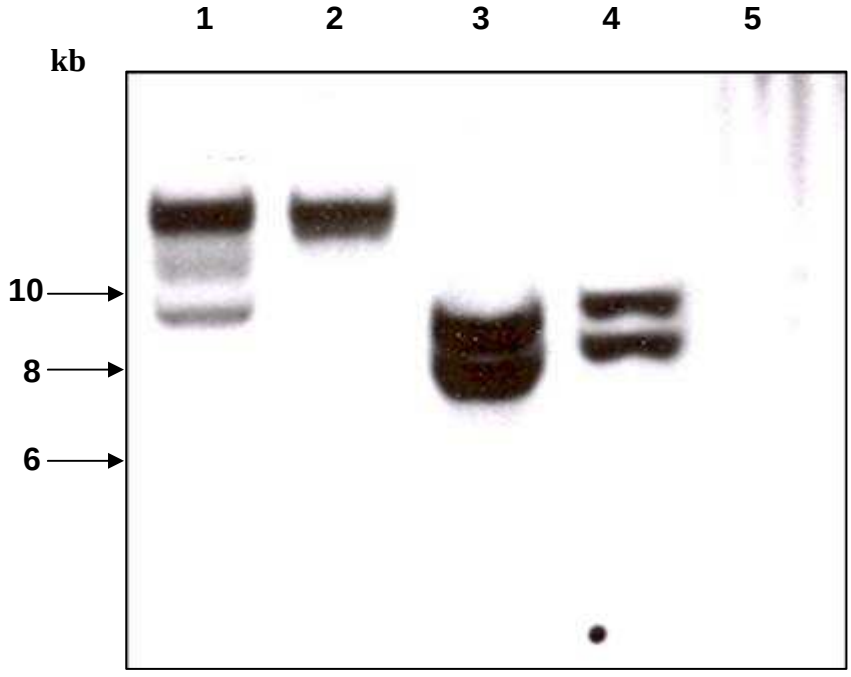

Fig. 3.2 Southern blot analysis of four representative transgenic tomato lines (1–4) carrying *Zep-gusA* and wild type tomato (lane 5). Tomato leaf genomic DNA (20 µg) was digested with *Eco*RI. An internal *gus*A gene fragment (512 bp) was used as a probe.

In the *Zep-gus*A plants, GUS activity in young and mature leaves, stems and roots was below the threshold of histochemical detection (**Fig. 3.3**). GUS activity was also undetectable in sepals and petals, but was detected in the ovary and pistils (**Fig. 3.4**). Low GUS activity was detected in the central column and placenta tissues of immature green fruits. GUS activity increased in mature green fruits, peaked in orange fruits and decreased slightly in red ripe fruits (**Fig. 3.3C**). A distinct spatiotemporal pattern of *gus*A expression was observed in the pericarp, with no expression in immature green fruits, but hogher levels later in development peaking in orange and ripe red fruits (**Fig. 3.3C**). Pericarp cells in young immature green fruits contain a large number of chloroplasts, but these differentiate progressively into chromoplasts which completely replace the chloroplasts in the pericarp cells of ripe red fruits (Forth and Pyke, 2006; Egea et al., 2010). The *Zep-gus*A reporter gene is therefore developmentally regulated in close association with chromoplast differentiation. In contrast to the above, high levels of GUS activity were observed in all tissues of the *35s-gus*A plants and throughout the pericarp during all ripening stages (**Figs 3.3** and **3.4**).

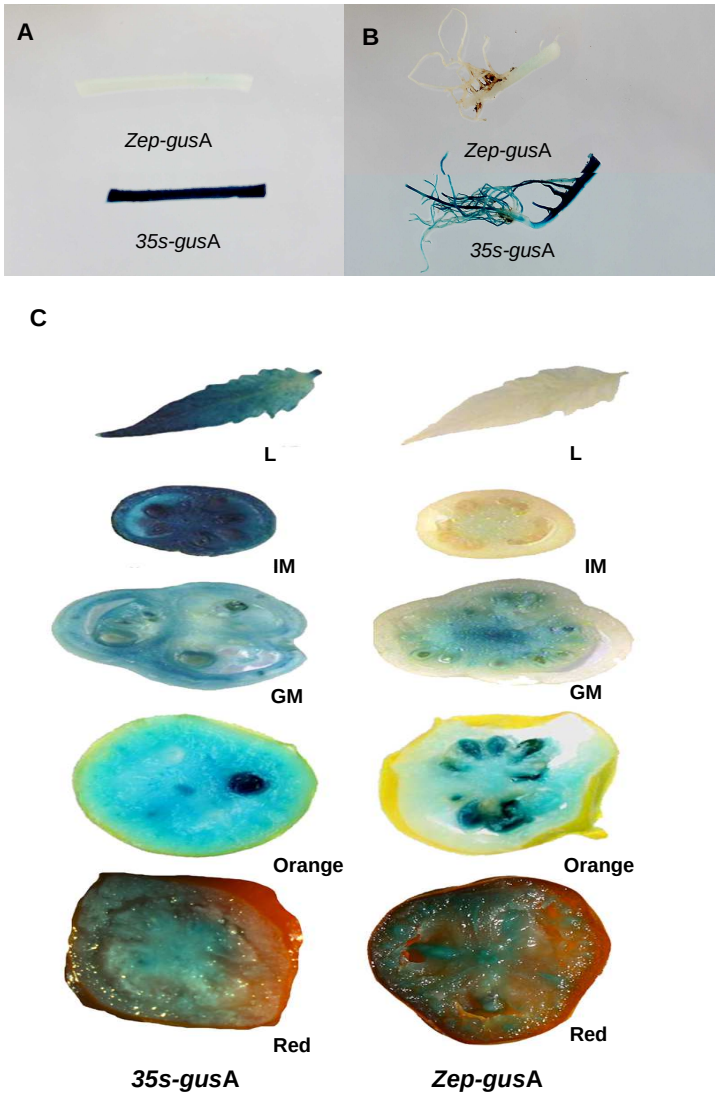

Fig. 3.3 Histochemical GUS staining of representative transgenic tomato plants carrying the *Zep-gus*A and *35S-gus*A constructs, respectively. **A** stems; **B** roots; **C** leaves and fruits. **L** leaf, **IM** immature green fruit, **MG** mature green fruit, **Orange** orange fruit, **Red** ripe red fruit; *Zep-gus*A, pBI-GlZEPPro-GUS; *35s-gus*A, pBI121. All four lines showed very similar staining patterns.

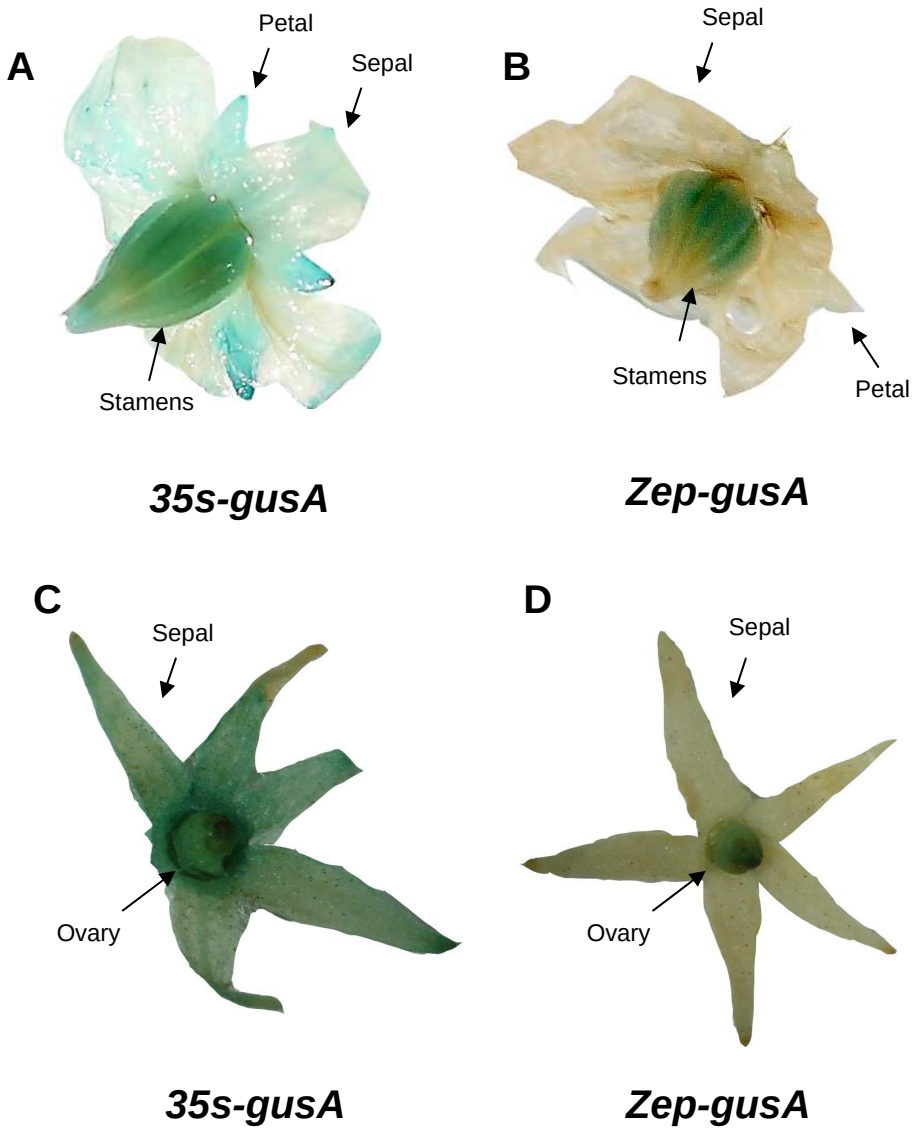

Fig. 3.4 Histochemical GUS staining in flowers of transgenic tomato plants carrying *Zep-gus*A and *35s-gus*A constructs, respectively. All four lines showed very similar staining patterns

Quantitative analysis of GUS activity in stably-transformed tomato plants

Quantitative analysis of GUS activity in *Zep-gus*A transgenic plants demonstrated that only low levels of GUS were present in the leaves, but higher levels were present in flower tissues such as stamens, pistils and petals **(Fig. 3.5**). GUS activity was low in immature green

fruits, but increased five-fold during development peaking in orange fruits. The quantitative and histochemical GUS assays were concordant (**Figs 3.3-3.5**).

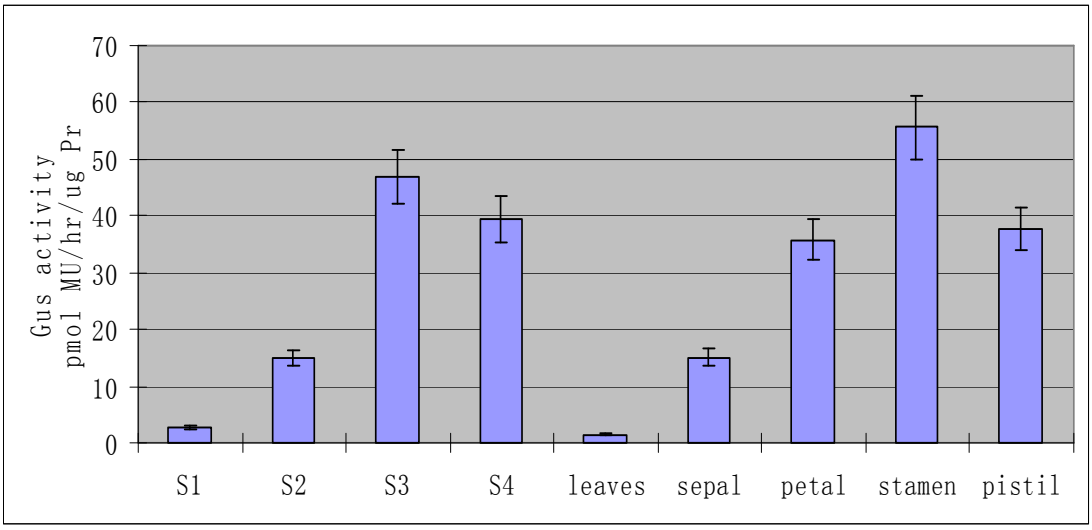

Fig. 3.5 GUS expression in different tissues of transgenic tomato plants carrying *Zep-gus*A. GUS activity was determined in triplicate measurements in four independent biological replicates (independent transgenic plants). Columns represent GUS activity expressed in pmoles MU/hr·µg of soluble protein in fruits at different stages of maturity (S1–S4) and leaves, sepals, petals, stamens and pistils. Error bars represent standard error of the mean. S1, fruits at immature green stage; S2, fruits at mature green stage; S3, fruits at orange fruit stage; S4, fruits at ripe red fruit stage.

Quantitative analysis of *gusA* gene expression in stably-transformed tomato plants

The transcriptional activity of the *GlZEP* promoter was analyzed in more detail by measuring *gusA* mRNA levels in different tissues of the *Zep-gus*A tomato plants by quantitative real-time RT-PCR (**Fig. 3.6**). These measurements in separate extracts from leaves, sepals, petals, stamens and pistils revealed relatively high *gus*A mRNA levels in stamens and the lowest levels in leaves. Expression studies during fruit development showed very low steady-state *gus*A mRNA levels in immature green fruits, an up to six-fold increase in orange fruits and a slight decrease in ripe red fruits (**Fig. 3.6**). These data were in strong agreement with the levels of GUS activity determined in the histochemical and fluorometric assays (**Figs 3.3-3.5**).

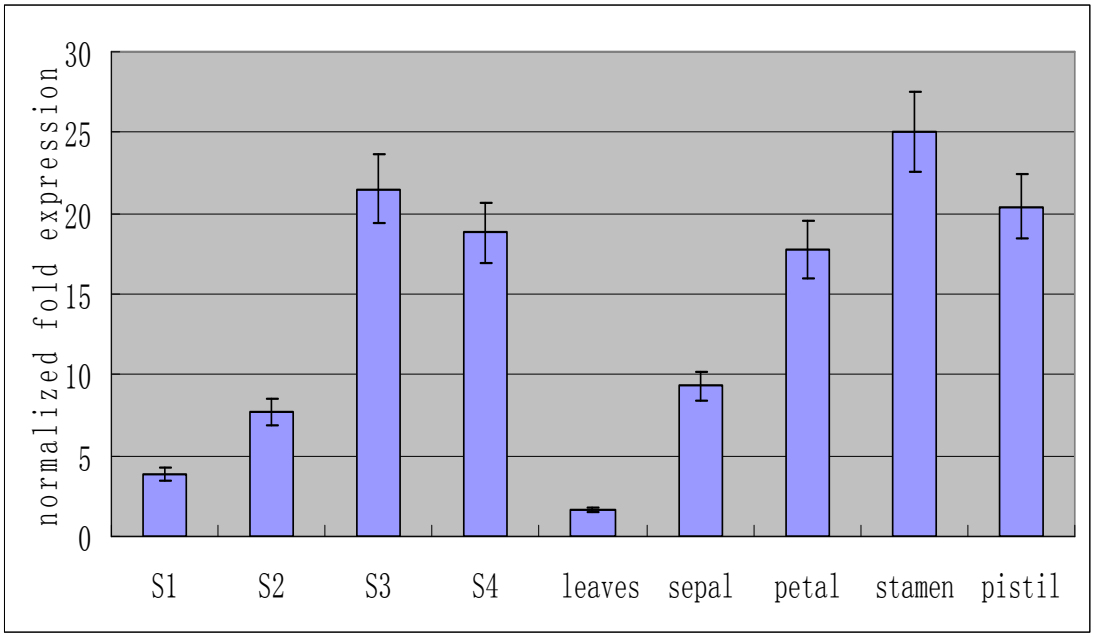

Fig. 3.6 Expression of *gus*A gene in different tissues of transgenic tomato plants carrying *Zep-gus*A. Quantitative real-time RT-PCR was performed with cDNA prepared from leaves, sepals, petals, stamens, pistils and different stage fruits. Relative expression was determined in triplicate measurements in four independent biological replicates. Columns represent the relative *gus*A expression levels normalized against *β-actin* mRNA with standard errors of the mean. S1, fruits at immature green stage; S2, fruits at mature green stage; S3, fruit at orange fruit stage; S4, fruit at red ripen fruit stage.

DISCUSSION

Carotenoid biosynthesis is differentially regulated in tissues containing chloroplasts and chromoplasts, reflecting important functional differences between these tissues and the different roles carotenoids fulfill in each setting (reviewed by Zhu et al., 2010). To ensure that green tissues and fruits/flowers can independently accumulate different carotenoids, many carotenogenic enzymes exist as multiple isoforms encoded by separate genes. The tomato genome, for example, encodes two isoforms of geranylgeranyl diphosphate synthase (*GGPPS*), phytoene synthase (*PSY*), lycopene *β*-cyclase (*LYCB*) and *β*-carotene hydroxylase (*BCH*), one set expressed preferentially in green tissues and the other expressed preferentially in flowers and fruits (Ronen et al., 2000; Galpaz et al., 2006). Chromoplast-specific isoforms of lycopene *β*-cyclase have also been identified in citrus (Alquezar et al., 2009; Dalal et al., 2010; Mendes et al., 2011), kiwifruit (Ampomah-Dwamena et al., 2009), saffron (Ahrazem et al., 2010) and papaya (Blas et al., 2010; Devitt et al., 2010).

There has been great interest in the investigation of carotenoid biosynthesis and its regulation in plants, primarily because of the dietary benefits of carotenoids and the drive to develop crops with higher levels of *β*-carotene. However, this has drawn attention away from the natural role of carotenoids in plants, i.e. the promotion of pollination and seed dispersal, which can only be investigated by looking at the expression profiles of carotenogenic genes in homologous and heterologous genetic backgrounds and linking the expression profiles of different enzymes to the carotenoids that accumulate in different tissues (Zhu et al., 2002; 2003; Li et al., 2010).

We have previously shown that the *GlZEP* gene is expressed strongly in chromoplast-rich mature petals of *G. lutea* plants, but only minimally in chloroplast-containing younger petals and leaves (Zhu et al., 2003), suggesting the promoter may be active in tissues containing chromoplasts and repressed in tissues lacking them. In agreement with this, no GUS activity was detected in tobacco plants (whose petals and fruits lack chromoplasts) expressing a *GlZEP-gus*A transgene so we sought to carry out similar analysis in tomato plants, which contain abundant chromoplasts in mature fruits and flowers. Chromoplasts in tomato fruits begin differentiating at the breaker stage, and full conversion of chloroplasts into chromoplasts occurs when the fruits are completely ripe (reviewed by Egea et al., 2010). Chloroplast-to-chromoplast differentiation can be conveniently assessed using pericarp pigmentation during tomato fruit development. We selected the dwarf tomato cultivar Micro-Tom (Scott and Harbaugh, 1989) as a model to investigate *GlZEP* promoter activity in chromoplast-containing tissues because of its small size and short life cycle (70–90

days from sowing to fruit ripening) (Meissner et al., 1997). This cultivar has previously been used for the analysis of metabolic and developmental pathways (Haroldsen et al., 2011; Carvalho et al., 2011).

We investigated *GlZEP* promoter activity by transient expression and stable transformation in tomato plants transformed with a range of *GlZEP-gus*A reporter constructs. We recovered 12 independent transgenic plant lines expressing the *GlZEP-gus*A construct, all of which demonstrated the same profile of GUS activity albeit with varying staining intensity, so we selected four representative lines for further analysis. We investigated the activity of full-length and truncated promoter constructs in leaves, stems, roots, flowers and fruits at different developmental stages. GUS activity in young and mature leaves, stems and roots was below the threshold for histochemical detection (**Fig. 3.3**). GUS activity was also undetectable in sepals and petals, but could be detected in the ovary and pistils (**Fig. 3.4**). Similar GUS staining was observed in the flowers of transgenic tomato plants expressing *gus*A driven by the tomato PDS promoter or the tomato *CYC-B* promoter (Corona et al., 1996; Dalal et al., 2010). In these reports there was no apparent link to chromoplast differentiation, possibly reflecting the conserved function of carotenoids in flower tissues.

The full-length *GlZEP* promoter (2,225 bp upstream of the transcriptional start site) was functional in the heterologous tomato environment and the expression profile of the reporter gene driven by the full-length promoter was identical to that observed in its homologous background (Zhu et al., 2003). High levels of GUS activity were observed in chromoplast-containing flowers and fruits, but there was only minimal activity in other tissues (fruits, sepals, leaves, stems and roots). Reporter gene activity was strictly correlated with fruit development and chromoplast differentiation, with minimal activity in immature green fruit but increasing activity in ripening orange fruit before falling off towards the end of the ripening process. The shortest *GlZEP* deletion construct (D677-*gus*A) contained 677 bp of upstream sequence but nevertheless resulted in only slightly lower levels of GUS activity compared to the full-length promoter (**Fig. 3.1**), suggesting that all *cis*-acting elements required for high level GUS activity in chromoplast-rich tissues are contained within the proximal 677 bp of the promoter.

All promoters contain *cis*-acting elements that confer spatiotemporal specificity and responsiveness to external stimuli (Peremarti et al., 2010). We characterized the *GlZEP* promoter fragment in more detail by searching the sequence for relevant *cis*-acting elements using the PlantCARE database (Lescot et al., 2002). We identified TATA and CAAT boxes that are typical in eukaryotic promoters, as well as multiple light-response elements (GT1,

box I, G-box, GAG motif, Box 4 and chs-CMA2a) that are likely to link carotenoid biosynthesis to flower development by integrating day-length cues and other stimuli (**Table 3.1**). Carotenoid biosynthesis is regulated by light (Bartley and Scolnik, 1993; Von Lintig et al., 1997; Simkin et al., 2003; Li et al., 2008; Welsch et al., 2008). A putative circadian responsive element was also found in the *GlZEP* promoter, which supports the diurnal rhythm in *zep* gene expression that has been reported in tobacco and tomato leaves (Audran et al., 1998; Thompson et al., 2000; Facella et al., 2008).

The *GlZEP* promoter also contains *cis*-acting elements involved in hormone biosynthesis and stress responses (**Table 3.1**). The presence of a drought-inducible MBS element agrees with previous reports suggesting that the expression of endogenous *zep* in tobacco and tomato roots is induced by drought stress (Audran et al., 1998; Thompson et al., 2000). Two ATCTA motifs are present as tandem repeats in the *GlZEP* promoter. Similar pairs have previously been identified in the *Arabidopsis thaliana PSY* promoter (mediating high-level basal transcription independent of light quality), and in the promoters of several genes related to photosynthesis (Welsch et al., 2003). Single copies of the ATCTA motif are found in other carotenogenic promoters such as Arabidopsis deoxy-xylulose-phosphate synthase (*DXS*) and *PDS* (Welsch et al., 2003), tomato and maize *PDS* (Welsch et al., 2003), and tomato *CYC-B* (Dalal et al., 2010). This motif is also present in several promoters involved in tocopherol biosynthesis (Welsch et al., 2003). In Arabidopsis, paired ATCTA motifs are recognized by AtRAP2.2, a member of the APETALA2/ERE-binding protein transcription factor family (Welsch et al., 2007). We engineered one of our constructs deliberately to eliminate one of the ATCTA motifs (*D1709-gus*A), and also generated two more substantially truncated constructs lacking both copies (*D1134-gus*A and *D677-gus*A). All three constructs performed similarly to the full-length promoter (*zep-gus*A) suggesting that neither motif contributes significantly to the basal activity of the full-length *GlZEP* promoter. In contrast, deletion of the RAP2.2 transcription factor binding site in the *ShCYC-B* full length promoter resulted in a considerable loss of promoter activity (Dalal et al., 2010). However, it is possible that the loss of adjacent sequences rather than the RAP2.2 element might be responsible for the fall in promoter activity and the only way to confirm the role of this element directly is to modify it by site-directed or linker-scanning mutagenesis.

The promoters of coexpressed genes often share common regulatory motifs and are potentially regulated by a common set of transcription factors. Therefore, the identification of relevant *cis*-acting regulatory elements in the promoter regions of important metabolic genes can provide leads that help uncover new mechanisms of transcriptional regulation (Liu et al.,

2005; Nilsson et al., 2010). The expression profile of *zep-gus*A in transgenic tomato plants is strikingly similar to that of tomato *PDS* (Corona et al., 1996) and *CYC-B* (Dalal et al., 2010), which have also been evaluated through the analysis of reporter gene fusions in transgenic tomato plants, suggesting all three may be regulated by a common mechanism. It is also important to emphasize that the *GlZEP* promoter is correctly regulated in a heterologous background, indicating strong conservation of the regulatory mechanisms across species. Common motifs in the three promoters include the CAAT box, Box 4 and RAP2.2 (Corona et al., 1996; Welsch et al., 2007; Dalal et al., 2010) but further analysis and comparisons are required to identify additional known and unknown motifs that are shared between co-regulated promoters and that may help us to unravel further underlying regulatory mechanisms.

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CHAPTER III

Towards the modulation of the C3 pathway in rice for enhanced photosynthetic efficiency

ABSTRACT

We introduced the *Escherichia coli* glycolate catabolic pathway into rice chloroplasts to reduce the loss of fixed carbon when phosphoglycolate, a by-product of photosynthesis, is recycled by photorespiration in C3 plants. Five chloroplast-targeted bacterial genes encoding the three subunits that comprise glycolate dehydrogenase (GDH), glyoxylate carboligase (GCL) and tartronic semialdehyde reductase (TSR) were used to generate transgenic plants containing all the necessary genes to complete the conversion of glycolate to glycerate inside the chloroplast and thus to improve the efficiency of carbon fixation. Introducing a bacterial glycolate catabolic pathway into C3 plants such as rice, to reduce photorespiratory losses and enhance carbon fixation, is equivalent to introducing a C4-like mechanism into C3 plants in that it should further our understanding of the basis of biomass accumulation, the carboxylation versus oxygenation activity of RuBisCO, and energy transfer in the photorespiratory pathway.

INTRODUCTION

Rice provides the major source of calories for most of the world's population, particularly in developing countries. If we are to provide food for the predicted global population of 9 billion people by 2050, we need to achieve substantial increases in the yield of rice and other staple food crops (Kajala et al., 2011). Currently, it is estimated that 925 million people suffer from chronic hunger and about 14,400 children die from hunger-related causes every day (FAO, 2010). Population growth in Asia will require a 60% increase in rice production and so each rice-producing hectare that currently feeds 27 people will need to provide food for 43 people in the very near future (Sheehy et al., 2008).

Photosynthesis is a fundamental chemical process in which the energy from sunlight or other light is utilized by green plants and blue-green algae to convert carbon dioxide and water into carbohydrates (Kebeish, 2006; Kebeish et al., 2007). In fact, most of the food we eat, the fuel we burn and the fibers we wear reflect the activity of green plants. We introduced the *Escherichia coli* glycolate catabolic pathway into rice chloroplasts to reduce the loss of fixed carbon by photorespiration in order to increase the biomass accumulation and efficiency of photosynthesis.

The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) fixes a vast amount of inorganic carbon into biomass, as well as molecular oxygen (Bowes et al., 1971). Photorespiration is the light-dependent uptake of O_2 with the concomitant release of CO_2 (**Fig. 4.1**). This gas exchange resembles respiration and is the reverse of photosynthesis, where CO_2 is fixed and O_2 released (Peterhansel et al., 2010). Serine resulting from the mitochondrial decarboxylation reaction is transported back to peroxisomes where it is converted to hydroxypyruvate and further to glycerate. Glycerate is transported back to the chloroplast where it is phosphorylated to phosphoglycerate and re-integrated into basal metabolism. During this reaction, one molecule of CO_2 and one molecule of NH_3 are lost. These losses mean that photorespiration is often described as a wasteful process (Wingler et al., 2000). On the other hand, the pathway rescues 75% of the carbon in phosphoglycolate that would be otherwise inaccessible for further metabolism. Thus, photorespiration can also be regarded as an important pathway that makes the best of a bad situation caused by the inevitable oxygenase activity of RuBisCO. There are several additional potential benefits of photorespiration in plant metabolism such as the removal of toxic phosphoglycolate, protection against photoinhibition, the promotion of defense reactions and integration into primary metabolism (Peterhansel et al., 2010 and references therein).

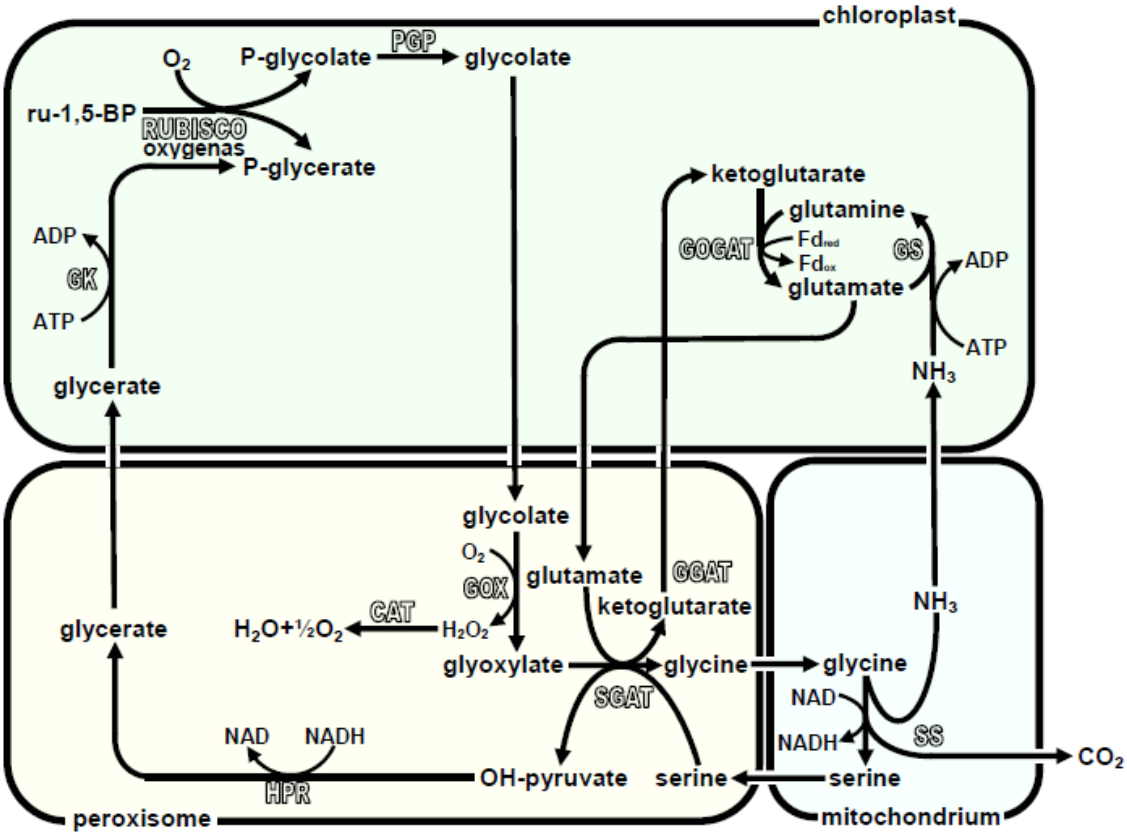

Fig 4.1 Representation of the photorespiration pathway in C3 plants
In this pathway, the oxygenation of RuBP by RuBisCO oxygenase results in the formation of one molecule of PGA and one molecule of PG. PGA enters in the Benson Calvin cycle to form carbohydrates and also to regenerate RuBP. PG is processed to form PGA in a reaction sequence occurring in the chloroplast, peroxisomes and mitochondria. PG is converted to glycolate by PGP. Glycolate is transported from the chloroplast into the peroxisome where it is oxidized by GOX to form glyoxylate. Glyoxylate is then converted to glycine by GGAT. Glycine is internally transported to the mitochondria where it is decarboxylated to form serine by GDC/SHMT. The serine is transported back to the mitochondria where it is converted to hydroxypyruvate by SGAT. Hydroxypyruvate is then converted to glycerate by HPR. Glycerate is then transported to the chloroplast where it is converted into PGA by GK. RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; PGP, phosphogycolate phosphatase; GOX, glycolate oxidase; CAT, catalase; GGAT, glyoxylate/glutamate amino transferase; GDC/SHMT, glycine decarboxylase/serine hydroxymethyl transferase; SGAT, serine/glutamate amino transferase; HPR, hydroxypyruvate reductase; GK, glycerate kinase; GS, glutamine synthetase; GOGAT, glutamate synthase (Kebeish, 2006).

The affinity of RuBisCO for CO_2 is about 100 times higher than for O_2, but the atmosphere contains much more O_2 than CO_2 resulting in a 4:1 ratio of CO_2 to O_2 fixation. RuBisCO oxygenase activity is much more common in C3 plants reflecting the absence of the CO_2 concentrating mechanism. C4 and CAM plants overcome photorespiration by concentrating CO_2 in the vicinity of RuBisCO (as described in the general introduction). C4

plants are therefore able to concentrate CO_2 in bundle sheath cells (which contain RuBisCO) at 3–20 times the level of atmospheric CO_2 (Jenkins, 1989; von Caemerer et al., 1999). Because of this CO_2 concentrating mechanism, C4 plants greatly reduce RuBisCO oxygenase activity (Hatch, 1987; 1992).

Carbon fixation via the Benson Calvin cycle and 2-phosphoglycolate (2-PG) recovery by photorespiration are conserved in all photosynthetic organisms. However, plants in agricultural production systems are selected for higher yield and better performance traits. Carbon metabolism can be optimized in agricultural production systems by genetic engineering. For example, the maize phosphoenolpyruvate carboxylase (PEPC) gene has been transferred into several C3 crops, including potato (Ishimaru et al., 1998) and rice (Matsuoka et al., 1998; Ku et al., 1999) in order to increase the overall level of carbon fixation. Transgenic rice plants were also produced expressing pyruvate orthophosphate dikinase (PPDK) and NADP-malic enzyme (Ku et al., 1999). Field trials in China and Korea demonstrated 10–30% and 30–35% yield increases for PEPC and PPDK transgenic rice plants, respectively, which was unexpected because only one C4 enzyme was expressed in each case. In the PEPC transgenic plants, there was also an unanticipated secondary effect in which RuBisCO inhibition by O_2 was reduced (Ku et al., 1999). In potato, the heterologous PPDK activity was enhanced 5.4-fold compared to wild type plants, inducing a partial C4 metabolism. However, there was no change in the photosynthetic characteristics of the plants and the PPDK activity was still low compared to maize. Strategies exploring the conversion of glycolate to malate instead of glycerate have also been evaluated (Maurino and Flugge, 2009). In this approach, glycolate is oxidized by glycolate oxidase and the resulting H_2O_2 is detoxified by catalase. Malate synthase forms malate from glyoxylate and acetyl-CoA present in the chloroplast. Through two additional endogenous reactions, acetyl-CoA is recycled and glyoxylate fully converted to CO_2. The installation of this pathway in Arabidopsis also enhanced the growth of the plant (Maurino and Flugge, 2009).

Transgenic Arabidopsis plants have been generated that bypass photorespiration (Kebeish et al. 2007, **Fig 4.2**) by expressing the three *Escherichia coli* enzymes glycolate dehydrogenase (GDH; Lord, 1972; Pellicer et al., 1996) glyoxylate carboligase (GCL; Chang et al., 1993) and tartronic semialdehyde reductase (TSR; Gotto and Kornberg, 1961). This involved the conversion of glycolate to glycerate in the chloroplast, shitfting CO_2 release from the mitochondria to the chloroplasts and avoiding the release of NH_3. Furthermore, the energy balance calculated for the bacterial pathway ws superior to photorespiration, mainly reflecting the fact that bacterial GDH does not use oxygen as an electron acceptor like the plant

glycolate oxidase, but instead transfers electrons to organic co-factors thus saving reducing power. The photorespiratory bypass in Arabidopsis therefore reduced flux through the photorespiration pathway, enhancing photosynthesis and biomass production (Kebeish et al., 2007).

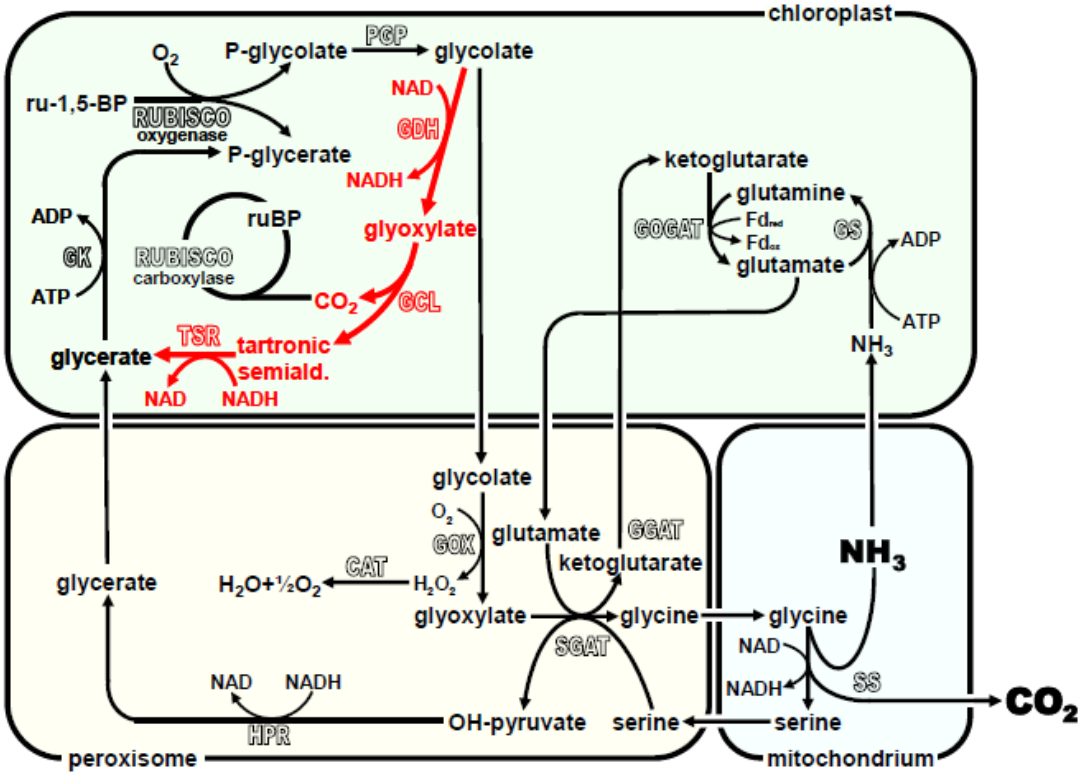

Fig 4.2 Representation of the photorespiratory pathway (black) in C3 plants and the proposed pathway (red) for the conversion of glycolate to glycerate.
The RuBisCO oxygenase reaction forms P-glycerate and P-glycolate, the latter dephosphorylated by PGP to form glycolate, which is in turn oxidized by GDH to form glyoxylate. Two molecules of glyoxylate are condensed by GCL to form tartronic semialdehyde, and CO_2 is released in the chloroplast. Tartronic semialdehyde is then reduced by TSR to form glycerate, which is phosphorylated by GK to form P-glycerate, which is used directly for carbohydrate biosynthesis via the Benson Calvin cycle. PGP = phosphoglycolate phosphatase; GDH = glycolate dehydrogenase; cTP-AtGDH = *A. thaliana* glycolate dehydrogenase fused to a chloroplast targeting peptide (cTP); GCL = glyoxylate carboxyligase; TSR = tartronic semialdehyde reductase; GK = glycerate kinase.

MATERIALS AND METHODS

Gene cloning and vector construction

Genes encoding *Escherichia coli* glcD, glcE and glcF (the three GDH subunits) as well as TSR and GCL, were cloned by R. Kebeish (Institute for Biology I, RWTH-Aachen, Germany). The genes were inserted into pTRAux_Cab7 vectors. The *glc*D gene (encoding subunit D of GDH) was under the control of the maize PPDK promoter and the PPDK-3'UTR terminator. The *glc*E gene (encoding subunit E of GDH) was under the control of the maize *RbcS* (RuBisCOsmall subunit) promoter and the RbcS-3'UTR terminator. The *glc*F gene (encoding subunit F of GDH) was under the control of the maize Ubi-1 promoter (with first intron) and the *Cauliflower mosaic virus* pA35S terminator. The *tsr* gene was under the control of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter and GAPDH-3'UTR terminator. The *glc* gene was under the control of the PEPC promoter and PEPC-3'UTR terminator. All vectors (**Fig. 4.3**) were kindly provided by Prof F. Kreuzaler, Institute for Biology I, RWTH-Aachen, Germany. We also cloned the eGFP (enhanced green fluorescent protein) gene under the control of the GAPDH promoter and GAPDH-3'UTR terminator to confirm enzyme localization. Plasmid constructs were maintained *E. coli* strain DH5α and were isolated uing Qiagen and Invitek plasmid DNA maxi and mini kits according to the manufacturer's instructions. We separated 2–5 μl of the eluted DNA by 1% agarose gel electrophoresis to confirm plasmid quality and quantity.

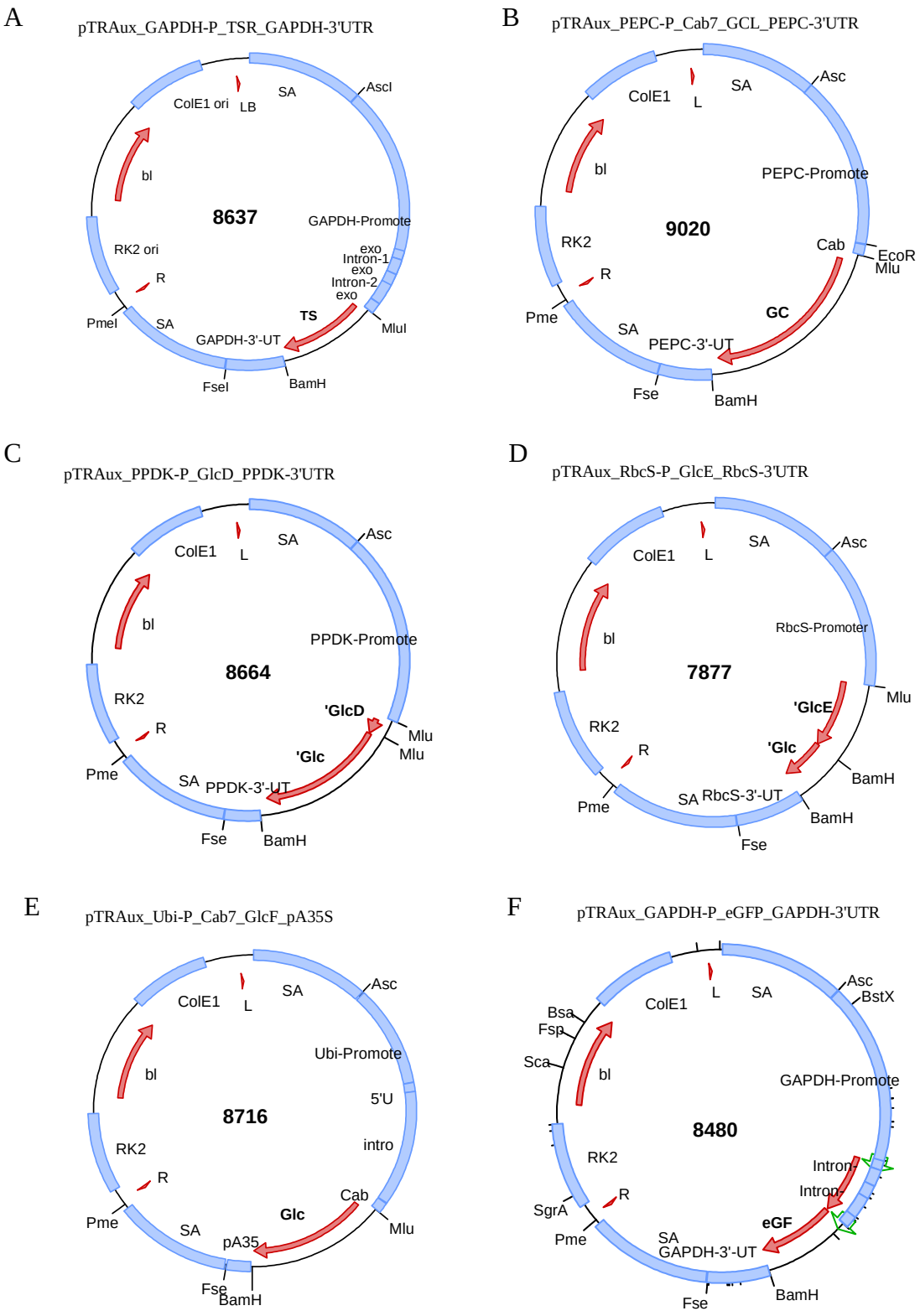

Fig. 4.3 Vectors carrying the *E. coli* glycolate catabolic pathway genes.
A: pTRAux_GAPDH-P_TSR_GAPDH-3'UTR contains the *tsr* gene encoding tartronic semialdehyde reductase (TSR). **B**: pTRAux_PEPC-P_Cab7_GCL_PEPC-3'UTR contains the

gcl gene encoding glyoxylate carboligase (GCL). **C**: pTRAux_PPDK-P_GlcD_PPDK-3'UTR contains the *glc*D gene encoding glycolate dehydrogenase subunit D. **D**: pTRAux_RbcS-P_GlcE_RbcS-3'UTR contains the *glc*E gene encoding glycolate dehydrogenase subunit E. **E**: pTRAux_Ubi-P_Cab7_GlcF_pA35S contains the *glc*F gene encoding glycolate dehydrogenase subunit F. **F**: pTRAux_GAPDH-P_eGFP_GAPDH-3'UTR contains the *eGFP* gene encoding enhanced green fluorescent protein.

Rice transformation

Mature rice embryos (*Oryza sativa* L. cv EYI 105) were excised and cultured as described (Sudhakar et al., 1998; Valdez et al., 1998). After 7 days of culture, bombardment was carried out using 40 µg of plasmid DNA coated gold particles (Christou et al., 1991). The rice embryos were incubated on high-osmoticum containing medium (0.2 M mannitol,) for 4 h prior to bombardment. For co-transformation, the gold particles (10 mg) were coated with 40 µg of the five glycolate catabolic pathway plasmids (7.4, 7.2, 7.5, 7.6 and 7.8 µg, respectively) mixed with a plasmid containing the hygromycin phosphotransferase (*hpt*) gene as selectable marker (2.5 µg). The particles were stored in 10 ml of ethanol (Sudhakar et al., 1998; Valdez et al., 1998). Bombarded embryos were cultured on MS medium supplemented with 50 mg/l hygromycin and 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in the dark. Embryogenic callus and plantlets were recovered on regeneration medium, in the light, as described (Sudhakar et al., 1998; Valdez et al., 1998). Plantlets were regenerated after callus selection using hygromycin-supplemented shooting and rooting medium. After regeneration, the plantlets were transferred to soil.

Genomic DNA extraction

Genomic DNA was extracted from 250 mg of frozen, ground leaves as described by Dellaporta et al. (1983). We added 5 ml of extraction buffer (500 mM NaCl, 100 mM Tris-HCl, 50 mM EDTA; pH 8) and 380 µl 20% SDS and incubated for 30 min at 65ºC before extracting with 5 ml (1:1) phenol:chloroform (Sigma, Steinheim, Germany) and centrifuging at 4500 rpm for 10 min at room temperature. The supernatant was transferred to a new fresh and incubated at 37ºC for 1 h with 20 µl 10 mg/mL RNase. After a second round of phenol:chloroform extraction, the supernatant was transferred to a fresh tube and genomic DNA was precipitated by incubating for 1 h at room temperature with 5 ml isopropanol and centrifuging at 5000 rpm for 30 min at room temperature. The supernatant was discarded and the genomic DNA pellet was washed with 1 ml 70% ethanol for 1 h on ice. After centrifugation at 5000 rpm for 30 min and removal of the ethanol, the DNA pellet was

air-dried and dissolved in 40 µl sterile water. The concentration of DNA was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and DNA quality was verified by 0.8% agarose gel electrophoresis.

DNA blot analysis

Rice genomic DNA (13 µg) was digested with *EcoR*I and fractionated by 0.8% TBE agarose gel electrophoresis (Sambrook et al., 1989). The DNA was transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) and fixed by UV cross-linking. DIG-labeling of the probe (**Table 4.1**) was carried out as described by Capell et al. (2004). The DIG-labeled probe was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and denatured at 68ºC for 10 min before 2-3 h pre-hybridation at 42ºC in 10 ml EasyHyb solution (Roche Diagnostics GmbH, Mannheim, Germany). The membrane was hybridized with appropriate probes overnight at 42ºC. Membranes were washed twice for 5 min in 2x SSC + 0.1% SDS at room temperature, twice (25 min) in 0.5x SSC + 0.1% SDS, once (15 min) in 0.2x SSC + 0.1% SDS, and once (10 min) in 0.1% SDS at 68ºC. Chemiluminescence was measured using the DIG Luminescent Detection Kit according to the manufacturer's instructions. After washing, the membranes were incubated with CSPD chemiluminescent substrate (Roche Diagnostics GmbH, Mannheim, Germany) and exposed to BioMax light film (Kodak, Steinheim, Germany) at 37ºC.

Table 4.1 Forward (F) and reverse primers (R) used for synthesizing the probes for DNA and mRNA blot analysis.

Primer name	Primer sequence
GlcD_F	5'-GGTGTGTTGTTGGTGATGGCGCGCTTT-3'
GlcD_R	5'-CTCCACGCCGTCCAGCTCGCATA-3'
GlcE_F	5'-GCGCTGCTGGAGCAGGTGAAT-3'
GlcE_R	5'-CACTCATGGCTTCTTGCAGGCTGATT-3'
GlcF_F	5'-GCCTGTGTTCACTGCGGATT-3'
GlcF_R	5'-CGGGCCACCAGGCATCAATA-3'
TSR_F	5'-GGCATTATGGGTACACCGATGGCCATTA-3'
TSR_R	5'-CGCACTTTGCAGTGCCAGGTTGAGAT-3'
GCL_F	5'-CTCAGCGATGCGTAAGCACGGCGGTATT-3'
GCL_R	5'-CACCATGTCAGACGCCAGCAGCGTT-3'

RNA extraction

Total RNA was extracted from 120 mg of frozen, ground transgenic rice leaves using 1.2 ml Trizol reagent (Invitrogen, Paisley, UK) for 5 min at room temperature, extracted with 240 µl chloroform and centrifuged at 13,000 rpm for 15 min at 4ºC. The supernatant was transferred to a fresh tube and precipitated with 600 µl isopropanol for 10 min at room temperature. After centrifugation at 13,000 rpm for 10 min at 4ºC, the supernatant was removed and the RNA pellet was washed with 1 ml 70% ethanol for 1 h. After repeating the centrifugation, the RNA pellet was dissolved in sterile double-distilled water containing 1 µl RNase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany). The RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and RNA quality was verified by 1.2% agarose gel electrophoresis.

RNA blot analysis

Total RNA (30 µg) was denatured and fractionated by 1.2% agarose-formaldehyde gel electrophoresis in 1x MOPS buffer (Sambrook et al., 1989). The RNA was transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) (Sambrook et al., 1989) and fixed by UV crosslinking. The labeled probe (**Table 4.1**) was purified using the QIAquick Gel Extraction Kit (Qiagen, UK) and denatured at 68ºC for 10 min prior to use. The membrane was hybridized with appropriate probes at 50ºC overnight using DIG EasyHyb (Roche Diagnostics GmbH, Mannheim, Germany). Membranes were washed twice for 5 min in 2 x SSC + 0.1% SDS at room temperature, twice (25 min) in 0.5 x SSC + 0.1% SDS, once (15 min) in 0,2 x SSC + 0.1% SDS, and once (10 min) in 0.1 x SSC + 0.1% SDS at 68ºC (high stringency washes). Chemiluminescent detection was carried out as described for the DNA gel blots.

Real-time RT-PCR

Total RNA was extracted from the leaves of six-week-old plants as described above and the mRNA was reverse transcribed into cDNA using the Omniscript® RT kit (Qiagen, Hilden, Germany) and oligo(dT) primers (Invitrogen, Carlsbad, USA). Specific primers were designed to amplify the *E. coli tsr*, *gcl*, *glc*D *glc*E and *glc*F genes and the rice *RAc1* (actin) gene as the internal control. Real-time RT-PCR was carried out using the Bio-Rad CFX96 sequence detector system (Foster City, California, USA) with 25-µl reactions performed in triplicate in 96-well optical reaction plates, containing 10 ng of cDNA, 1x iQ SYBR green supermix (BioRad, Hercules, CA) and 0.2 µM forward and reverse primers (**Table 4.2)**. The

cycling conditions consisted of a single incubation step at 95ºC for 5 min followed by 40 cycles of 95ºC for 10 s, 58ºC for 35 s and 72ºC for 15 s. Specificity was confirmed by product melt curve analysis over the temperature range 50–90ºC with fluorescence acquired after every 0.5ºC increase. The fluorescence threshold value and gene expression data were calculated with the BioRad CFX96TM software.

Table 4.2 Sequences of forward (F) and reverse (R) primers used for real-time RT-PCR.

Primer name	Primer sequence
GlcD_F	5'-CGATGAAATCACGACCTTCCATGCGG-3'
GlcD_R	5'-TGCACATGCATGGCACCAAATTCAGC-3'
GlcE_F	5'-ATGCGACCCGCTTTAGTGCCGG-3'
GlcE_R	ACATGCGACCGGGGTTAAACACGC-3'
GlcF_F	5'-GCACGCCAGCTGCGGGATAACA-3'
GlcF_R	5'-ATCCAGTGACGCACAGAGGTACGA-3'
Actin_rice_F	5'-GGAAGCTGCGGGTATCCATGAG-3'
Actin_rice_R	5'-CCTGTCAGCAATGCCAGGGAAC-3'
TSR_F	5'-TGCGCTGAACCTGCCAAACACTGC-3'
TSR_R	5'-CGAGGGCCAGTTTATGGTTAGCCA-3'
GCL_F	5'-TATCGGGTACCGGTAGTCGTGGA-3'
GCL_R	5'-CAGGTTTCAGTCGGTGCGTCCG-3'

Fluorescent localization of the transgene expression

Leaf samples were collected from 4-week-old seedlings expressing eGFP and fluorescence was detected using a Leica DFC 300 FX (excitation/emission at 475 and 510 nm, respectively) with a DM 4000 B lens. Fluorescence images were analyzed with Leica Application Suite v3.1.0.

Protein analysis

Proteins were extracted from ground wild-type and transgenic rice leaves in two volumes of extraction buffer (0.2 M Tris-HCl pH7.5, 5 mM EDTA, 0.1% Tween-20). The samples were vortexed for 1 h at 4ºC and centrifuged for 10 min at 13,800 rpm. The protein

concentration in the supernatant was measured using the Bradford method (1957) with BSA as the standard. For immunoblotting, 20 µg of protein were fractionated by SDS-PAGE (10% acrylamide) according to Laemmli (1970) and transferred to Immun-Blot PVDF membranes (BioRad, Hercules CA, USA). Membranes were blocked with 5% non-fat milk overnight and then washed three times for 15 min with PBS containing 0.1% Tween-20, and then twice for 15 min with PBS. A purified GlcD polyclonal antibody (prepared in chickens at the Institute for Biology I, RWTH-Aachen. Germany) was diluted 1:2000 with PBS and incubated with the membrane at room temperature for 2 h with agitation. The membranes were washed three times for 15 min with TBS containing 0.1% Tween-20 and twice for 15 min with TBS. The secondary antibody, a rabbit anti-chicken alkaline phosphatase conjugate (Sigma, St. Louis, USA) was diluted 1:20,000 in TBS and incubated with the membrane at room temperature for 2 h with agitation. The membranes were washed three times for 15 min with TBS and the signal was detected with Sigma-Fast reagent (Sigma, St. Louis, USA) for 4–5 min before submerging the membrane in water to stop the reaction.

Photosynthetic parameters

All photosynthetic parameters were measured with the Li-Cor Li-6400 Portable Photosynthesis system (Lincoln, Nebraska, USA) and a corresponding leaf chamber 6400-40 LCF (Leaf Chamber Fluorometer) connected to an infrared analyzer. The measurements were taken at the Institute of Plant Sciences, JülichResearch Center, Germany.

Oxygen inhibition andCO_2 compensation point

Oxygen inhibition and the CO_2 compensation point were measured at 27°C, a flow rate of 200 µmol/s (oxygen inhibition) or 1000 µmol/m^2/s (CO_2 compensation point) and a photon flux density (PFD) of 1000 µmol/m^2/s. The blue component of light was 10% and the humidity ~70%. Gas mixtures with 2% ($A_{2\%}$) and 21% ($A_{21\%}$) oxygen, 400 ppm CO_2 and nitrogen were used to measure oxygen inhibiton, and a 30-min adaptation phase allowing stomatal conductance to reach a constant value was introduced before measurement commenced (**Table 4.3**). The oxygen inhibition was calculated as follows:

$$O_2\text{-Inhibition}(\%) = \frac{A_{2\%}-A_{21\%}}{A_{2\%}} * 100\%$$

Table 4.3 Measurement of oxygen inhibition

	Data point recording	Mixture of gases
30 min	at 30 s	21% O_2
30 min	at 30 s	2% O_2

RESULTS

DNA analysis

We selected six from 30 lines of T0 plants and analyzed DNA blots using the 700-bp PCR product described above. We digested 13-µg genomic DNA samples with *Eco*RI, which cuts once within the plasmid backbone, and confirmed the integration of the genes encoding TSR, GCL, glcD, glcE and glcF in all six lines, with the exception of line 21, which lacked a copy of *glc*E (**Fig. 4.4**). We observed that each line displayed a unique banding pattern, confirming they originated from independent transformation events.

RNA analysis

RNA isolated from the leaves of T0 transgenic plants was analyzed by RNA blot analysis and quantitative real-time RT-PCR. The RNA blots showed that most lines expressed the *tsr* and *gcl* transcripts, albeit at different levels (**Fig. 4.5A**). For example, the highest levels of *tsr* expression were observed in lines 5 and 12, but line 5 expressed *gcl* at a low level. Lines 12 and 13 accumulated the highest levels of *gcl* mRNA. The expression of *glc*D, *glc*E and *glc*F was not detected by RNA blot analysis (data not shown). Quantitative real-time RT-PCR showed that all five transgenes were expressed at different levels in the different transgenic lines (**Fig. 4.5B**). The *tsr* and *gcl* transgenes were expressed at much higher levels than the three genes representing the GDH subunits, and the relative differences in *tsr* and *gcl* expression levels observed among the transgenic lines supported the RNA blot data, although were easier to quantify. Thus we detected a 10-fold difference in *tsr* mRNA levels between lines 5 and 12. Differences in the expression levels of the GDH subunit genes were also observed by quantitative real-time RT-PCR but the highest expression level was still 10 times lower than *tsr* and *gcl*.

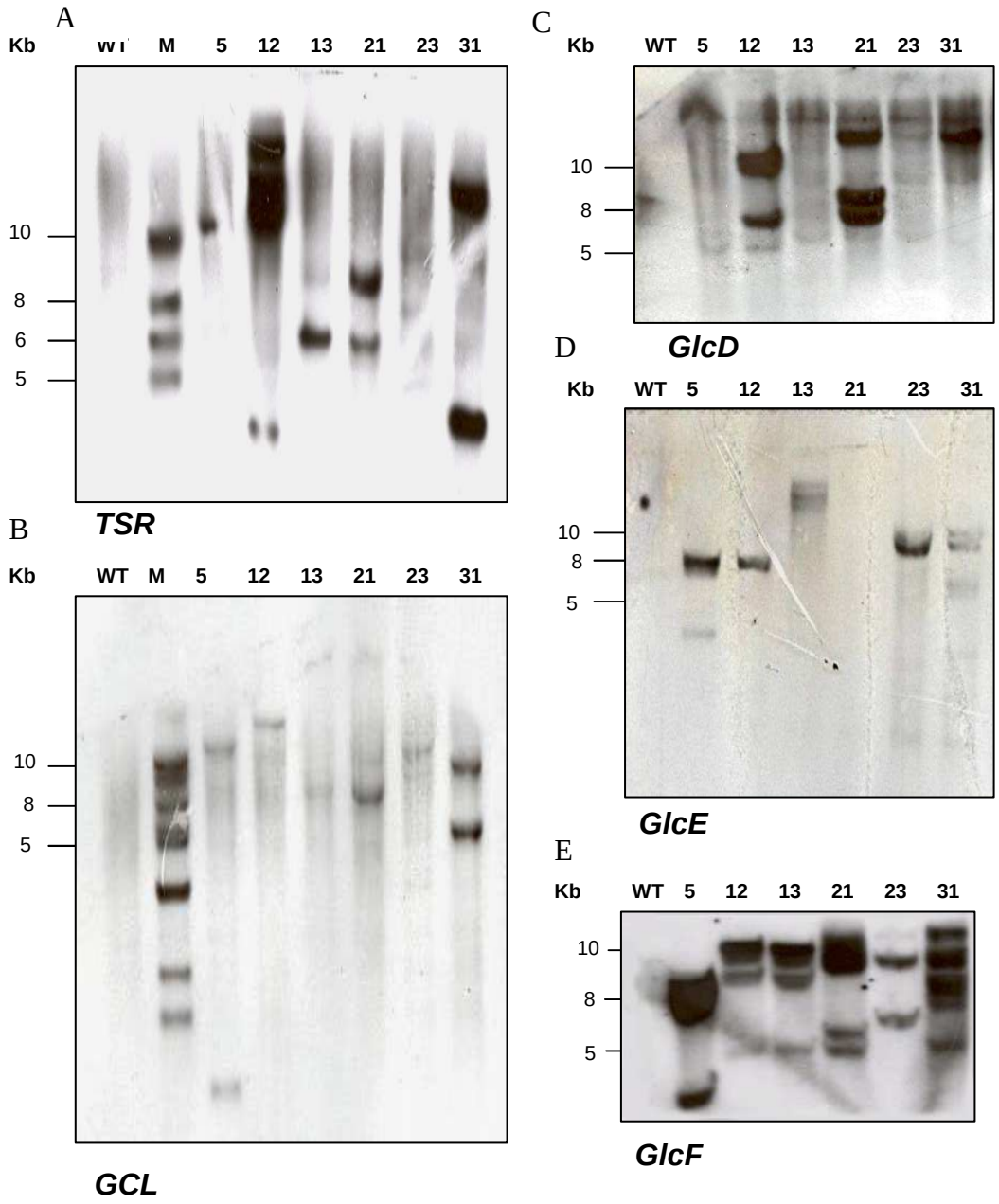

Fig 4.4 DNA blot analysis of T0 transgenic plants

Rice genomic DNA was digested with with *EcoR*I, separated by agarose gel electrophoresis, blotted onto nylon membranes and hybridized with specific probes for each transgene. **A**: *tsr* **B**: *gcl* **C**: *glc*D **D**: *glc*E **E**: *glc*F. WT = wild type plants, other lanes represent different transgenic lines, M = size markers.

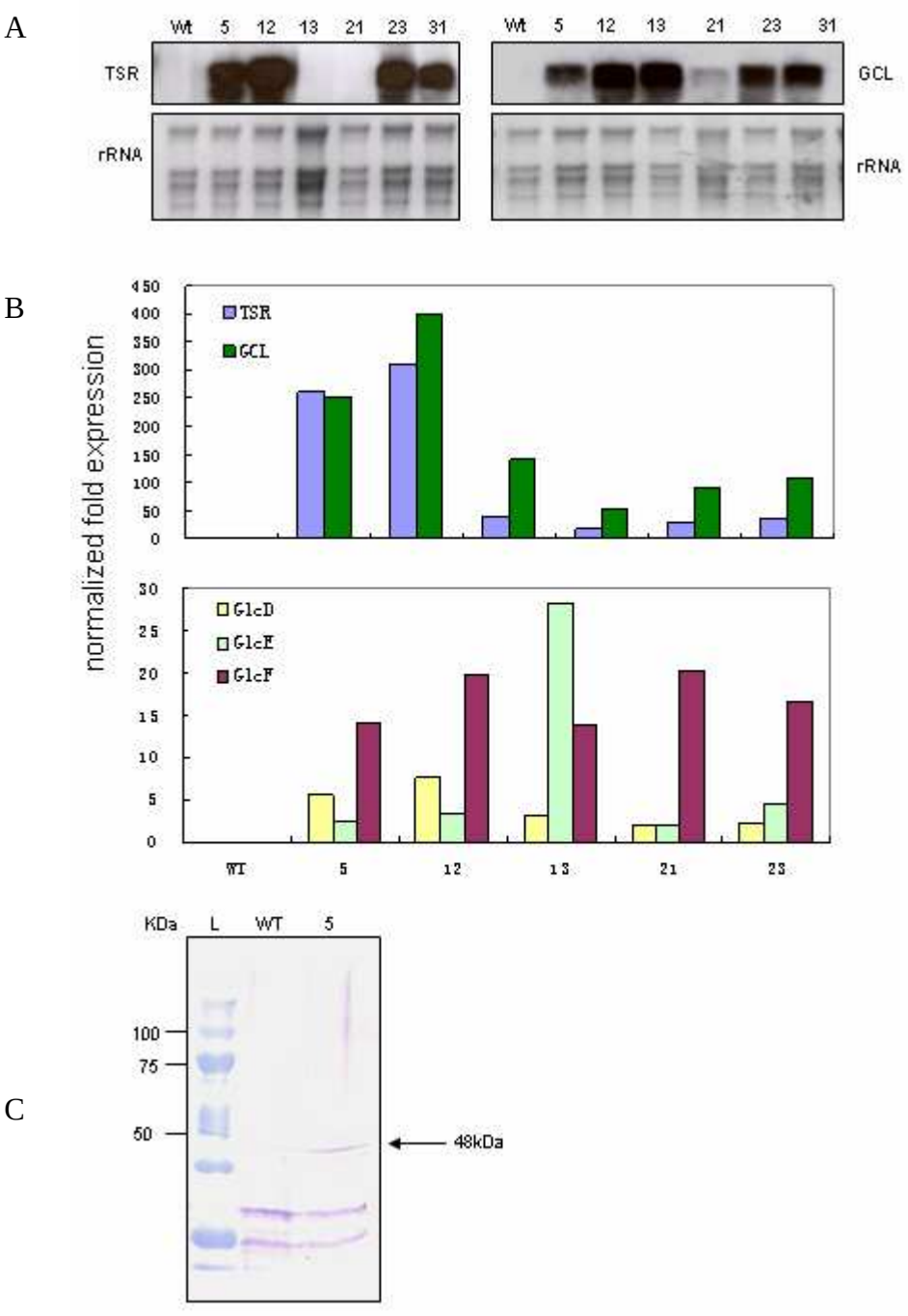

Fig. 4.5 mRNA and protein expression in T0 transgenic plants.

A. RNA blots of six independent T0 lines (5, 12, 13, 21, 23 and 31) showing *tsr* and *gcl* expression. **B:** Quantitative real-time RT-PCR. Relative expression levels were determined by taking triplicate measurements from four independent biological replicates. Columns show relative gene expression levels normalized against *β*-actin with standard errors of the mean. **C**: glcD protein accumulation in leaves from line 5. L = protein size ladder (glcD = 48 kDa).

Protein analysis

glcD protein levels in the leaves of T0 transgenic plants from line 5 were determined by western blot. The glcD protein (48 kDa) was detected in the transgenic leaves but not in the wild-type leaves (**Fig. 4.5C**). Antibodies for the other heterologous proteins were not available.

Fluorescence assay for enzyme localization

We generated 10 transgenic lines expressing the GAPDH-eGFP construct and took leaf samples from 4-week-old plants to analyze the fluorescence signal. Fluorescence was detected in the chloroplasts, showing that the promoter we used was functional and led to the import of heterologous proteins into the plastids (**Fig. 4.6**).

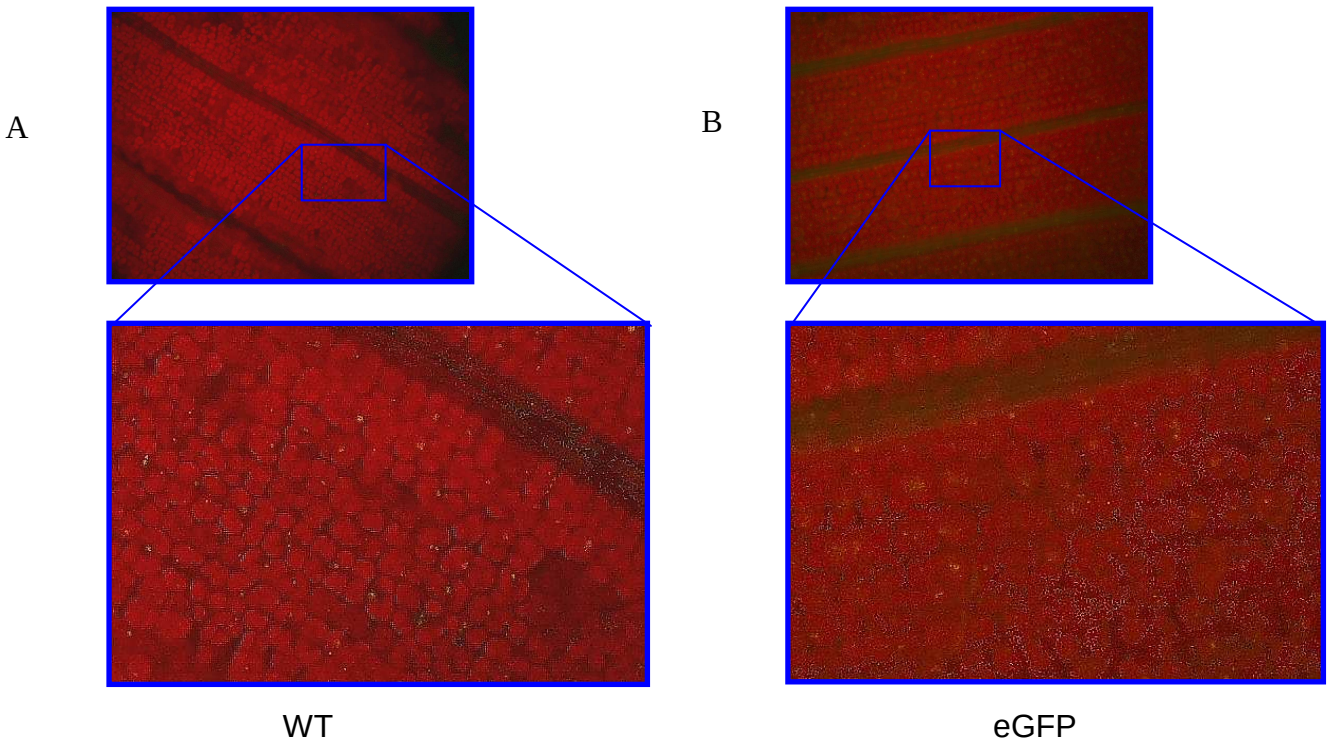

Fig. 4.6 Detection of fluorescence in the chloroplasts of transgenic plants.

Leaf samples were taken from 4-week-old wild-type plants and transgenic plants expressing eGFP under the control of the GAPDH promoter. Fluorescence was detected at 475 and 510 nm. **A:** No fluorescence was detected in the chloroplast or the veins of wild-type (WT) leaves. **B:** Fluorescence was detected in the chloroplasts and in the veins of transgenic leaves expressing eGFP under the control of the GAPDH promoter.

Oxygen inhibition and CO_2 compensation point

Oxygen inhibition and CO_2 compensation point analysis was carried out in 6-week-old T0 transgenic plants. Oxygen inhibition determines the ratio of CO_2 to O_2 in the vicinity of RuBisCO, and we found no significant differences between the wild-type and transgenic plants. The CO_2 compensation point is the CO_2 concentration at which photosynthetic CO_2 uptake is equal to respiratory CO_2 release, showing how efficiently plants use CO_2. We found no significant differences between the wild type and transgenic plants.

DISCUSSION

Photorespiration lowers photosynthetic efficiency, particularly in C3 plants (including major crops such as rice and wheat). The photorespiration rate in isolated bundle sheath strands from C4 plants under ambient atmospheric conditions have been estimated at 3–7% of the rate of CO_2 fixation (Farineau et al., 1984). However, Maroco et al. (1998) demonstrated that in C4-cycle limited mutants, atmospheric levels of O_2 (20 kPa) resulted in a more severe inhibition of photosynthesis because of the higher levels of photorespiration. Thus, photorespiration in C4 plants is considered insignificant because the RuBisCO oxygenase reaction is suppressed by concentrating CO_2 in the bundle sheath cells (Edwards et al., 2001). Several groups have attempted to establish components of the C4 CO_2 concentrating mechanism in Arabidopsis and other C3 plants in order to reduce photorespiration (Peterhansel et al., 2010 and references therein).

The purpose of our novel pathway is to convert glycolate into glycerate inside the chloroplast. The proposed mechanism will not completely switch off the photorespiratory pathway but it should compete with the existing photorespiratory pathway and reduce photorespiratory CO_2 loss by modifying the fate of the products of RuBisCO oxygenase activity. This is achieved by installing the bacterial glycolate catabolic pathway in the chloroplast. Bacteria such as *E. coli* can grow on glycolate as a sole carbon source (Pellicer et al., 1996; Lord et al., 1972). Three enzymes (GDH, GCL and TSR) are required to convert glycolate to glycerate, with the concomitant release of CO_2 (Lord, 1972; Pellicer et al., 1996; Chang et al., 1993; Gotto and Kornberg, 1961). This strategy has two major advantages compared to the endogenous photorespiratory pathway. First, the plastidal glycolate pathway does not release NH_3 that needs to be refixed (this consumes energy and reducing equivalents during conventional photorespiration) and second, the novel pathway does not consume ATP, an advantage compared to C4-like pathways where ATP is used to regenerate the CO_2 acceptor molecule.

We introduced the five genes encoding the three enzymes needed to reconstitute the *E. coli* glycolate pathway into transgenic rice plants plus a selectable marker gene conferring hygromycin resistance. DNA blot analysis verified the presence of the *tsr*, *gcl*, *glc*D, *glc*E and *glc*F genes in six of the 30 regenerated lines, and the integration patterns confirmed that the six lines originated from independent transformation events. We also measured the accumulation of the five corresponding transcripts by RNA blot and quantitative real-time RT-PCR, revealing significant differences among the six transgenic lines (**Figs. 4.5A,B**). Only the *tsr* and *gcl* transcripts could be detected in RNA blots, and the RT-PCR assay

showed they were were more than 10-fold more abundant than transcripts encoding the three GDH subunits (*glc*D, *glc*E and *glc*F). These differences could reflect differential regulation at the level of transcription level (the promoters used for transgene expression or the positon of integration) or post-transcription regulation (e.g. mRNA stability).

Western blot and fluorescent localization experiments were used to investiate the accumulation of the enzymes. Despite the low mRNA levels, we found that the glcD could be detected in western blots, but unfortunately antibodies against the other four enzymes were not available (**Fig. 4.5C**). The combination of the GAPDH promoter and the eGFP protein showed clearly that the enzymes were imported into the chloroplasts. Previous reports have shown that chloroplasts are able to metabolize glycolate and glyoxylate, and that CO_2 is produced during the course of these reactions (Goyal and Tolbert, 1996; Kisaki and Tolbert, 1969; Zelitch, 1972). This enhanced photosynthesis induces the accumulation of starch which in turn improves growth and yield (Leakey et al., 2009; Long et al., 2004).

We also attempted to quantify the photorespiratory and gas exchange activity of the transgenic plants by determining the oxygen inhibition and CO_2 compensation point in the mithocondrial glycine decarboxylase reaction. However, we detected no differences between wild type plants and those containing the heterologous glycolate pathway. This might reflect the low-level expression of the three GDH subunits as suggested by the RT-PCR experiments, which could be addressed by expressing these genes under the control of the GAPDH promoter. We confirmed that the GADPH promoter achieves the strong expression of eGFP, which is imported into the chloroplast. Further transformation experiments will be required to confirm the enhanced expression of these genes, and we will also measure the carbohydrate content and chlorophyll fluorescence in leaves.

We have succeeded in establishing a novel pathway with the potential to increase the concentration of CO_2 in the vicinity of RuBisCO in transgenic rice plants, thus reducing CO_2 loss resulting from photorespiration in C3 plants. The novel pathway involves the metabolism of glycolate produced in the chloroplast during photorespiration and competes with the existing photorespiratory pathway to reduce photorespiratory CO_2 loss by controlling the fate of the products generated by RuBisCO oxygenase activity. Future work will focus on the creation of transgenic plants with the five transgenes expressed using the GAPDH promoter and the analysis of transgene expression, protein accumulation, enzyme activity and photosynthetic efficiency in those plants.

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CHAPTER IV

The potential impact of plant biotechnology on the Millennium Development Goals

ABSTRACT:

The eight Millennium Development Goals (MDGs) are international development targets for the year 2015 that aim to achieve relative improvements in the standards of health, socioeconomic status and education in the world's poorest countries. Many of the challenges addressed by the MDGs reflect the direct or indirect consequences of subsistence agriculture in the developing world, thus plant biotechnology has an important role to play in helping to achieve MDG targets. In this chapter, I discuss each of the MDGs in turn, provide examples to show how plant biotechnology may be able to accelerate progress towards the stated MDG objectives, and offer my opinion on the likelihood that technology will be implemented. In combination with other strategies, plant biotechnology can make a contribution towards sustainable development in the future although the extent to which progress can be made in today's political climate depends on how current barriers to adoption are addressed.

Introduction

The Millennium Development Goals (MDGs) are a set of eight ambitious international development targets for the year 2015, which were agreed by 192 members of the United Nations as well as numerous non-governmental organizations (NGOs) at the Millennium Summit in 2000 (**Talbe 5.1**). The aim of the MDGs is to improve standards of health, socioeconomic status and education by tackling poverty, hunger and disease, increasing educational opportunities and creating a global development partnership (UN, 2010a).

Goal 1		Eradicate extreme poverty and hunger
	Target 1A:	Halve the proportion of people living on less than $1 a day
	Target 1B	Achieve Decent Employment for Women, Men, and Young People
	Target 1C	Halve the proportion of people who suffer from hunger
Goal 2		Achieve universal primary education
	Target 2A	By 2015, all children can complete a full course of primary schooling, girls and boys
Goal 3		Promote gender equality and empower women
	Target 3A	Eliminate gender disparity in primary and secondary education preferably by 2005, and at all levels by 2015
Goal 4		Reduce child mortality rate
	Target 4A	Reduce by two-thirds, between 1990 and 2015, the under-five mortality rate
Goal 5		Improve maternal health
	Target 5A	Reduce by three quarters, between 1990 and 2015, the maternal mortality ratio
	Target 5B	Achieve, by 2015, universal access to reproductive health
Goal 6		Combat HIV/AIDS, malaria, and other diseases
	Target 6A	Have halted by 2015 and begun to reverse the spread of HIV/AIDS
	Target 6B	Achieve, by 2010, universal access to treatment for HIV/AIDS for all those who need it
	Target 6C	Have halted by 2015 and begun to reverse the incidence of malaria and other major diseases
Goal 7		Ensure environmental sustainability
	Target 7A	Integrate the principles of sustainable development into country policies and programs; reverse loss of environmental resources
	Target 7B	Reduce biodiversity loss, achieving, by 2010, a significant reduction in the rate of loss

	Target 7C	Halve, by 2015, the proportion of people without sustainable access to safe drinking water and basic sanitation
	Target 7D	By 2020, to have achieved a significant improvement in the lives of at least 100 million slum-dwellers
Goal 8		Develop a global partnership for development
	Target 8A	Develop further an open, rule-based, predictable, non-discriminatory trading and financial system
	Target 8B	Address the Special Needs of the Least Developed Countries (LDC)
	Target 8C	Address the special needs of landlocked developing countries and small island developing States
	Target 8D	Deal comprehensively with the debt problems of developing countries through national and international measures in order to make debt sustainable in the long term
	Target 8E	In co-operation with pharmaceutical companies, provide access to affordable, essential drugs in developing countries
	Target 8F	In co-operation with the private sector, make available the benefits of new technologies, especially information and communications

Talbe 5.1 The Millennium Development Goals in full (UN 2010a)

The program is now more than two-thirds complete, and progress towards the goals has been patchy, with significant improvements in the rising economies such as China and India, but little progress in some other countries, particularly in sub-Saharan Africa (UN 2010b). China has almost halved its poverty-stricken population over the last decade and is well on the way to realizing all the MDGs by 2015. In contrast, the major target countries in sub-Saharan Africa have reduced the level of poverty by less than 1% and seem unlikely to meet any of the MDGs (UN, 2010b).

The success of China and India has much to do with their economic growth, but growth is not a prerequisite for the achievement of MDG targets. Bangladesh, for example, has shown that progress can be made with little or no growth simply by adopting and rolling out inexpensive solutions on a large scale, including national vaccination campaigns and nutritional supplementation programs (UNICEF, 2010). Tying the MDGs to expensive solutions that in turn depend on either economic growth or donations in aid cannot be maintained indefinitely, and it is therefore imperative that inexpensive but scalable solutions are deployed as rapidly as possible to provide a sustainable basis for development. In this context, plant biotechnology has a role to play by providing healthier and more nutritious crops and also new platforms to produce inexpensive vaccines and drugs. However, the impact of plant biotechnology is not limited to augmenting or replacing expensive

intervention programs. Biotechnology can create plants that reduce the impact of weeds, insect pests, diseases and harsh environments, providing a basis not only for the reduction of hunger through more successful subsistence agriculture but also the stimulation of economic prosperity by providing higher yields of better-quality crops that increase the wealth as well as the health and wellbeing of poor agricultural workers. Although plant biotechnology is not a panacea for the world's socioeconomic woes, it is already being used in numerous ways to address the Millennium Development Goals. There remain significant barriers to adoption that are largely political in character, with little or no rational scientific basis. Overcoming these political hurdles in the short term is a more challenging objective than achieving technological progress (Farre et al., 2009).

MDG1: Eradicate extreme poverty and hunger

Overview

The number of people living in hunger currently oscillates around one billion, which represents nearly one in every seven people in the world (FAO, 2009b). Hunger can be defined as an insufficient daily intake of energy (the average requirement being 2,000 kcal per day), and the figure of one billion therefore excludes those who receive sufficient calories but are nevertheless malnourished due to the absence of essential vitamins and minerals. The world's hungry populations have limited access to food but not because of insufficient production. Indeed, there is plenty of food, enough to support a much higher global population than exists today, but there is inadequate food distribution, and the world's poorest people cannot afford to purchase the food that is available. Hunger, at least at present, is therefore caused by poverty and poor distribution rather than insufficient global production (DFID, 2010).

The World Bank defines extreme poverty as living on less than US $1.25 per day. MDG1 is therefore expressed in the form of three objectives, the first to reduce the number of people living in poverty by 50%, the second to improve employment opportunities (particularly for women and young people) and the third to reduce the level of hunger (**Fig. 5.1**). These are interlinked objectives, and they need to be tackled simultaneously to see improvements in all the three. Progress towards MDG1 is also important to ensure progress towards most of the other MDGs, particularly those that aim to reduce the burden of disease and improve education. Poverty and hunger both lead to poor health and lost opportunity, creating a vicious cycle in which people are forced to endure a monotonous existence that focuses solely on survival (Islam, 2008).

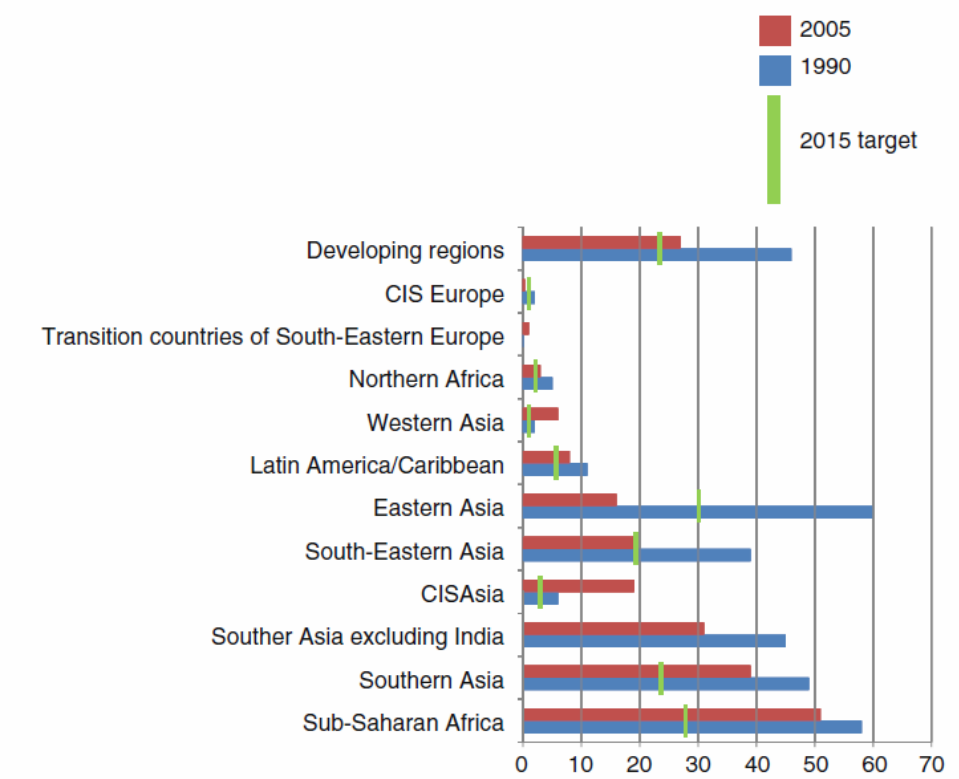

Fig. 5.1 Proportion of people living on less than $US1.25/day by region, 1990 and 2005, compared to 2015 MDG targets. Source: UN (2010).

Although urban poverty is a growing problem, most of the world's poorest people are rural dwellers and depend on subsistence agriculture (Fan et al., 2005). Strategies to address extreme poverty in rural areas should therefore focus on improving agricultural productivity to allow the poor to produce enough food to survive, the remainder being marketed and generating income. Short-term solutions such as providing food aid will not provide long-term and sustainable progress towards MDG1. Instead, there needs to be a drastic shift in socioeconomic policy focusing on agricultural and commercial development, with modern seed varieties playing an important role because they generate the most vigorous crops (Sanchez, 2009). Most subsistence calories are obtained from cereal crops, particularly rice and maize. These two crops are the staple diet for more than 75% of the human population (FAO, 2009a). Maize also provides much of the fodder for livestock in the countries where it is grown, including both developed countries such as the US, and many countries in Africa. The short-term objective should therefore be to reduce the yield gap in cereal crops (the gap between potential yields and actual yields) to reduce hunger, improve health and create

economic prosperity. In the longer term, it will be necessary to apply the same solutions to diverse fruit and vegetable crops as well as cash crops such as cotton, tobacco and coffee.

The role of plant biotechnology

Plant biotechnology can help to achieve MDG1 through the deployment of high-yielding genetically engineered varieties that are resistant to weeds, insect pests and diseases caused by viruses, bacteria and fungi, and that are able to withstand harsh environmental constraints such as drought (Farre et al., 2009). Weeds, insect pests and pathogens can reduce yields either by adversely affecting plant growth and development, or by consuming and/or spoiling the products of food crops in the field or in storage. Globally, this reduces crop yields by up to 30%, but the impact in developing countries can be much higher because the climatic conditions favor the survival and breeding of insect pests and disease vectors. After pests and diseases, unfavorable environmental conditions such as drought, poor soil quality and (in Asia) flooding also have a devastating effect. The development of crops with an inbuilt capacity to withstand these effects could help to stabilize crop production and hence significantly contribute to food security and economic prosperity (Christou and Twyman, 2004).

Weeds

Weed management is the largest single input into agriculture in both industrialized and developing countries. However, whereas weed management in the developed world is highly mechanized and has benefited extensively from the technological advantages provided by genetically engineered herbicide-resistant crops and broad-spectrum herbicides, developing country agriculture currently relies on an army of laborers, mostly women, who tend the land and spend long hours removing weeds manually (Akobundu, 1991).

Two issues compound the impact of weeds in developing countries—the lack of resources to adopt technological solutions that are taken for granted in the developed world, and the disinterest shown by research organizations in the west to tackle weed species that are specific to Africa and Asia (Gressel et al., 2004). In Africa, maize and sorghum crops are often infested by *Striga*, a genus of parasitic flowering plants that is very difficult to control once established because it builds up a resilient seed bank in the soil (Parker, 2009). *Striga* represents such a severe constraint to maize production that controlling this weed is seen as the key to resolving Africa's dependence on subsistence agriculture (Hearne, 2009). There has been some recent success in the conventional breeding of resistant sorghum varieties by

combining traits that make the sorghum plants poor inducers of *Striga* germination and poor hosts for colonization (Ejeta et al., 2007), but it has not been possible to achieve the same goals in maize. Progress towards the selective control of *Striga* in maize has been made through mutation and conventional breeding for imazapyr resistance (Kanampiu et al., 2002), which has been implemented as StrigAway technology co-developed by CIMMYT, BASF and the Weizmann Institute (Mataruka et al., 2010). Although this requires the application of herbicides, it is not necessary for farmers to spray their crops because the herbicide can be applied directly to the seed. A complementary biotechnology solution is to introduce herbicide resistance directly into maize. Glyphosate-resistant transgenic maize has been adopted in South Africa, which allows one worker with a backpack sprayer to control weeds over several hectares. Although South Africa does not suffer from *Striga* infestations to the same extent as other countries in the region, the use of glyphosate resistance for general weed control shows that it could also be applied to tackle *Striga* infestations (Gressel and Valverde, 2009).

The industrialization of rice cultivation in Asia has also generated an emerging problem with weeds. The switch from transplanting rice plantlets into flooded paddies (weed control by water) to direct seeding (weed control by herbicides) has led to the emergence of herbicide-resistant feral rice species (Valverde, 2005) and *Echinochloa* species that were formerly quite easy to control with selective herbicides (Valverde and Itoh, 2001). Here, transgenic strategies need to be applied with care because the rapid evolution of herbicide resistance has already been documented, presenting a likelihood that transgenes conferring herbicide resistance could introgress into weedy rice species and eliminate the selective difference between weedy and cultivated rice (Gealy, 2005).

Insect pests, insect-borne diseases and the consequences of pest infestations

Many of our crop plants are attacked by insect pests, and devastating losses occur throughout the world due to pest infestations either in the field or in stored products. In the developing world, about half of all crop production is thought to be lost to insects, 15% of these losses occurring due to post-harvest consumption and spoilage (Christou et al., 2006). Insects not only cause direct yield losses by damaging and consuming plants but also act as vectors for many viral diseases, and the damage they inflict encourages bacterial and fungal infections, the latter resulting in contamination with mycotoxins.

A good example of the positive impact of plant biotechnology is the development of pest-resistant crops expressing insecticidal toxin genes from the soil bacterium *Bacillus*

thuringiensis (Bt). Different strains of Bt produce different toxins which are both potent and highly specific against narrow taxonomic groups of insects, making them harmless to mammals and to beneficial insects (Sanahuja et al., 2011). In developing countries, Bt crops have been extraordinarily successful and beneficial, increasing yields, reducing the use of pesticides and the fuel needed for spraying, and improving the economic status of farmers while at the same time preserving biodiversity (James, 2010; Brookes and Barfoot, 2010).

The adoption of Bt crops in India provides strong support for the role of plant biotechnology in the progress towards MDG1. In 2009, more than 5.5 million small-scale farmers planted a total of 8.4 million hectares of Bt cotton, representing nearly 90% of the national total (James, 2010). More than half of these crops contained multiple Bt genes providing resistance against different pests, and for the first time locally-developed varieties were planted instead of varieties developed in the US, therefore keeping all the agricultural profits within India's economy rather than servicing foreign royalty payments. India is now the world's largest cotton exporter (having been a net importer at the beginning of the decade), and it is estimated that rural farmers have benefitted from the technology through yield improvements to a total amount exceeding US $5 billion. Net yields per hectare have doubled in 10 years while agrochemical inputs have halved (APCoAB, 2006; Manjunath, 2008). The widespread adoption of Bt cotton in India has also helped to address the concerns of critics, who highlight the potential for resistant pests to evolve under intense selection pressure. Against these expectations, the first generation of Bt crops has maintained efficacy against nearly all targeted pest populations for more than a decade (Bourguet, 2004). The scarcity of resistant populations despite the lack of integrated pest management suggests that resistance may attract a fitness penalty in the absence of the Bt toxins (Sanchis and Bourguet, 2008). Resistant populations have appeared for a small number of pests, such as pink bollworm (*Pectinophora gossypiella*) which has evolved resistance to Bollgard I cotton (expressing the Cry1Ac toxin) in the Amreli, Bhavnagar, Junagarh and Rajkot areas of Gujarat. Resistance is anticipated because each toxin binds to a specific receptor in the brush border of midgut epithelial cells, and point mutations affecting toxin/receptor interactions would be strongly favored under selection. However, no resistance has been observed in fields growing the Bollgard II variety, which expresses the Cry1Ac and Cry2Ab toxins simultaneously (Monsanto, 2010). These toxins bind different receptors, and the likelihood of mutations occurring in genes for both receptors is much lower than the likelihood of a single mutation, so this strategy of 'pyramiding' resistance genes (i.e. expressing multiple toxins with different targets in the pest) is a very powerful approach to prevent the evolution of resistant pest

populations.

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

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

premium in some developing countries, providing another impetus to lift farmers out of poverty (Yorobe, 2004).

Drought

Agriculture is highly dependent on water, and access to fresh water is therefore as important for agricultural productivity as the quality of the seeds and the soil. With fresh water resources dwindling, the impact of drought can be devastating on crops, and the use of biotechnology to develop varieties that require less water and that are tolerant to drought conditions is now becoming as important as pest and disease resistance.

Drought stress in crops induces a number of response pathways including protection against reactive oxygen species, the active export of sodium ions and the synthesis of small molecules called osmoprotectants that increase the osmotic potential of cells causing them to retain water. Efforts focusing on direct responses such as the introduction of transgenes encoding antioxidant enzymes, enzymes that synthesize antioxidant compounds, genes encoding sodium transporters, and enzymes that synthesize osmoprotectants have resulted in many laboratory strains of transgenic plants that survive in concentrated salt solutions (Bhatnagar-Mathur et al., 2008). Other researchers have targeted the genes that regulate stress pathways (receptors, intracellular signaling molecules and transcription factors) which may be more useful because they, in turn, regulate a large number of protective genes (Bhatnagar-Mathur et al., 2008).

A drought-tolerant variety of maize co-developed by Monsanto and BASF is to be launched in the US in 2012 (James, 2010). This expresses a stress-responsive transcriptional regulator that increases yields by up to 35% under water limiting conditions (Nelson et al., 2007). Stress-responsive transcription factors are one of three key classes of regulators that have been used to develop drought-tolerant varieties, the others being proteins that control signaling and post-translational modification in stress pathways, and regulators of osmoprotectant synthesis and metabolism such as the *Bacillus subtilis* chaperone CspB which is expressed in another drought-tolerant variety developed by Monsanto (Castiglioni et al., 2008). Although Texas in the US suffered its worst drought for 50 years in 2009 (with estimated losses of US $3.5 billion, approximately one-sixth of the agriculture market value), the situation in Africa and parts of Asia is much worse, with regular harvest failures due to insufficient rainfall and the absence of an irrigation infrastructure. Monsanto is part of WEMA (Water Efficient Maize program for Africa) which also includes the Gates Foundation, the Howard Buffet Foundation, CIMMYT and several stakeholders in

sub-Saharan Africa, and it is committed to donating a royalty-free drought-tolerant maize variety for humanitarian use by 2017 (Mataruka et al., 2010). Under moderate drought conditions in Africa the yield expected from the tolerant variety should provide an additional 12 million tons of maize, providing food for over 20 million people who would otherwise depend on food given in aid.

MDG2 and MDG3: Achieve universal primary education, promote gender equality and empower women

Overview

Many developing countries are close to providing universal primary education, with the total number of primary-age children not attending school falling from 115 million in 2002 to 72 million in 2007, even with growing populations. Again, however, the picture is less encouraging in sub-Saharan Africa and South Asia, with 41 and 31.5 million primary-age children out of school, respectively (UN, 2010a). In all developing regions, children in rural areas are twice as likely to be out of school as children living in urban areas and children with disabilities and special needs are the least likely of all to receive a school education **(Fig. 5.2a**).

The underlying reasons for the trends discussed above reflect the direct costs of sending children to school, as well as the impact of losing potential workers on family farms. Achieving universal education therefore requires a shift in attitudes as well as the provision of educational opportunities, and also requires that children are healthy, adequately fed and well nourished. Abolishing school fees and subsidizing costs (e.g. for textbooks, uniforms and transportation) will make primary education more affordable for parents. Programs that link education, health and nutrition, such as school meal programs and social protection measures are necessary to achieve these aims, ultimately leading back to effective governance (Sachs and McArthur, 2005). It is also important to encourage parental involvement in achieving MDG2.

Girls are less likely to be educated than boys throughout the developing world, and the prevailing culture is male dominated, a trend exacerbated in rural areas (**Fig. 5.2b,c**). Therefore, the level of illiteracy is higher in women, they are less likely to be employed, they tend to fill low-paid positions if they are employed and they are often excluded from positions of authority (UN, 2010a). Women overall suffer more from poverty and are often completely dependent on men financially. Furthermore, women are more likely to suffer from poor health and malnutrition, and more women than men in developing countries are HIV positive

(UNAIDS, 2008).

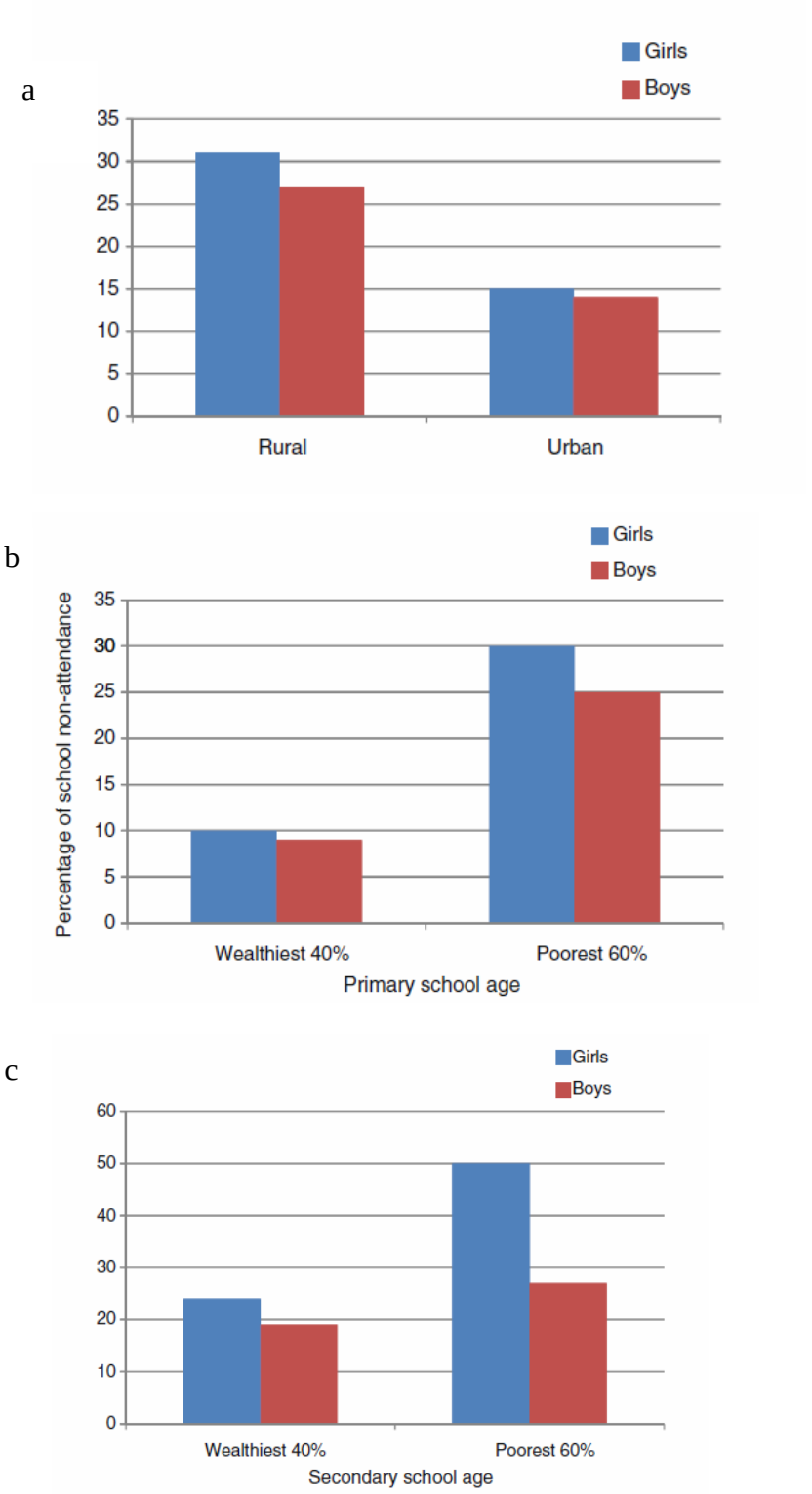

Fig 5.2 Percentage of out-of-school children by gender, in 42 countries, up to 2008. (a) All children, by area of residence (rural or urban). (b) Primary age children, by household wealth. (c) Secondary age children, by household wealth. Source: UN (2010).

As with MDG2, a change in attitude is important to achieve MDG3, focusing on the rights of women to play an equal role to men in society. Overlapping with MDG2, one of the

objectives of MDG3 is to strengthen opportunities for the education of girls and women, while meeting the above-mentioned commitments to universal primary education. Other objectives are to guarantee women's sexual and reproductive health rights, their property and inheritance rights, and their access to infrastructure; to strive for gender equality in employment, increase women's influence in local and national governance, and combat domestic violence.

The role of plant biotechnology

Plant biotechnology cannot directly contribute to progress in either MDG2 or MDG3, but it can help by making numerous indirect impacts to improve health, wealth and wellbeing, and by providing educational opportunities. The role of plant biotechnology in the achievement of MDG1 as discussed above is pertinent because this reduces hunger and poverty. Many children from rural communities do not attend school because their parents cannot afford to send them, so increasing the wealth-generating potential of rural farmers by providing them with better crops is one way to increase the proportion of children going to school. Furthermore, transgenic crops make tillage, pesticide spraying and weeding unnecessary and release women and children who would otherwise be forced to work on the land, allowing them the opportunity for education (Gressel, 2009).

It is often women that carry out the laborious agricultural work such as soil preparation, planting, weeding and harvesting, either for subsistence farming (as unpaid family workers) or as a service without financial security or social benefits, so the reduction in labor requirements has a disproportionately positive impact on women and girls, simultaneously addressing MDGs 2 and 3.

The widespread adoption of Bt cotton in India is one of the primary reasons for the dramatic increase in school attendance by primary-age children over the last decade, but its impact on girls and women has been even more remarkable (Subramanian et al., 2010; Subramaniam and Qaim, 2010). Comparing Bt and conventional cotton, the average wage per hectare increased by US $40, with women experiencing a greater income gain (55% average), equivalent to 424 million additional days of employment for women (**Fig. 5.3**). The potential role of plant biotechnology in reducing the nutritional and health burden on women is discussed under MDGs 4 and 5 below.

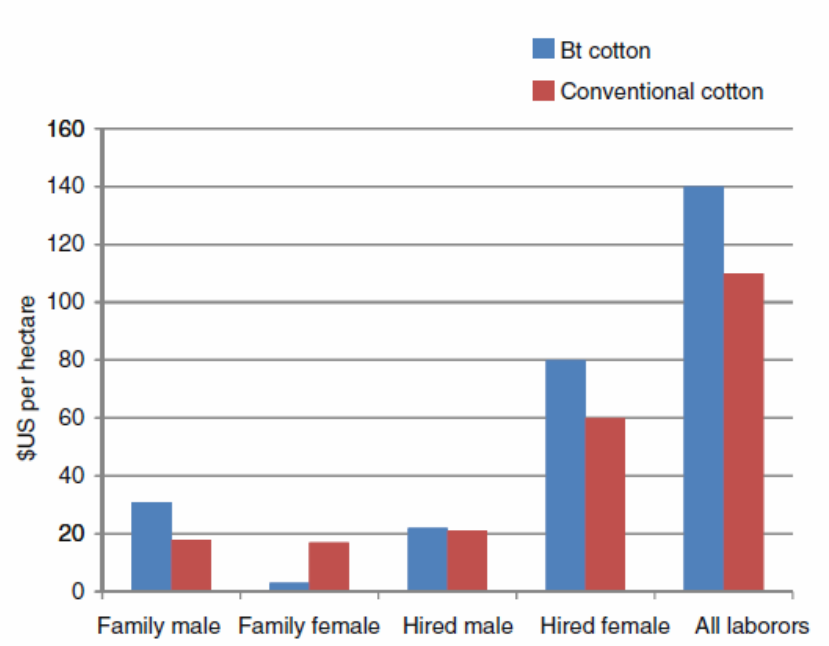

Fig 5.3 Remuneration ($US/ha) from labor on farms with Bt and conventional cotton in rural India (Subramanian et al., 2010).

MDGs 4 and 5: Reduce child mortality and improve maternal health

Overview

Nearly 9 million children under the age of 5 years die every year, 40% during their first month of life, and most of these deaths are concentrated in the world's poorest countries in sub-Saharan Africa and South Asia (UN, 2010a). The deaths are predominantly caused by diseases that could be prevented or treated, and the mortality rate is exacerbated by poor maternal health usually reflecting underlying chronic malnutrition. MDG4 aspires to reduce the infant mortality rate in developing countries by two thirds based on the number of deaths before the age of 5 years per 1000 live births, specifically targeting the number of deaths before first birthday and specifically mentioning the fight against measles (**Fig. 5.4**).

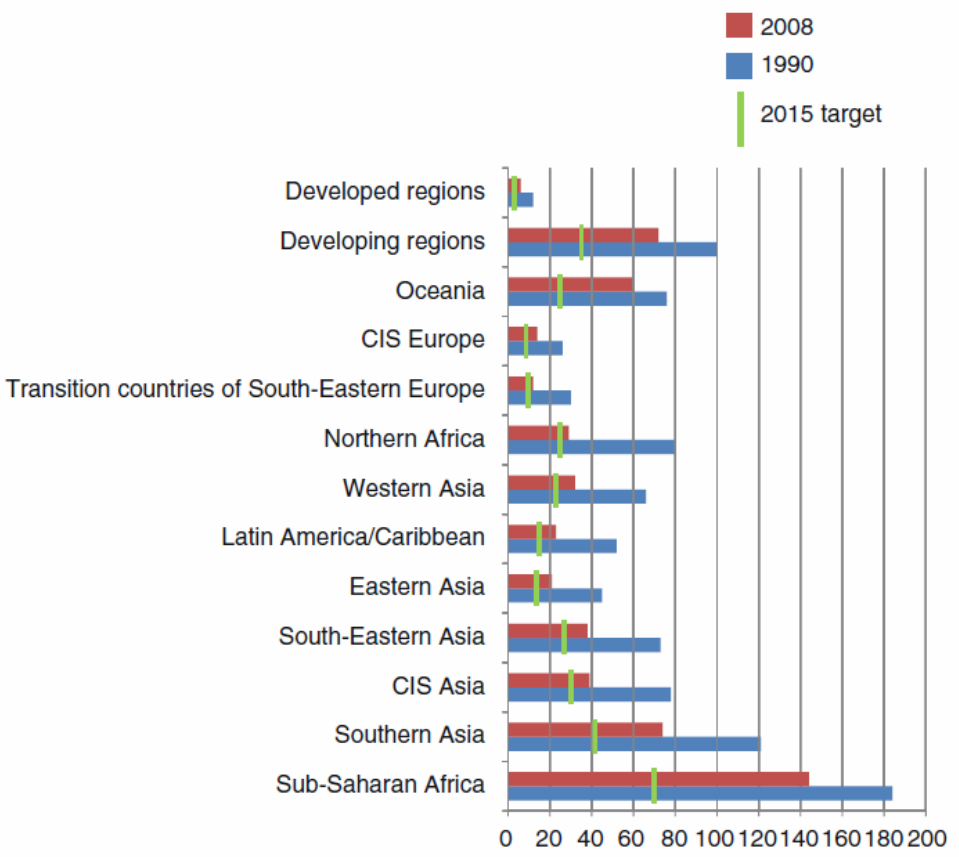

Fig 5.4 Under-five mortality rate per 1000 live births, by region, 1990 and 2008, compared to 2015 MDG targets. Source: UN (2010).

The causes of infant mortality are diverse, but the leading factors are pneumonia, diarrhea, malaria and HIV/AIDS, which together accounted for 43% of all infant deaths worldwide in 2008. I defer the discussion of HIV/AIDS and malaria to MDG6, which specifically focuses on those diseases. Pneumonia and diarrhea together account for a third of all under-five deaths, and most of these lives could be saved through low-cost prevention and treatment measures, including antibiotics for acute respiratory infections, oral rehydration for diarrhea, vaccination against pneumococcal pneumonia and rotavirus, and nutritional supplements. Proper nutrition is essential to fight disease effectively because malnutrition weakens the immune system and reduces resistance to diseases. Iron, zinc and vitamin A deficiencies have the severest impact on child morbidity and mortality, and these are also the most prevalent in developing countries because staple crops such as rice and white maize are naturally deficient in these compounds (Freedman et al., 2005).

There has been strong progress towards MDG4 in some parts of the world, such that the overall infant mortality rate fell from 12.4 million children per year in 1990 to 8.8 million

in 2008, a drop of 28% (UN, 2009; 2010a). The greatest improvements have been seen in North Africa, Eastern and Western Asia, Latin America and the Caribbean, with substantial progress in some of the world's poorest countries (Bangladesh, Bolivia, Eritrea, Ethiopia, Lao People's Democratic Republic, Malawi, Mongolia, Mozambique, Nepal and Niger). However, the rest of sub-Saharan Africa has fallen well behind and now accounts for 50% of all infant deaths. Also, 1 in 14 children still die before the age of five in South Asia.

As stated above, neonatal and under-five mortality is influenced by maternal health, i.e. the health of women during pregnancy, childbirth and the postpartum period (especially during breast feeding). Approximately one in six women die in pregnancy or childbirth in developing countries, compared to 1 in 30,000 in Europe (WHO/ UNICEF, 2010). Over half of the deaths result from hemorrhage and hypertension, 20% involve comorbidity factors such as malaria and HIV, and 10% result from complications due to the lack of skilled midwives. MDG4 aims to reduce maternal deaths by 75% and increase the availability of skilled medical personnel attending childbirth. Progress towards MDG4 has been rapid in some countries (particularly Bolivia, China, Ecuador and Egypt), but progress in others has been poor, with more than 50% of all maternal deaths now concentrated in six countries (Afghanistan, Democratic Republic of Congo, Ethiopia, India, Nigeria and Pakistan). Global rates are listed in **Table 5.1**, with southern sub-Saharan Africa performing worst: the maternal mortality ratio (the ratio of the number of maternal deaths per 100,000 live births) in that region increased from 171 in 1990 to 381 in 2008 (UN, 2009).

	1990	2000	2008
Asia-Pacific	14 (13-15)	10 (9-11)	8 (8-9)
Asia, central	72 (68-77)	60 (56-64)	48 (45-52)
Asia, east	86 (76-98)	55 (48-62)	40 (35-46)
Asia, south	560 (391-794)	402 (293-555)	323 (232-444)
Asia, southeast	248 (187-337)	212 (155-293)	152 (112-212)
Australasia	7 (6-8)	6 (5-7)	6 (5-7)
Caribbean	348 (234-518)	323 (218-483)	254 (168-372)
Europe, central	34 (31–37)	18 (17–20)	13 (12–14)
Europe, eastern	43 (39–48)	41 (37–45)	32 (29–35)
Europe, western	10 (10–11)	8 (8–9)	7 (7–8)

Latin America, Andean	229 (176–295)	156 (116–205)	103 (77–134)
Latin America, central	85 (77–94)	70 (64–78)	57 (51–63)
Latin America, southern	54 (49–60)	44 (39–49)	41 (36–45)
Latin America, tropical	113 (66–184)	71 (47–107)	57 (37–87)
North Africa/Middle East	183 (154–218)	111 (92–135)	76 (61–94)
North America, high income	11 (10–12)	13 (11–15)	16 (14–18)
Oceania	416 (252–649)	329 (202–518)	279 (174–434)
Sub-Saharan Africa, central	732 (488–1101)	770 (535–1108)	586 (392–839)
Sub-Saharan Africa, east	690 (574–842)	776 (639–948)	508 (430–610)
Sub-Saharan Africa, southern	171 (132–222)	373 (280–499)	381 (288–496)
Sub-Saharan Africa, west	582 (485–709)	742 (608–915)	629 (508–787)

Table 5.2 Maternal mortality ratio (uncertainty interval) per 100,000 live births by region and country (Hogan et al., 2010).

The role of plant biotechnology: improved nutrition

Malnutrition contributes to poor maternal health and (both directly and indirectly) to poor childhood health. Various strategies have been proposed to deal with micronutrient deficiencies including the provision of mineral supplements, the fortification of processed food, the biofortification of crop plants at source with mineral-rich fertilizers, the implementation of breeding programs to generate mineral-rich varieties of staple crops, and the use of biotechnology for nutritional improvement (Gomez-Galera et al., 2010). Among these approaches, only conventional breeding and genetic engineering provide germplasm as a permanent and sustainable resource, and only genetic engineering allows the introduction of genes from any source directly into local varieties.

Perhaps, the best example of genetic engineering for nutrient enhancement in a developing country context is Golden Rice, which is enriched for β-carotene (pro-vitamin A). This compound can be converted into retinal (the major functional form of vitamin A) by humans and other herbivorous/omnivorous mammals. Non-engineered cereal grains including rice and maize are poor sources of β-carotene, and polished rice grains contain no β-carotene at all. Vitamin A is required for vision and a healthy immune system. Vitamin A deficiency affects 127 million people in developing countries, including 25% of pre-school children, causing more than half a million cases of permanent blindness in children and 2.2 million deaths per year (UNICEF, 2006). Therefore, many researchers have attempted to elevate

β-carotene levels in staple cereals by introducing the corresponding metabolic pathway. The first significant advance was 'Golden Rice 1, where the entire *β*-carotene biosynthetic pathway was reconstructed in the endosperm by expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase and lycopene *β*-cyclase, and a bacterial (*Erwinia uredovora*) phytoene desaturase; the resulting grains contained up to 1.6 μg/g of carotenoids by dry weight (Ye et al., 2000). Later, the daffodil phytoene synthase gene was substituted with the equivalent gene from maize, resulting in Golden Rice 2, in which the total carotenoid content of the endosperm increased to 37 μg/g dry weight (Paine et al., 2005). Both Golden Rice lines were donated to the Golden Rice Humanitarian Board, and up to six events of Golden Rice 2 were developed in the background of the American Kaybonnet variety, with one event selected for regulatory approval and commercialization. This line provides enough β-carotene in a 100-g portion of milled rice to achieve the recommended daily intake (RDI) of vitamin A for a child under five (Virk and Barry, 2009) and could therefore prevent vitamin A deficiency (VAD) if consumed on a regular basis. Local popular rice varieties have been selected in several countries with widespread VAD (Bangladesh, India, Indonesia, Philippines and Vietnam), and it is likely Golden Rice will be commercially available by late 2012 in at least the Philippines and Bangladesh, the other countries following later (Zeigler, 2009). There has been widespread criticism of the length of time it has taken to achieve regulatory approval and the barriers that have to be overcome to achieve adoption, a subject discussed in detail below (Potrykus, 2010).

Another key nutrient relevant in MDG4 and MDG5 is folic acid. Deficiency for folic acid in pregnancy leads to neural tube defects in the fetus and a greater chance of abortion or complications during delivery. Pregnant women require at least 600 mg of folate per day, but rice and maize provide nowhere near adequate amounts. Whereas processed food is supplemented with folic acid in the west, developing countries have not implemented sustainable folic acid supplementation programs. Folate synthesis in plants involves two separate pathways (the pterin and para-aminobenzoate branches) whose products are eventually conjugated together. Folate biofortification in rice seeds has been achieved by overexpressing two *Arabidopsis thaliana* genes, one from each of the pathways, resulting in a 100-fold enhancement. This means that 100 g of polished grains contains four times the RDI for folate (Storozhenko et al., 2007).

Although plants engineered to accumulate single nutrients are beneficial, they address only individual micronutrient deficiencies and would ultimately serve to displace rather than prevent malnutrition. For example, in the future where individual rice varieties with higher

levels of *β*-carotene, folate, iron, zinc and other micronutrients are approved and widely available, people might have to choose between nutrients because it would be difficult to eat enough rice to cover all requirements. Two solutions offer themselves, i.e. the creation of nutritionally improved varieties that have such high levels of nutrients that only small portions are required (allowing a mixed meal of different varieties to satisfy all nutritional requirements) or the creation of varieties simultaneously enhanced for multiple nutrients. The latter would be simpler and more economical although the technical hurdles would be more difficult to overcome.

In an effort to address this issue, transgenic maize plants simultaneously enhanced for carotenoids, folate and ascorbate provide the first example of a nutritionally enhanced crop targeting three entirely different metabolic pathways (Naqvi et al., 2009). This was achieved by transferring four genes into a white maize variety resulting in a 407-fold elevation of β-carotene levels (57 μg/g dry weight), a 6.1-fold increase in ascorbate levels (106.94 μg/g dry weight) and a 2-fold increase in folate levels (200 μg/g dry weight). The decision to engineer three pathways at the same time rather than crossing lines individually engineered to increase the level of single nutrients was taken because the crossing strategy is slow and inefficient (Zhu et al., 2008). The simultaneous transformation strategy results in all the transgenes integrating at a single locus, which therefore remains stable through subsequent generations.

Pregnant women and infants tend to have higher mineral requirements and particularly fall victim to deficiencies in iron (recommended daily allowance/adequate intake = 8 mg/day for males but 18 mg/day for women of reproductive age and 27 mg/day in pregnancy), zinc (RDA/AI = 8–13 mg/day for all) and calcium (RDA/AI = 1000–1300 mg/day for all). Calcium is essential for bone development, iron is needed for the synthesis of hemoglobin and the activity of many enzymes, and zinc is a cofactor for numerous enzymes and transcription factors. Mineral biofortification requires different strategies to vitamin biofortification because minerals are not synthesized *de novo* like organic compounds and must be sequestered from the environment (Gomez-Galera et al., 2010). One notable recent report describes the hyperaccumulation of iron in rice plants transformed with two genes, one encoding nicotianamine synthase (which is required for iron transport through the vascular system) and the other ferritin (which increases the capacity for iron storage) (Wirth et al., 2009). Many of the channels and transporters that process iron also process zinc, often resulting in co-accumulation. Calcium levels in carrot roots and lettuce leaves were enhanced by 30–100% by overexpressing the H^+/Ca^{2+} transporter sCAX1, and this is another strategy

that could be transferred to cereal crops (Morris et al., 2008; Park et al., 2009).

The role of plant biotechnology: using plants to produce inexpensive (oral) vaccines

Plants have been used for medicinal purposes for hundreds of years, but it is only recently that they have been deliberately engineered to produce specific pharmaceutical products (Twyman et al., 2005). Two broad strategies are envisaged. In the first, plants are simply an expression platform like any other (e.g. bacteria, yeast or mammalian cells), and the product is purified and formulated in the standard manner. In the second, plants are used as both the expression platform and the delivery vehicle, and this category includes the use of plants to produce oral vaccines. The principle is that a recombinant subunit vaccine is expressed in an edible plant organ such as potato tubers or cereal seeds and then administered as part processed food (e.g. puree or juice) which would be suitable for the large-scale immunization of adults and children in developing country settings (Yusibov and Rabindran, 2008). Plants have been used to produce many different vaccine candidates that have been successful in phase I clinical trials in humans, including oral vaccines to prevent hepatitis B, cholera, rabies and diarrheal diseases (Tiwari et al., 2009). The main technical challenge with oral vaccines is to induce a sufficient immunological response through mucosal immunity, which can be achieved by linking the antigen to a mucosal adjuvant such as the labile enterotoxin B subunit (LTB). The LTB protein was the first plant-derived recombinant oral antigen to be tested in clinical trials (Tacket et al., 1998).

Because diarrheal diseases account for a large proportion of under-five deaths in developing countries, the use of plant-derived oral vaccines to prevent sickness and diarrhea is the most relevant application of the technology in the context of MDG4. As proof of this concept, Tacket et al. (2000) developed an oral vaccine against Norwalk virus (which causes travelers' sickness), and the results of the phase I trials were similar to those with LTB, with nearly all of the volunteers who participated in the trial showing significant increases in the numbers of IgA-antibody forming cells (AFCs) and six also showing increases in IgG AFCs. There were also noticeable increases in serum IgG and stool IgA against the virus.

The provision of edible vaccines against common diseases in school children in developing countries could give parents additional encouragement to bring their children to school. For example, an oral vaccine comprising the cholera toxin subunit (CTB) expressed in rice under the control of an endosperm-specific promoter, induced antigen-specific mucosal and systemic immune responses in mice, and would be an excellent candidate to develop for human use in the developing world (Nochi et al., 2007). Advantages of vaccines delivered in

cereal grains include the increased stability in storage and after administration, addressing distribution problems and the lack of a cold chain, and also prolonging the window of opportunity to induce an effective immune response after administration. The rice/CTB vaccine could be stored at room temperature for more than 18 months without degradation, and once administered it resisted the harsh environment in the stomach because it accumulated in endosperm storage organelles known as protein bodies which provided shielding (bioencapsulation). Oral immunization induced CTB-specific serum IgG and mucosal IgA, and conferred protection because serum from immunized mice prevented cholera toxin binding to GM1-ganglioside, which causes severe diarrhea.

As well as their use in humans, oral vaccines produced in plants also provide an inexpensive and convenient way to prevent diseases in domestic animals, which would also help to increase the productivity and economic prosperity of farmers. Hundreds of vaccines for animal diseases have been expressed in plants, many proving efficacious in challenge studies. One worth particular mention is the recently-developed vaccine against Newcastle disease in poultry, which was developed by Dow AgroSciences and became the first plant-derived vaccine to receive USDA approval. This product was developed to test the regulatory pathway and has not yet been marketed, but it has cleared the way for other vaccines produced using the same platform technology.

Despite the efficacy of plant-derived vaccines, their deployment in human populations seems unlikely at present. The approval process for Golden Rice indicates that there is an unwritten tiered approach to acceptability, with crops engineered to prevent pests and diseases now widely accepted (at least outside Europe), those with improved nutritional traits receiving guarded approval but still distrust, and those with value-added products such as pharmaceuticals mired in an uncertain regulatory environment (Spok et al., 2008). There is a general regulatory consensus that crops producing pharmaceutical products would need to be segregated from food crops to prevent adventitious exposure to the bioactive substance and reduce the likelihood of outcrossing (Spok et al., 2008). An additional challenge specific to oral vaccines is the achievement of consistent doses of the antigen when delivering it as part-processed food or feed/fodder. Paul and Ma (2010) present a critical review of plant-derived oral vaccines and the challenge of developing effective delivery strategies.

MDG 6: Combat HIV/AIDS, malaria and other diseases

Overview

HIV/AIDS, malaria and tuberculosis represent the major public health challenges in

the world's poorest countries (**Fig. 5.5**). HIV is transmitted not only through sexual contact but also by intravenous drug use and from mother to child. The disease has caused more than 25 million deaths since it was first recognized in 1981, and 33.4 million people are currently thought to be HIV positive, 95% of whom live in developing countries (UNAIDS/WHO, 2009). AIDS remains the leading cause of adult mortality in Africa today, and the sixth leading cause of death in the world. MDG6 aims to halt and reverse the spread of HIV/AIDS by 2015 and provide wider access to HIV drugs. Malaria is caused by parasites of the genus *Plasmodium*, transmitted by mosquitoes. It affects 350–500 million people each year, and one million die from the disease, particularly children under five and pregnant women. As for HIV/AIDS, the poor are disproportionately affected and make up the vast majority of the 40% of the world's population living in high-risk areas. MDG6 aims to reduce the incidence of malaria globally and provide access to drugs and mosquito nets. Tuberculosis is a respiratory disease transmitted by aerosol, caused by the bacterium *Mycobacterium tuberculosis*. More than one third of the world's population is thought to be infected, and the disease kills 1.7 million people each year, predominantly in developing countries (Elías-López et al., 2008). An approved tuberculosis vaccine, BCG (Bacille Calmette Guérin), is used worldwide and is administered to approximately 100 million infants per year providing good protection against the most severe childhood forms of the disease, and antibiotics can also be used to treat infections. However, these resources are not easily accessible in developing countries, hence the prevalence of the disease. HIV activates dormant tuberculosis, and more than 10 million people worldwide are infected with both HIV and tuberculosis.

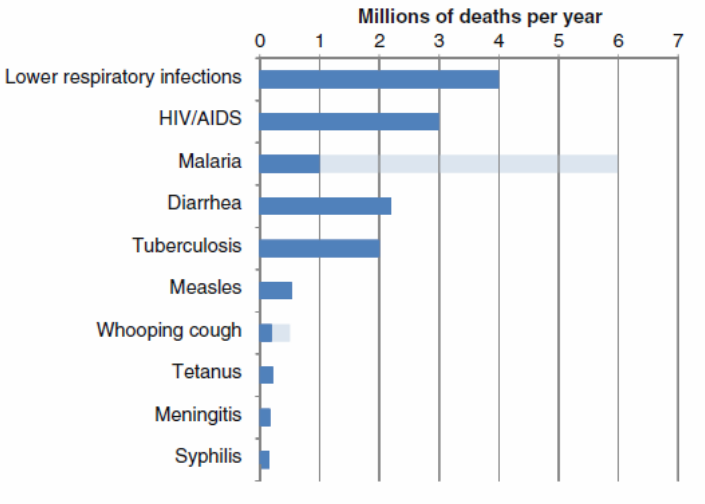

Fig 5.5 Ranking of fatal diseases in the developing world (millions of deaths per year). Where accurate figures are not known, the two bars represent minimum and maximum estimates. Source: World Health Organization.

The role of plant biotechnology (HIV)

Barrier methods help to prevent new HIV infections as well as other diseases and unplanned pregnancies, and one of the objectives under the HIV component of MDG6 is to increase education about the disease and the availability of condoms and other barrier devices. However, gender inequality and cultural preferences (see MDG3) place many women in the position of being unable to negotiate condom use without male cooperation, even if the male is known to be HIV positive (Population Council, 2000; Padian et al., 1998).

Microbicides that are applied well in advance of sexual intercourse would place the means to control HIV infection in the hands of monogamous women. Several candidate products have been developed based on surfactants, HIV-neutralizing antibodies and lectins, alone or in combination with anti-retroviral drugs (Ramessar et al., 2010). One drawback of this approach is that antibodies must be used in very high doses (up to 1 g per application) because of their stoichiometric mechanism of action and to ensure enough of the active ingredient survives the harsh mucosal environment. The microbicide would need to be applied daily, perhaps several times a day, and with the anticipated demand this would require the relevant antibodies to be produced on a multi-ton scale which is several orders of magnitude above current global production capacities. Antibodies are generally produced by fermentation in mammalian cells and are therefore among the most expensive biopharmaceuticals on the market. In order to supply microbicides to impoverished women in the rural communities of sub-Saharan Africa and South Asia, a revolutionary change in production technology would be necessary.

Plant biotechnology has a role to play in this scenario because plants provide a key advantage over animal cells for the production of biopharmaceuticals—the economy of scale. Increasing the scale of production in animal cells requires larger fermenters and facilities, whereas plants can be scaled up much more readily through additional land or greenhouse space (Ma et al., 2003; Twyman et al., 2005; Ramessar et al., 2008a,c). Many promising microbicide compounds have been successfully expressed in transgenic plants, including the antiviral lectins griffithsin (O'Keefe et al., 2009) and cyanovirin-N (Sexton et al., 2006). Plant-derived griffithsin showed broad-spectrum activity against HIV at picomolar concentrations, was directly virucidal by binding to HIV envelope glycoproteins, and was capable of blocking cell-to-cell HIV transmission. It was also non-irritating and non-inflammatory in human cervical explants and *in vivo* in the rabbit vaginal irritation model. Cyanovirin-N was produced using hydroponic cultures and was shown to bind HIV gp120 and protect T cells from HIV infection *in vitro*.

HIV-neutralizing antibodies have also been produced in plants, including 2G12 produced in tobacco and maize (Ramessar et al., 2008b; Rademacher et al., 2008; Strasser et al., 2009), 2F5 produced in tobacco (Floss et al., 2009) and 4E10 produced in tobacco. The HIV-neutralizing activity of tobacco and maize 2G12 was equal or superior to that of the same antibody produced in CHO cells, and 2G12 has now been produced under GMP conditions in preparation for phase I clinical trials (the first plant-derived antibody to reach clinical development, through a publicly funded initiative)(Fischer et al., 2012). Another interesting example is the production of a combined microbicide candidate to minimize the risk of viral adaptation, prevent the evolution of resistant strains and provide sufficient cross-clade protection (Ramessar et al., 2010). Sexton et al. (2009) combined the HIV-neutralizing antibody b12 with cyanovirin-N and produced the fusion protein in transgenic tobacco. The fusion protein was more potent against HIV than either individual component.

The role of plant biotechnology (malaria)

Plants have also been used to express malarial antigens in an attempt to develop an inexpensive vaccine candidate, but such products are at a very early stage in development and would not be expected in the clinic for at least 5 years. However, plants are not solely used for the production of recombinant proteins—they are also valuable sources of antimalarial drugs, such as artemisinin. The cost of extracting artemisinin from its source means the drug is too expensive for the poorest people in developing countries, those most in need of it. The cost could be reduced by recreating the metabolic pathway leading to artemisinin in a plant species that is more accessible or easy to culture although there is currently insufficient knowledge of the enzymatic steps in the pathway (Ma et al., 2009).

The role of plant biotechnology (tuberculosis)

Plant-derived vaccine candidates against tuberculosis have been produced in tobacco and Arabidopsis, with some evidence that they generate immune correlates of protection. For example, Rigano et al. (2004) produced transgenic Arabidopsis plants expressing the immuno-dominant tuber culosisantigen ESAT-6 fused to a mucosal adjuvant and fed the oral vaccine to mice. They found that the fusion protein induced an immune response but unfortunately not enough toreduce the bacterial load and to protect mice against disease challenge. More recently, ESAT-6 and Ag85B were expressed in tobacco as fusions with an elastin-like peptide to increase their accumulation (Floss et al., 2010). Purified TBAg-ELP

was obtained by inverse transition cycling and tested in mice and piglets for safety and efficacy. Antibodies recognizing mycobacterial antigens were produced in both species. A T-cell immune response recognizing the nativemyco bacterial antigens was detected in mice.

In a related approach, Elías-López et al. (2008) produced transgenic tomato plants expressing interleukin-12, a key cytokine. Oral delivery studies in which crude fruit extracts (lyophilized preparations) were fed to mice infected with various strains of the tuberculosis agent showed that the animals were more resistant to the disease and suffered less lung tissue damage having ingested the tomato extracts.

MDG 7: Ensure environmental sustainability

Overview

The objectives under MDG7 are to integrate the principles of sustainable development into country policies and programs and reverse the loss of environmental resources, reduce biodiversity loss significantly by 2010, reduce the proportion of the population without sustainable access to safe drinking water and basic sanitation to 50% of initial levels by 2015, and achieve a significant improvement in the lives of at least 100 million slum dwellers by 2020.

Sustainable development requires that natural resources are conserved, and while progress is being made in all areas, the rate of environmental destruction is still alarmingly high. Although urbanization and industrialization play an important role in this process, agriculture also has a major impact. As discussed above, access to safe water is limited in many countries because of pollution with both pathogens and chemical residues, particularly agrochemical te run-off. The 2010 target for biodiversity conservation has been missed, and key habitats for threatened species are not being adequately protected (UN, 2010a). The rate of deforestation is slowing but even so averaged 5.2 million hectares per year over the last decade. More carbon dioxide is being released into the atmosphere than ever before, 35% more than 10 years ago. This trend needs to be stabilized and reversed if MDG7 is to be achieved.

The role of plant biotechnology

Plant biotechnology has a critical role to play in the improvement of environmental sustainability. Some of the major impacts have already been discussed in the context of other MDGs and will only be summarized here. These are: (1) the development of crops that require less water (drought-tolerant crops), thereby releasing more fresh water resources for

drinking and for infrastructure development; (2) the development of high-yielding crops that produce adequate yields on smaller plots, thereby reducing the need for forests to be cut down to provide agricultural land; (3) the development of crops that are resistant to weeds, insect pests and pathogens to reduce chemical use and fuel consumption. The deployment of Bt crops has reduced the use of pesticides, also saving on fossil fuels required for spraying. The deployment of herbicide-tolerant crops has reduced fuel use and CO_2 emissions by limiting the need for plowing, and conserving soil and moisture by encouraging tilling-free agriculture. The cumulative reduction in pesticide use for the period 1996–2008 was approximately 356,000 tons (8.4%), which is equivalent to a 16.1%reduction in the associated net environmental impact as measured by the environmental impact quotient (EIQ). The corresponding data for 2008 alone revealed a reduction of 34,600 tons of pesticides (9.6%) and a reduction of 18.2% in EIQ (Brookes and Barfoot, 2010). In countries such as India, China, Argentina and Brazil, which are the most enthusiastic adopters of Bt agriculture after the US and Canada, the greatest impact of Bt has been the reduction in the number of pesticide sprays (Naranjo, 2009). In India, for example, the reduction is from 16 down to 2–3 sprays per growing season (Qaim et al., 2006; Karihaloo and Kumar, 2009).

MDG8: Develop a global partnership for development

The MDGs represent a global partnership for development, and developing countries must take on the primary responsibility to work towards achieving the first seven MDGs. They must do their part to ensure greater accountability and efficient use of resources. But for developing countries to achieve this, it is absolutely critical that developed countries deliver on their end of the bargain with more effective aid, more sustainable debt relief and fairer trade rules, well in advance of 2015.

The objectives in MDG8 are to (a) address the special needs of yhe least developed countries, landlocked countries and small island developing states; (b) develop an open, predictable, nondiscriminatory trading and financial system; (c) deal comprehensively with developing country debt; (d) make available the benefits of new technologies in cooperation with the private sector, especially information and communications. In terms of plant biotechnology, the fourth objective is the most relevant, and there are already several examples of how this has been put into practice with the Golden Rice Humanitarian Board and WEMA (see above). These programs form the basis for technology donation for humanitarian purposes, where technology can be used royalty-free for subsistence agriculture or to alleviate poverty, hunger, malnutrition and disease. The two examples cited above focus

in one case on nutritional improvement and in the other on the avoidance of starvation during drought, but the same principles apply to pharmaceutical plants. For example, all the partners in the Pharma-Planta consortium (http://www.pharma-planta.net), which established the regulatory pathway necessary to produce HIV-neutralizing antibodies in plants (Spok et al., 2008), have signed up to a humanitarian use clause which allows all the technology developed in the project (as well as any necessary background IP) to be used royalty-free for humanitarian purposes. The Harvest Plus Challenge Program is a similar concept although focusing on conventional biofortification strategies and mostly eschewing genetic engineering.

Because MDG8 will depend on political cooperation between developed and developing countries, this is the appropriate juncture to discuss the role of politics in plant biotechnology and the barriers to adoption that have been erected (Farre et al., 2009). Plant biotechnology is one of a raft of strategies that can be combined to make progress towards the MDGs, and many of the technological barriers have been overcome. However, the impact of this scientific progress is being neutralized by the unwillingness of politicians to see beyond immediate popular support and to take politically controversial decisions that would in the short to medium term save millions of lives and in the long term would make a significant impact on the health, well being and economic prosperity of the world's poorest people. The problem is essentially that whereas political decision-making should be based on rational scientific evidence, it is more often dictated by certain organizations, with dubious agendas, and the media, which thrives on sensationalism (Farre et al., 2010). Unfortunately, this feeds back in such a way that those charged with regulating biotechnology are pressured into implementing excessive regulation, which extends development times unnecessarily and results in many more lives being put at risk (Farre et al., 2010).

Conclusions and outlook

Each of the MDGs reflects one or more fundamental aspects of socioeconomic development in countries that depend predominantly on subsistence agriculture to feed their populations. Therefore, it seems natural that the improvement of agricultural productivity should form the keystone upon which the frameworks of progress can be built. In this context, technological solutions to improve agricultural productivity and sustainability can be regarded as a valuable approach to ensure rapid progress towards the MDGs, particularly technologies that improve yield, vigor and nutritional value in staple crops and allow the production of added-value products such as pharmaceuticals.

The prospects of implementation vary considerably, with some products already deployed and having a strong impact, others on the verge of approval, and others unlikely to see large-scale deployment by 2015 if at all. The success of Bt crops in India and China is likely to be repeated in Africa and South Asia as these have reduced hunger and led to economic prosperity within a remarkably short time. Many additional Bt varieties are waiting in the wings, and perhaps even more exciting is the prospect of multi-trait crops simultaneously protected against a range of pests and viral and microbial diseases, as well as drought and other environmental factors. Within the next 2 years, we should also see the first commercial release of Golden Rice, and this will hopefully open the door for a range of additional nutritionally enhanced crops that will address food insecurity in a sustainable manner. The prospect of more ambitious technologies such as the use of plants to produce vaccines and drugs is unlikely to have an immediate impact in the developing world because the regulatory burden would be high and the construction of contained facilities would provide no further advantage compared to production in the west. In the short term, it is more likely that plant-derived pharmaceuticals will fill niche markets in the west and then spread to high-volume, low-margin products as yields improve, but the royalty-free donation of technologies and products may lower the cost of goods to the extent required to meet the demands of local health authorities in developing countries.

Most importantly, it is clear that the irrational political handling of plant biotechnology must be resolved so that developing countries are not put in the position of choosing between principles and lives. National and international funding agencies and charitable organizations should encourage collaborative projects with universities and other research organizations in target countries so that capacity-building programs can prepare a generation of local experts to establish their own research facilities, enabling them to operate independently, without political pressure, to develop sustainable solutions for their own populations. Most importantly, there must be leadership from the top—the EU needs to stop pandering to activists and the media, and should take decisions based on rational scientific evidence in order to help the world's most vulnerable people. Only when bold decisions are made in Europe and elsewhere in the industrialized world, can the fruits of our scientific endeavor be used to accelerate progress towards the MDGs.

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GENERAL CONCLUSIONS

Conclusions

1. The promoter regions of two paralogous rice genes (*OsADC1* and *OsADC2*) encoding the enzyme arginine decarboxylase (ADC) were cloned and characterized.
2. Promoter-*gus*A fusion constructs in transgenic rice plants showed that the promoters were inducible by drought stress resulting in a higher level of GUS activity in stressed plants.
3. A potential TATA box and numerous putative stress-response elements were identified in the two ADC promoters. The *OsADC1* and *OsADC2* promoters are therefore useful as tools to develop and implement more precise and targeted strategies for the creation of plants tolerant to abiotic stress.
4. The *Gentiana lutea zeaxanthin epoxidase* (*GlZEP*) gene promoter was cloned and characterized.
5. Promoter-*gus*A fusion constructs in transgenic tomato plants revealed high levels of *gus*A mRNA accumulation and GUS activity in chromoplast-containing flowers and fruits, but minimal levels in immature green fruits containing chloroplasts, and in sepals, leaves, stems and roots.
6. *GlZEP-gus*A expression was strictly associated with fruit development and chromoplast differentiation, suggesting an evolutionarily-conserved link between *GlZEP* and the differentiation of organelles that store carotenoid pigments.
7. The *Escherichia coli* glycolate catabolic pathway was introduced into rice chloroplasts in an effort to reduce photorespiration.
8. A population of transgenic plants was recovered expressing five chloroplast-targeted bacterial genes encoding: (a) the three subunits that comprise glycolate dehydrogenase (GDH); (b) glyoxylate carboligase (GCL); and (c) tartronic semialdehyde reductase (TSR).
9. This population was characterized at the DNA, RNA and to some extent the protein level and sets the stage for further more detailed experiments to determine the potential of this approach.
10. Technological solutions to improve agricultural productivity and sustainability are required as part of a broader strategy to ensure progress to meet the MDGs, but political barriers to adoption must also be overcome.

ANNEX

Functional characterization of the Gentiana lutea *zeaxanthin epoxidase (GlZEP) promoter in transgenic tomato plants*

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ORIGINAL PAPER

Functional characterization of the *Gentiana lutea* zeaxanthin epoxidase (*GlZEP*) promoter in transgenic tomato plants

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Abstract The accumulation of carotenoids in plants depends critically on the spatiotemporal expression profiles of the genes encoding enzymes in the carotenogenic pathway. We cloned and characterized the *Gentiana lutea* zeaxanthin epoxidase (*GlZEP*) promoter to determine its role in the regulation of carotenogenesis, because the native gene is expressed at high levels in petals, which contain abundant chromoplasts. We transformed tomato (*Solanum lycopersicum* cv. Micro-Tom) plants with the *gusA* gene encoding the reporter enzyme β-glucuronidase (GUS) under the control of the *GlZEP* promoter, and investigated the reporter expression profile at the mRNA and protein levels. We detected high levels of *gusA* expression and GUS activity in chromoplast-containing flowers and fruits, but minimal levels in immature fruits containing green chloroplasts, in sepals, leaves, stems and roots. *GlZEP-gusA* expression was strictly associated with fruit development and chromoplast differentiation, suggesting an evolutionarily-conserved link between ZEP and the differentiation of organelles that store carotenoid pigments. The impact of our results on current models for the regulation of carotenogenesis in plants is discussed.

Keywords *Gentiana lutea* · Zeaxanthin epoxidase · Promoter · β-Glucuronidase · Transgenic tomato · Carotenoid · Chromoplast

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Introduction

Carotenoids are abundant isoprenoid pigments produced by all photosynthetic organisms as well as certain non-photosynthetic bacteria and fungi (Goodwin 1980). In chloroplasts, carotenoids are accessory light-harvesting pigments that protect the photosynthetic apparatus from photo-oxidation (Frank and Cogdell 1996; Demmig-Adams and Adams 2002). They also act as precursors for the plant hormones abscisic acid (ABA) (Creelman and Zeevart 1984) and strigolactone (Gomez-Roldan et al. 2008; Umehara et al. 2008). Chromoplasts are specialized plastids

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found in flowers and fruits that have adapted to store carotenoids. The accumulation of carotenoids confers a range of pigmentation in the yellow-orange-red spectrum that attract animals and therefore facilitate the dispersal of pollen and seeds (Bartley and Scolnik 1995).

There is significant interest in the regulation of carotenoid biosynthesis in plants because of their health-promoting antioxidant activity (Kloer and Schulz 2006) and the specific nutritional importance of carotenoids such as β-carotene (Von Lintig and Vogt 2004; Giuliano et al. 2008; Farre et al. 2010; Bai et al. 2011). However, this has shifted attention away from the key roles that carotenoids play in the continuation of the plant life cycle by attracting pollinating insects and herbivores that distribute seeds. Therefore, relatively little is known about the regulation of carotenoid biosynthesis in petals and fruits, and the link between carotenoid synthesis and chromoplast differentiation.

Gentiana lutea flowers contain large amounts of lutein, violaxanthin, antheraxanthin and β-carotene (Zhu et al. 2003). The chromoplasts in *G. lutea* petals originate either from pre-existing fully-developed chloroplasts or from immature proplastids (He et al. 2002). There is a strong temporal correlation during flower development between the accumulation of carotenoids and the formation of chromoplasts, which coincides with the induction of carotenogenic gene expression (Zhu et al. 2002, 2003). Zeaxanthin epoxidase (ZEP) catalyzes the conversion of zeaxanthin to violaxanthin via antheraxanthin, and is therefore the key enzyme responsible for the accumulation of antheraxanthin and violaxanthin in *G. lutea* petals (Zhu et al. 2003). ZEP is also the first committed enzyme in the ABA biosynthesis pathway (Marin et al. 1996; Seo and Koshiba 2002). Expression profiling in *G. lutea* has shown that *GlZEP* mRNA is abundant in fully-developed petals that contain mature chromoplasts but only minimal amounts are present in younger petals that still contain chloroplasts, and in leaves and stems (Zhu et al. 2003; and unpublished data). Steady-state *GlZEP* mRNA levels increase 1.8-fold between the hard bud stage (S1) and the fully-open flower stage (S5) (Zhu et al. 2003).

To gain insight into the regulation of *GlZEP* during petal development and chromoplast differentiation, we isolated the *GlZEP* promoter and evaluated different constructs for their activity in transgenic tomato plants by fusing them to the *gusA* reporter gene. Histochemical GUS assays revealed that a construct containing 677 bp of the *GlZEP* upstream promoter were sufficient to confer strong GUS activity in chromoplast-rich tissues but not in tissues containing chloroplasts, similar to the expression profile of the native gene in *G. lutea*. These data indicate that the 677-bp *GlZEP* promoter contains evolutionarily-conserved sequences that confer high level expression in chromoplast-rich tissues.

Materials and methods

Plant material

Gentiana lutea leaves, stems and flowers were obtained from the Hokkaido Experimental Institute of Health Science (Japan). The tissues were frozen in liquid nitrogen immediately after harvesting and then stored at −80°C.

Tomato (*Solanum lycopersicum* cv. Micro-Tom) plants were grown in the greenhouse at 25°C with a 16-h photoperiod. The leaves, stems, roots, flowers and fruits of wild-type and T4 homozygous transgenic plants were used for histochemical GUS assays immediately after harvesting, or were frozen in liquid nitrogen and stored at −80°C until required for GUS staining or *gusA* mRNA profiling. Fruits were harvested at four different stages: immature green (IG), mature green (MG), orange and ripe red.

Isolation of genomic DNA and RNA

Genomic DNA was extracted from 5 g of leaf tissue as described by Sambrook et al. (1989). Total RNA was extracted using TRIZOL® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and DNA was digested with RNase-free DNase I (QIAGEN, Valencia, CA, USA). Total RNA was quantified using a NANODROP 1000 spectrophotometer (Thermo Scientific, Vernon Hills, Illinois, USA).

Cloning of the *GlZEP* promoter

Gentiana lutea genomic DNA (20 μg) was completely digested with *Ban*II and ligated using 10 Weiss units of T4 DNA Ligase (Invitrogen, Carlsbad, CA) to generate circular molecules. These were used as templates for amplification of the *GlZEP* promoter

region by long accurate (LA) PCR with the Takara LA PCR kit (Takara, Shuzo, Japan), using forward primer FP1 (5′-CCC TAA ACC CTT CAA CAT CAC TGG TTT CAA GAT TCC-3′, positions +311 to +346 where position +1 is the first nucleotide of *ZEP* cDNA) and reverse primer RP1 (5′-GAA TGA GAG CCA ATC CAA GGA CAT GAA GCA GCA CCA-3′, positions +119 to +154) based on the GenBank *GlZEP* cDNA sequence (accession number EF203254). The product was transferred to vector PCR® II TOPO® (TA Cloning Kit, Invitrogen, Carlsbad, CA) for sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit on a 3130 × 1 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Promoter-GUS constructs

GlZEP promoter fragments were fused to the *gusA* gene in vector pBI101 (Clontech Laboratories, Mountain View, CA, USA) (Jefferson et al. 1987). The full-length *GlZEP* promoter region was amplified from *G. lutea* genomic DNA using forward primer 5′-GTC GAC CCT TAA TGG CGG TAA TTA TGT TCT GTT ATC-3′ (positions −2225 to −2194; *Sal*I restriction site underlined) and reverse primer 5′-GGA TCC TAA TCC AAT TAC AAA AGA GTG AAA AGA-3′ (positions −27 to −1; *Bam*HI restriction site underlined). The 2,225-bp amplified promoter fragment was transferred to the PCR® II TOPO® vector using the Invitrogen TA Cloning® kit, to generate plasmid pCR-GlZEPPro. Both plasmids (pCR-GlZEPPro and pBI101) were digested with *Sal*I and *Bam*HI, allowing the GlZEPPro fragment to be inserted upstream of *gusA* in the pBI101 vector, yielding the final construct pBI-GlZEPPro-GUS (*Zep-gusA*).

Three 5′ deletions of the *GlZEP* promoter region were also created by PCR. Primers 5′-GTC GAC CCT TAA TGG CGG TAA TTA TGT TCT GTT ATC-3′ (positions −2225 to −2195) and reverse primer 5′-GGA TCC TTC TTG CTT CAA TTT AGT TAC AAT TTG CTA G-3′) (positions 252–283) were used to amplify the full-length *GlZEP* promoter with 5′-UTR for pBI-GlZEPPro-5UTR-GUS (*Zep5utr-gusA*). Then forward primers D1 (5′-GTC GAC TTA TGA GTA CCG AGG TAT GCC TT-3′) (positions −1709 to −1684), D2 (5′-GTC GAC GAG TGC AGG TCT GTT ACA GTC AG-3′) (positions −1134 to −1109) and D3 (5′-GTC GAC GAT TCG AAT TGA GCG AAT AGT C-3′) (positions −677 to −655) were combined with reverse primer (5′-GGA TCC TAA TCC AAT TAC AAA AGA GTG AAA AGA-3′) (positions −27 to −1) to generate the three stepwise deletions, with *Sal*I and *Bam*HI restriction sites as discussed above. The amplified promoter was transferred to PCR®II TOPO® then pBI101 upstream of *gusA* using the strategy described above. The vectors were named pBI-GlZEPProD1-GUS (*D1709-gusA*), pBI-GlZEPProD2-GUS (*D1134-gusA*) and pBI-GlZEPProD3-GUS (*D677-gusA*). The integrity of all intermediate and final constructs was confirmed by sequencing.

Transient expression of promoter-GUS constructs in tomato

Plasmids pBI101, pBI121 (*35s-gusA*), pBI-GlZEPPro-GUS (*Zep-gusA*), pBI-GlZEPPro-5UTR-GUS (*Zep5utr-gusA*), pBI-GlZEPProD1-GUS (*D1709-gusA*), pBI-GlZEPProD2-GUS (*D1134-gusA*) and pBI-GlZEPProD3-GUS (*D677-gusA*) were transferred to *Agrobacterium tumefaciens* strain LBA 4404 by electroporation (Mattanovich et al. 1989). Individual colonies were seeded into 5-ml aliquots of YEM medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM $MgSO_4$, pH 7.2) containing 50 μg/ml kanamycin and 25 μg/ml rifampicin, and were shaken at 300 rpm, 28°C overnight. Each culture was then used to inoculate 50 ml induction medium (YEM medium supplemented with 20 μM acetosyringone, 10 mM MES, pH 5.6) containing 50 μg/ml kanamycin and 25 μg/ml rifampicin, incubated as above. Bacteria were recovered by centrifugation (2,700×*g*), resuspended in infiltration medium (10 mm $MgCl_2$, 10 mM MES, 200 μM acetosyringone, pH 5.6) to an OD_{600} of ∼1.0, and then incubated at room temperature with gentle agitation (20 rpm) for 3 h. Approximately 600 μl of the infiltration medium was then injected into fruits at the mature green (MG) stage (25–30 days after anthesis) through the stylar apex using a 1-ml syringe with needle (Orzaez et al. 2006). Injected fruits were left on the vine for 3 days, and then harvested and sectioned for histochemical staining.

Stable tomato transformation

Tomato stems from 1-month-old sterile plants were transformed using the procedure described by Pfitzner (1998). Briefly, 0.5–1 cm stems from plants growing

on sterile germination medium (MS salts containing 0.6% agar) were severed, placed on MSOZR medium (MS salts, MS Fe-EDTA, B5 vitamins and 30 g sucrose supplemented with 5 μM acetosyringone, 2 mg/l zeatin riboside and 0.6% agar) and pre-incubated for 24 h in a growth chamber (25°C, 16-h photoperiod). The stems were then dipped into the bacterial suspension in MSO medium, blotted on sterile paper and placed back on the same MSOZR plates. After 2 days in the growth chamber as above, the stems were transferred to plates containing selective shoot regeneration medium (MSOZR medium supplemented with 50 μg/ml kanamycin, 500 μg/ml carbenicillin and 0.6% agar) and incubated in the growth chamber as above for 2 weeks. The shoots were subcultured on fresh medium for another 2 weeks and then transferred to selective shoot regeneration medium (MSOZR medium supplemented with 50 μg/ml kanamycin, 250 μg/ml carbenicillin and 0.6% agar) to regenerate shoots from proliferating callus. Shoots up to 1 cm in length were excised from callus and transferred to 5 × 10 cm containers containing selective root medium (MSO medium supplemented with 1 mg/l zeatin riboside, 50 μg/ml kanamycin, 250 μg/ml carbenicillin and 0.6% agar). Plantlets with roots appeared after 2–3 weeks and were transferred to soil in the greenhouse (25°C, 16-h photoperiod). Transgenic tomato lines were advanced to the T_4 homozygous generation for promoter analysis.

Histochemical and fluorimetric GUS assays

Histochemical GUS assays were carried out according to Jefferson et al. (1987) with minor modifications. Leaves, flowers, hand-cut stem and root segments, and sectioned fruits at different developmental stages were incubated at 37°C overnight (12 h) in the dark in 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) in 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% (v/v) Triton X-100 and 20% (v/v) methanol to eliminate endogenous GUS activity (Kosugi et al. 1990). After 12 h staining, tissues were destained in an ethanol series (50, 70, 80 and 95%) to remove chlorophyll, and then stored in 70% (v/v) ethanol, and photographed with a digital camera.

Fluorometric GUS assays were carried out as described by Jefferson et al. (1987) with minor modifications. Plant tissues (100 mg) were ground to powder under liquid nitrogen, dispersed in 0.8 ml extraction buffer (50 mM Na_2HPO_4 pH 7.0, 10 mM EDTA, 0.1% (v/v) sodium dodecanoyl(methyl)aminoacetate, 10 mM 2-mercaptoethanol and 0.1% (v/v) Triton X-100) and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant (250 μl) was mixed with 250 μl 2 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) on ice and 250 μl was transferred immediately to a fresh tube containing 2 ml of GUS stop buffer (0.2 M Na_2CO_3) to serve as a control. GUS assays were performed at 37°C for 1 h before the reaction was stopped by adding 2 ml of GUS stop buffer. The released fluorescent product, 4-methylumbelliferone (MU), was measured on an FP-750 spectrofluorometer (JASCO, Germany) with excitation at 365 nm and emission at 455 nm. The protein content of extracts was determined as described by Bradford (1976). GUS enzyme activity was expressed in pmoles MU/h·μg of soluble protein. Each assay was carried out twice.

DNA blot analysis

Leaf genomic DNA (20 μg) was digested with *Eco*RI, fractionated by 0.8% (w/v) agarose gel electrophoresis and transferred to a positively-charged nylon membrane (Roche, Mannheim, Germany) according to the manufacturer's instructions. Nucleic acids were fixed by UV crosslinking and hybridized with a digoxigenin-labeled 512-bp *gusA* probe at 42°C overnight using DIG Easy Hyb buffer (Roche Diagnostics GmbH, Mannheim, Germany). The probe was synthesized by PCR using the PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany), forward primer 5′-CCT GTA GAA ACC CCA ACC CGT GA-3′, reverse primer 5′-ACG CTG CGA TGG ATT CCG GCA TA-3′ and pBI121 as the template. The membrane was washed twice for 5 min in 2× SSC, 0.1% (w/v) SDS at room temperature, twice for 20 min in 0.2× SSC, 0.1% (w/v) SDS at 68°C, and then twice for 10 min in 0.1× SSC, 0.1% (w/v) SDS at 68°C. After immunological detection with anti-DIG-AP (Fab-Fragments Diagnostics GmbH, Germany) chemoluminescence generated by chloro-5-substituted adamantyl-1,2-dioxetane phosphate (CSPD) (Roche, Mannheim, Germany) was detected on Kodak BioMax light film (Sigma–Aldrich, St. Louis, USA).

Quantitative real-time PCR

First strand cDNA was synthesized from 2 µg total RNA using Ominiscript Reverse Transcriptase in a 20-µl total reaction volume following the manufacturer's recommendations (QIAGEN, Valencia, CA, USA). Quantitative real-time PCR was performed on a BIO-RAD CFX96^{TM} system using a 25-µl mixture containing 10 ng cDNA, 1× iQ SYBR Green Supermix (BIO-RAD) and 0.2 µM of each primer. For the amplification of *gusA* (GenBank accession no. U12639; Jefferson et al. 1987) we used forward primer 5′-CGT GGT GAT GTG GAG TAT TGC-3′ and reverse primer 5′-ATG GTA TCG GTG TGA GCG TC-3′. For the internal tomato *β*-actin control (GenBank accession no. U60482; Agarwal et al. 2009) we used forward primer 5′-GCT GGA TTT GCT GGA GAT GAT GC-3′ and reverse primer 5′-TCC ATG TCA TCC CAA TTG CTA AC-3′. To calculate relative expression levels, serial dilutions (0.2–125 ng) were used to produce standard curves for each gene. PCRs were performed in triplicate using 96-well optical reaction plates, comprising a heating step for 3 min at 95°C followed by 40 cycles of 95°C for 10 s, 57°C for 30 s and 72°C for 20 s. Amplification specificity was confirmed by melt curve analysis of the final PCR products in the temperature range 50–90°C with fluorescence acquired after each 0.5°C increment. The fluorescence threshold value and gene expression data were calculated using the CFX96^{TM} system software.

Results

Cloning of the *GlZEP* promoter

The *GlZEP* promoter was cloned by inverse PCR using cleaved and circularized *G. lutea* genomic DNA as the template and outward-facing primers based on the *GlZEP* cDNA sequence (GenBank accession number EF203254). After sequencing the resulting product, a 2,637-bp fragment was isolated directly from genomic DNA using gene-specific primers based on the new template. This fragment (Genbank accession number: EF203262) comprised 2,225 bp of the upstream promoter and 412 bp of the *GlZEP* cDNA. The 2,225 bp promoter fragment was designated the full-length *GlZEP* promoter, and position +1 was assigned to the first nucleotide of the *GlZEP* cDNA (Zhu et al. 2003). The PlantCARE database (Lescot et al. 2002, http://www.dna.affrc.go.jp/PLACE/signalscan.html) was used to identify putative *cis*-acting regulatory elements, revealing two potential TATA boxes at positions −72 and −84 as well as six CAAT boxes, which are known to play an important role in enhancing eukaryotic promoter efficiency (Table 1). We identified several elements that respond to light, including a GT1 motif, two box I motifs, three G-boxes, a GAG motif, four box 4 motifs (which form part of a conserved DNA module involved in light responsiveness) and a chs-CMA2a motif (Table 1). The multitude of light response elements is likely to regulate *GlZEP* expression according to day length and other cues involved in the control of flower development. We also identified several hormone/stress response elements including one ethylene response element (ERE), two CGTCA motifs (methyl jasmonate sensitive), two MYB binding sites involved in drought stress, a heat shock element, three Box-W1 motifs that responds to fungal elicitors, and a circadian control element (Table 1).

Transient expression of *GlZEP* promoter in tomato fruits using different promoter constructs

Tomato fruits at the mature green (MG) stage were injected with bacterial cultures carrying the vectors pBI101 (promoterless-*gusA*), pBI121 (*35s-gusA*) and *Zep-gusA*. Fruits were harvested 3 days later and transverse sections were stained for GUS activity. As expected, fruits expressing *gusA* controlled by the CaMV35S promoter (*35s-gusA*) or the full-length *GlZEP* promoter (*Zep-gusA*) showed substantial GUS activity (Fig. 1) whereas no GUS activity was detected in fruits containing the promoterless control vector pBI101 (Fig. 1).

The promoter was characterized in more detail by generating a series of 5′ stepwise truncations containing 1,709, 1,134 and 677 bp of upstream sequences respectively and inserting these fragments upstream of the *gusA* gene into vector pBI101. We also created a construct containing the full-length *GlZEP* promoter plus the 5′-UTR (*Zep5utr-gusA*). We evaluated the four new constructs by transient expression in tomato fruits as above, using the *35s-gusA* vector as a control. Histochemical GUS assay showed that all four constructs behaved in a similar manner in terms of expression patterns to the full-length *GlZEP* promoter (Fig. 1). The GUS activity (identified as blue color) of

Table 1 Putative *cis*-acting regulatory elements identified in the 2,225 bp *GlZEP* promoter region using the PlantCARE database (Lescot et al. 2002)

Function	*Cis*-element	Sequence	Position from cDNA	Origin of isolated promoter
Common *cis*-acting element in promoter and enhancer regions	CAAT-box	CAAT	−1957, −349, −221	*Hordeum vulgare*
		CAAAT	−2157, −1369	*Brassica rapa*
		CAATT	−1496	*Glycine max*
Cis-acting regulatory element involved in light responsiveness	Box I	TTTCA	−2160	*Pisum sativum*
		AA	−275	
	Box 4	ATTAAT	−599, −262, −237, −215	*Petroselinum crispum*
	chs-CMA2a	TCACT	−1567	*P. crispum*
		TGA		
	GAG-motif	AGAGAGT	−1068	*Arabidopsis thaliana*
	G-box	CACATGG	−1613	*Solanum tuberosum*
		CACGTC	−1585, −1355	*Zea mays*
	GT-1-motif	GCGGTA	−2217	*Oryza sativa*
		ATT		
Ethylene-responsive element	ERE	ATTTCAAA	−276	*Dianthus caryophyllus*
Cis-acting regulatory element involved in MeJA-responsiveness	CGTCA-motif	CGTCA	−2064, −1527	*H. vulgare*
MYB biding site involved in drought-inducibility	MBS	CAACTG	−1191, −997	*A. thaliana*
Cis-acting element involved in heat stress responsiveness	HSE	CNNGAANNTT	−2123	*Lycopersicon esculentum*
		CNNG		
Fungal elicitor responsive element	Box-W1	TTGACC	−2082, −746, −100	*P. crispum*
Cis-acting regulatory element involved in circadian control	Circadian	CAANNNNNATC	−822	*L. esculentum*

Zep5utr-gusA construct was slightly higher than that of the full-length *GlZEP* promoter (*Zep-gusA* construct) indicating that the 5′-untranslated region contains sequences necessary for high levels of expression. The GUS activities of *D1709-gusA* and *D1134-gusA* constructs were at a similar level compared to that of the full-length *GlZEP* promoter (*Zep-gusA*). The *D667-gusA* construct exhibited a slightly reduced GUS activity compared to that of the full-length *GlZEP*, *D1709-gusA* and *D1134-gusA* constructs (Fig. 1). Nevertheless the GUS expression pattern of the *D667-gusA* construct assessed histochemically exhibited similar expression to that of the full-length promoter indicating that all *cis*-acting elements necessary to confer high-level GUS activity in tomato fruits are contained within the proximal 677 bp of the *GlZEP* promoter sequence.

Histochemical analysis of GUS activity in stably transformed tomato plants

Tomato plants were stably transformed with two of the constructs described above: the full-length *GlZEP* promoter-GUS fusion (*Zep-gusA*) and the positive control pBI121 (*35s-gusA*). Histochemical GUS assays were carried out on 12 primary transformants expressing *Zep-gusA* and eight expressing *35s-gusA*. In the *Zep-gusA* plants, GUS activity was detected in fruits from the mature green stage onwards, but not in leaves, sepals, petals or immature green (IG) fruits. The distribution of GUS activity was the same in all 12 independent lines, although the intensity differed significantly. In contrast, GUS activity was detected in all the tissues of all eight *35s-gusA* plants.

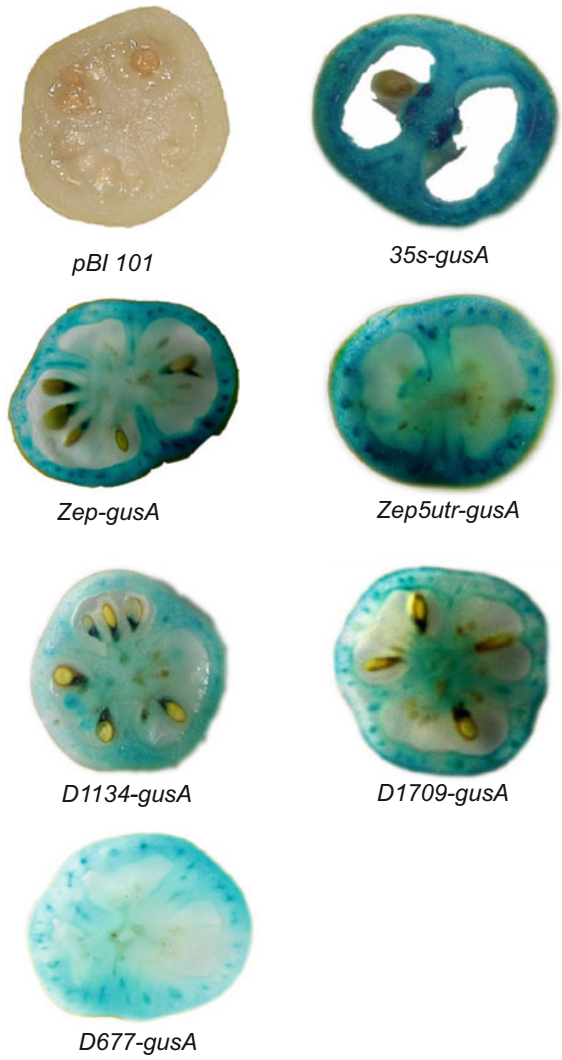

Fig. 1 GUS activity in tomato fruits transiently expressing promoterless-*gusA* (*pBI101*), *35s-gusA*, *Zep-gusA*, *Zep5utr-gusA*, *D1709-gusA*, *D1134-gusA*, *D667-gusA*, respectively

Four representative independent *Zep-gusA* lines exhibiting 3:1 segregation of the *gusA* gene and little variation in GUS activity were analyzed by DNA blot using *gusA* as the probe (data not shown). All four lines contained one or two transgene copies in the tomato genome. One *Zep-gusA* line (no. 4) and one *35S-gusA* line also showing 3:1 segregation were selected to produce T4 homozygous lines for further analysis. Plants from these lines were grown to maturity and GUS activity was assessed in different tissues.

In the *Zep-gusA* plants, GUS activity in young and mature leaves, stems and roots was below the threshold for histochemical detection (Fig. 2). GUS activity was also undetectable in sepals and petals, but could be detected in the ovary and pistils (Fig. 3). Low GUS activity was detected in the central column and placenta tissues of immature green fruits, increased in mature green fruits, peaked in orange fruits and decreased slightly in red ripe fruits (Fig. 2c). A distinct spatiotemporal pattern of GUS activity was observed in the pericarp, with no activity in immature green fruits but increasing activity later in development, peaking in orange and ripe red fruits (Fig. 2c). Pericarp cells in young immature green fruits contain a large number of regular-sized chloroplasts, but these differentiate progressively into chromoplasts and completely replace the chloroplasts in the pericarp cells of ripe red fruits (Forth and Pyke 2006; Egea et al. 2010). The *Zep-gusA* reporter gene is therefore developmentally regulated in close association with chromoplast differentiation. In contrast to the above, high levels of GUS activity were observed in all the tissues of the *35s-gusA* plants and throughout the pericarp during all ripening stages (Figs. 2, 3).

Quantitative analysis of GUS activity in stably transformed tomato plants

The quantitative analysis of GUS activity in *Zep-gusA* transgenic plants indicated that only low levels of GUS were present in the leaves, but higher levels were present in flower tissues such as stamens, pistils and petals (Fig. 4). GUS activity was low in immature green fruits, but increased five-fold during development peaking in orange fruits. The quantitative and histochemical GUS assays were concordant (Figs. 2, 3, 4).

Quantitative analysis of *gusA* gene expression in stably transformed tomato plants

The transcriptional activity of the *GlZEP* promoter was analyzed in more detail by measuring *gusA* mRNA levels in different tissues of the *Zep-gusA* tomato plants by quantitative real-time PCR (Fig. 5). These measurements in separate extracts from leaves, sepals, petals, stamens and pistils revealed relatively high *gusA* levels in stamens and the lowest levels in leaves. Expression studies during fruit development showed very low steady state *gusA* mRNA levels in immature green fruits, an up to sixfold increase in

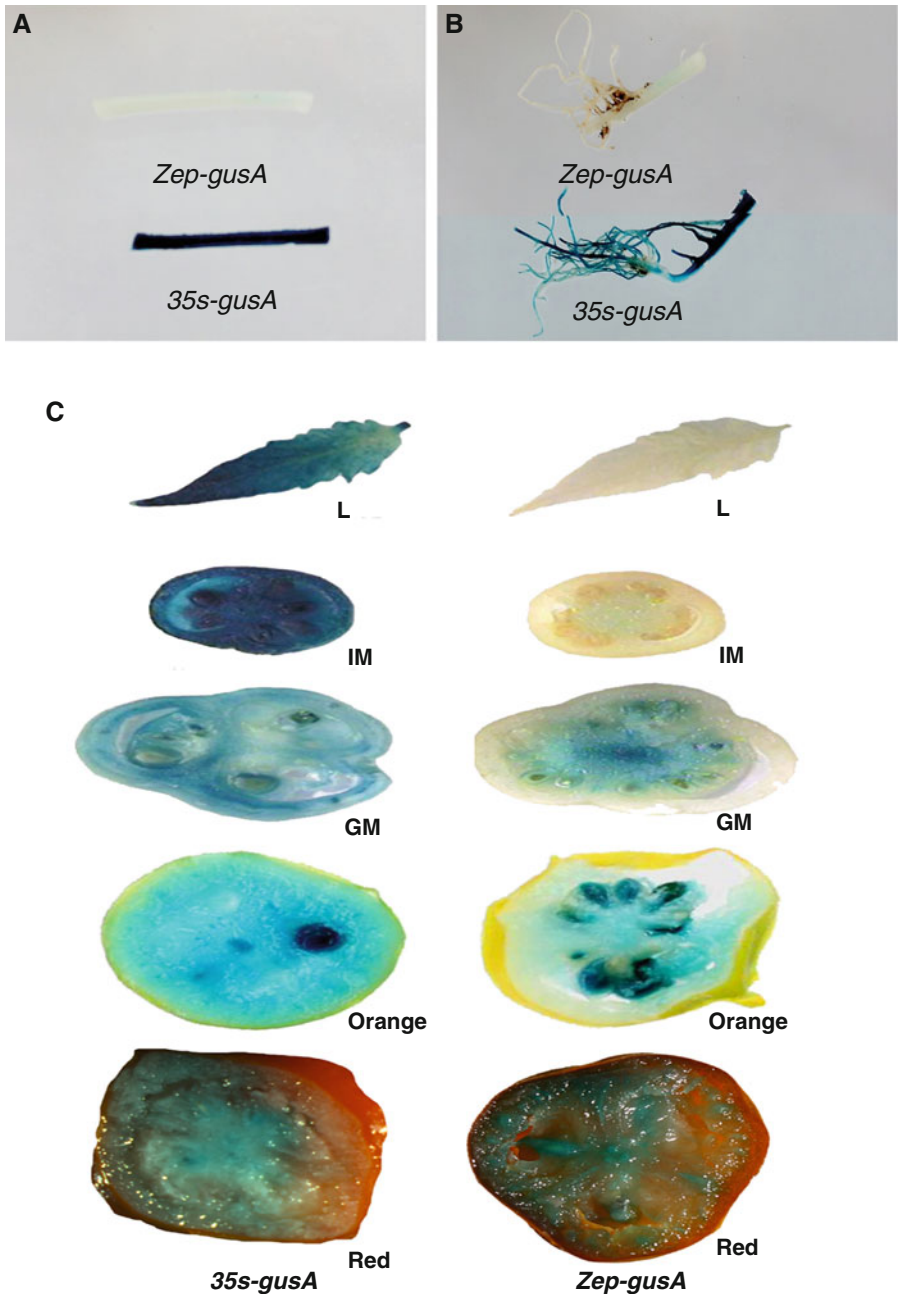

Fig. 2 Histochemical GUS staining of typical transgenic tomato plant carrying *Zep-gusA* and *35S-gusA* constructs, respectively. **a** stems; **b** roots; **c** leaves and fruits. *L* leaf, *IM* immature green fruit, *MG* mature green fruit, *Orange* orange fruit, *Red* red ripen fruit; *Zep-gusA*, pBI-GlZEPPro-GUS; *35s-gusA*, pBI121. All *four lines* tested exhibited very similar staining patterns

orange fruits and a slight decrease in red ripe fruits (Fig. 5). These data were in full agreement with the levels of GUS activity determined in the histochemical and fluorometric assays (Figs. 2, 3, 4).

Discussion

Carotenoid biosynthesis is differentially regulated in tissues containing chloroplasts and chromoplasts,

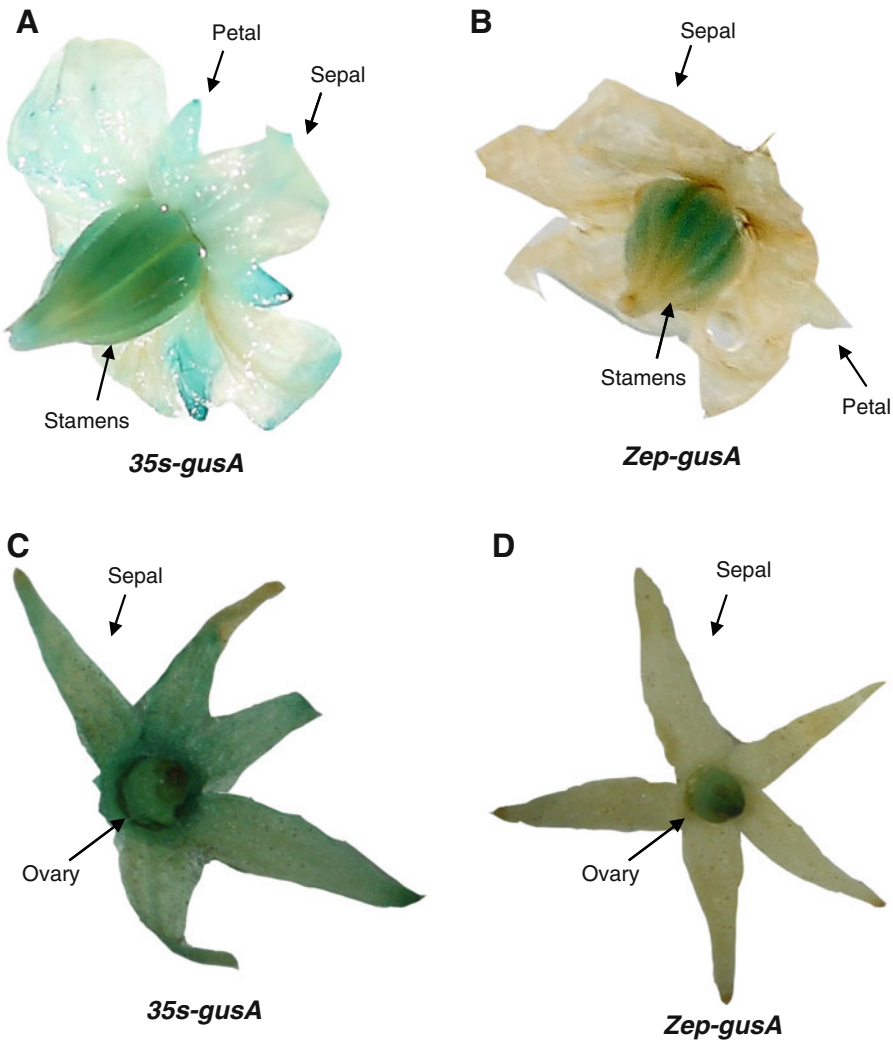

Fig. 3 Histochemical GUS staining in flowers of transgenic tomato plants carrying *Zep-gusA* and *35s-gusA* constructs, respectively. All *four lines* tested exhibited very similar staining patterns

reflecting important functional differences between these tissues and the different roles carotenoids fulfill in each setting (reviewed by Zhu et al. 2010). To ensure that green tissues and fruits/flowers can independently accumulate different carotenoids, many carotenogenic enzymes exist as multiple isoforms encoded by separate genes. The tomato genome, for example, encodes two isoforms of *GGPPS* (geranylgeranyl diphosphate synthase), *PSY* (phytoene synthase), *LYCB* (lycopene β-cyclase) and *BCH* (β-carotene hydroxylase), one set expressed preferentially in green tissues and the other expressed preferentially in flowers and fruits (Ronen et al. 2000; Galpaz et al. 2006). Chromoplast-specific isoforms of lycopene β-cyclase have also been identified in *Citrus* (Alquezar et al. 2009; Dalal et al. 2010; Mendes et al. 2011), kiwifruit (Ampomah-Dwamena et al. 2009), saffron (Ahrazem et al. 2010) and papaya (Blas et al. 2010; Devitt et al. 2010).

There has been great interest in the investigation of carotenoid biosynthesis and its regulation in plants, primarily because of the dietary benefits of carotenoids and the drive to develop crops with higher levels of β-carotene. However, this has drawn attention away from the natural role of carotenoids in plants, i.e. the promotion of pollination and seed dispersal, which can only be investigated by looking at the expression profiles of carotenogenic genes in homologous and

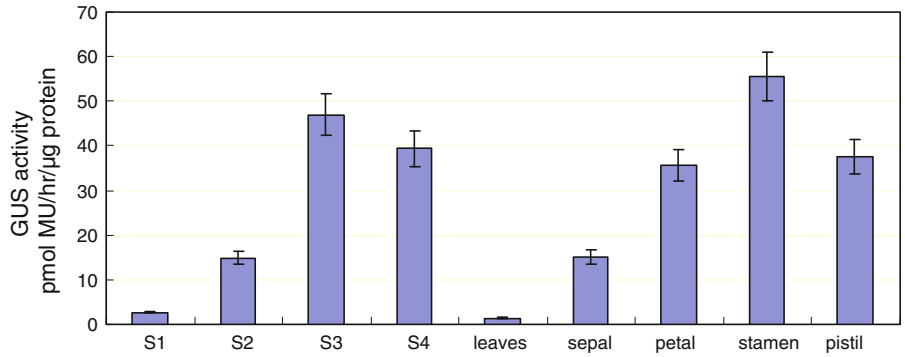

Fig. 4 GUS expression in different tissues of transgenic tomato plants carrying *Zep-gusA*. GUS activity was determined in triplicate measurements in four independent biological replicates (independent transgenic plants). *Columns* represent GUS activity expressed in pmoles MU/h·µg of soluble protein in fruits at different stages of maturity (S1–S4) and leaves, sepals, petals, stamens and pistils. Error bars represent standard error of the mean. S1, fruits at immature green stage; S2, fruits at mature green stage; S3, fruits at orange fruit stage; S4, fruits at red ripen fruit stage

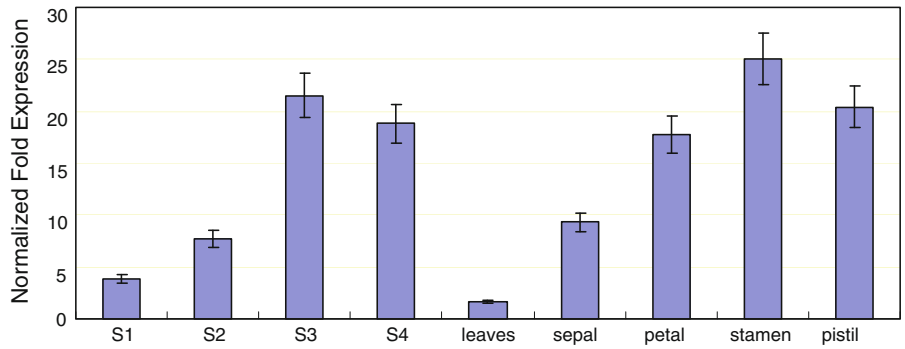

Fig. 5 Expression of *gusA* gene in different tissues of transgenic tomato plants carrying *Zep-gusA*. Quantitative real-time PCR was performed with cDNA prepared from leaves, sepals, petals, stamens, pistils and different stage fruits. Relative expression was determined in triplicate measurements in four independent biological replicates. *Columns* represent the relative *gusA* expression levels normalized against *β*-actin gene with standard errors. Error bars represent standard error of the mean. S1, fruits at immature green stage; S2, fruits at mature green stage; S3, fruit at orange fruit stage; S4, fruit at red ripen fruit stage

heterologous genetic backgrounds and linking the expression profiles of different enzymes to the carotenoids that accumulate in different tissues (Zhu et al. 2002, 2003; Li et al. 2010).

We have previously shown that the *G. lutea* zeaxanthin epoxidase gene (*GlZEP*) is expressed strongly in chromoplast-rich mature petals of *G. lutea* plants, but only minimally in chloroplast-containing younger petals and leaves (Zhu et al. 2003), suggesting the promoter may be active in tissues containing chromoplasts and repressed in tissues lacking them. In agreement with this, no GUS activity was detected in tobacco plants (whose petals and fruits lack chromoplasts) expressing a *GlZEP-gusA* transgene (data not shown) so we sought to carry out similar analysis in tomato plants, which contain abundant chromoplasts in mature fruits and flowers. Chromoplasts in tomato fruits begin differentiating at the breaker stage, and full conversion of chloroplasts into chromoplasts occurs when the fruits are completely ripe (reviewed by Egea et al. 2010). Chloroplast to chromoplast differentiation can be conveniently assessed using the pericarp pigmentation during tomato fruit development. We selected the miniature dwarf tomato cultivar Micro-Tom (Scott and Harbaugh 1989) as a model to investigate *GlZEP* promoter activity in chromoplast-containing tissues because of its small size and short life cycle (70–90 days from sowing to fruit ripening) (Meissner et al. 1997). This cultivar has previously been used for the analysis of metabolic and

developmental pathways (Haroldsen et al. 2011; Carvalho et al. 2011).

We investigated *GlZEP* promoter activity by transient expression and stable transformation in tomato plants transformed with a range of *GlZEP-gusA* reporter constructs. We recovered 12 independent transgenic plant lines expressing the *GlZEP-gusA* construct, all of which demonstrated the same profile of GUS activity albeit with varying staining intensity, so we selected four representative lines for further analysis. We investigated the activity of full-length and truncated promoter constructs in leaves, stems, roots, flowers and fruits at different developmental stages. GUS activity in young and mature leaves, stems and roots was below the threshold for histochemical detection (Fig. 2). GUS activity was also undetectable in sepals and petals, but could be detected in the ovary and pistils (Fig. 3). Similar observation of GUS staining was observed in the flowers of transgenic tomato expressing tomato *PDS* promoter- or tomato *CYC-B* promoter-driven *gusA* reporter gene (Corona et al. 1996; Dalal et al. 2010). In this case there is no apparent link to chromoplast differentiation, possibly reflecting the conserved function of carotenoids in flower tissues. The full-length *GlZEP* promoter (2,225 bp upstream of the transcriptional start site) was functional in the heterologous tomato environment and the expression profile of the reporter gene driven by the full-length promoter was identical to that observed in its homologous background (Zhu et al. 2003). High levels of GUS activity were observed in chromoplast-containing flowers and fruits, but there was only minimal expression in other tissues (fruits, sepals, leaves, stems and roots). Reporter gene activity was strictly correlated with fruit development and chromoplast differentiation, with minimal activity in immature green fruit but increasing activity in ripening orange fruit before falling off towards the end of the ripening process. The shortest *GlZEP* deletion construct (*D677-gusA*) contained 677 bp of upstream sequence but nevertheless resulted in only slightly lower levels of GUS activity compared to the full-length promoter (Fig. 1), suggesting that all *cis*-acting elements required for high-level GUS activity in chromoplast-rich tissues are contained within the proximal 677 bp of the promoter.

All promoters contain *cis*-acting elements that confer spatiotemporal specificity and responsiveness to external stimuli (Peremarti et al. 2010). We characterized the *GlZEP* promoter fragment in more detail by searching the sequence for relevant *cis*-acting elements using the PlantCARE database (Lescot et al. 2002). We identified TATA and CAAT boxes that are typical in eukaryotic promoters, as well as multiple light response elements (GT1, box I, G-boxes,GAG motif, Box 4 and chs-CMA2a) that are likely to link carotenoid biosynthesis to flower development by integrating day-length cues and other stimuli (Table 1). Carotenoid biosynthesis is regulated by light (Bartley and Scolnik 1993; Von Lintig et al. 1997; Simkin et al. 2003; Li et al. 2008; Welsch et al. 2008). A putative circadian responsive element was also found in the *GlZEP* promoter, which supports the diurnal rhythm in *ZEP* gene expression that has been reported in tobacco and tomato leaves (Audran et al. 1998; Thompson et al. 2000; Facella et al. 2008).

The *GlZEP* promoter also contains *cis*-acting elements involved in hormone biosynthesis and stress responses (Table 1). The presence of a drought-inducible MBS element agrees with previous reports that the expression of endogenous *ZEP* in tobacco and tomato roots is induced by drought stress (Audran et al. 1998; Thompson et al. 2000). Two ATCTA motifs are present as tandem repeats in the *GlZEP* promoter. Similar pairs have previously been identified in the *Arabidopsis thaliana PSY* promoter (mediating high-level basal transcription independent of light quality), and in the promoters of several genes related to photosynthesis (Welsch et al. 2003). Single copies of the ATCTA motif are found in other carotenogenic promoters such as Arabidopsis *DXS* (deoxy-xylulose-phosphate synthase) and *PDS* (Welsch et al. 2003), tomato and maize *PDS* (Welsch et al. 2003), and tomato *CYC-B* (Dalal et al. 2010). This motif is also present in several promoters involved in tocopherol biosynthesis (Welsch et al. 2003). In Arabidopsis, paired ATCTA motifs are recognized by AtRAP2.2, a member of the APETALA2/ERE-binding protein transcription factor family (Welsch et al. 2007). We engineered one of our constructs deliberately to eliminate one of the ATCTA motifs (*D1709-gusA*), and also generated two more substantially truncated constructs lacking both copies (*D1134-gusA* and *D677-gusA*). All three constructs performed similarly to the full-length promoter (*Zep-gusA*) suggesting that neither motif contributes significantly to the basal activity of the full-length *GlZEP* promoter. In contrast, deletion of the RAP2.2

transcription factor binding site in the *ShCYC-B* full-length promoter resulted in a considerable loss of promoter activity (Dalal et al. 2010). However, it is possible that the loss of adjacent sequences rather than the RAP2.2 element might be responsible for the fall in promoter activity and the only way to confirm the role of this element directly is to modify it by site-directed or linker-scanning mutagenesis.

The promoters of coexpressed genes often share common regulatory motifs and are potentially regulated by a common set of transcription factors. Therefore, the identification of relevant *cis*-acting regulatory elements in the promoter regions of important metabolic genes can provide leads that help uncover new mechanisms of transcriptional regulation (Liu et al. 2005; Nilsson et al. 2010). The expression profile of *Zep-gusA* in transgenic tomato plants is strikingly similar to that of tomato *PDS* (Corona et al. 1996) and *CYC-B* (Dalal et al. 2010), which have also been evaluated as reporter gene fusions in transgenic tomato plants, suggesting all three may be regulated by a common mechanism. It is also important to emphasize that the *GlZEP* promoter is correctly regulated in a heterologous background, indicating strong conservation of the regulatory mechanisms across species. Common motifs in the three promoters include the CAAT box, Box 4 and RAP2.2 (Corona et al. 1996; Welsch et al. 2007; Dalal et al. 2010) but further analysis and comparisons are required to identify additional known and unknown motifs that are shared between co-regulated promoters and that may help us to unravel further underlying regulatory mechanisms.

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OPINION PAPER

The potential impact of plant biotechnology on the Millennium Development Goals

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Abstract The eight Millennium Development Goals (MDGs) are international development targets for the year 2015 that aim to achieve relative improvements in the standards of health, socioeconomic status and education in the world's poorest countries. Many of the challenges addressed by the MDGs reflect the direct or indirect consequences of subsistence agriculture in the developing world, and hence, plant biotechnology has an important role to play in helping to achieve MDG targets. In this opinion article, we discuss each of the MDGs in turn, provide examples to show how plant biotechnology may be able to accelerate progress towards the stated MDG objectives, and offer our opinion on the likelihood of such technology being implemented. In combination with other strategies, plant biotechnology can make a contribution towards sustainable development in the future although the extent to which progress can be made in today's political climate depends on how we deal with current barriers to adoption.

Keywords Plant biotechnology · Millennium Development Goals · Poverty · Hunger · Malnutrition · HIV/AIDS · Agriculture · Developing countries

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Introduction

The Millennium Development Goals (MDGs) are a set of eight ambitious international development targets for the year 2015, which were agreed by 192 members of the United Nations as well as numerous non-governmental organizations (NGOs) at the Millennium Summit in 2000 (Appendix 1). The aim of the MDGs is to improve standards of health, socioeconomic status and education by tackling poverty, hunger and disease, increasing educational opportunities and creating a global development partnership (UN 2010a).

We are now more than two-thirds of the way through the program, and progress towards the goals has been patchy, with significant improvements in the rising economies such as China and India, but little progress in some other countries, particularly in sub-Saharan Africa (UN 2010b).

China has almost halved its poverty-stricken population over the last decade and is well on the way to realizing all the MDGs by 2015. In contrast, the major target countries in sub-Saharan Africa have reduced the level of poverty by less than 1% and seem unlikely to meet any of the MDGs (UN 2010b).

The success of China and India has much to do with their economic growth, but growth is not a prerequisite for the achievement of MDG targets. Bangladesh, for example, has shown that progress can be made with little or no growth simply by adopting and rolling out inexpensive solutions on a large scale, including national vaccination campaigns and nutritional supplementation programs (UNICEF 2010). Tying the MDGs to expensive solutions that in turn depend on either economic growth or donations in aid cannot be maintained indefinitely, and it is therefore imperative that inexpensive but scalable solutions are deployed as rapidly as possible to provide a sustainable basis for development. In this context, plant biotechnology has a role to play by providing healthier and more nutritious crops and also new platforms to produce inexpensive vaccines and drugs. However, the impact of plant biotechnology is not limited to augmenting or replacing expensive intervention programs. Biotechnology can create plants that reduce the impact of weeds, insect pests, diseases and harsh environments, providing a basis not only for the reduction of hunger through more successful subsistence agriculture but also the stimulation of economic prosperity by providing higher yields of better quality crops that increase the wealth as well as the health and wellbeing of poor agricultural workers. Although plant biotechnology is not a panacea for the world's socioeconomic woes, it is already being used in numerous ways to address the Millennium Development Goals. There remain significant barriers to adoption that are largely political in character, with little or no rational scientific basis. Overcoming these political hurdles in the short term is a more challenging objective than achieving technological progress (Farre et al. 2009).

MDG1: Eradicate extreme poverty and hunger

Overview

The number of people living in hunger currently oscillates around one billion, which represents nearly one in every seven people in the world (FAO 2009b). Hunger can be defined as an insufficient daily intake of energy (the average requirement being 2,000 kcal per day), and the figure of one billion therefore excludes those who receive sufficient calories but are nevertheless malnourished due to the absence of essential vitamins and minerals (we return to this topic later). The hungry has limited access to food but not because of insufficient production. Indeed, there is plenty of food, enough to support a much higher global population than exists today, but there is inadequate food distribution, and the world's poorest people cannot afford to purchase the food that is available. Hunger, at least at present, is therefore caused by poverty and poor distribution rather than insufficient global production (DFID 2010).

The World Bank defines extreme poverty as living on less than US $1.25 per day. MDG1 is therefore expressed in the form of three objectives, the first to reduce the number of people living in poverty by 50%, the second to improve employment opportunities (particularly for women and young people) and the third to reduce the level of hunger (Fig. 1). These are interlinked objectives, and they need to be tackled simultaneously to see improvements in all the three. Progress towards MDG1 is also important to ensure progress towards most of the other MDGs, particularly those that aim to reduce the burden of disease and improve education. Poverty and hunger both lead to poor health and loss of opportunity, creating a vicious cycle in which people are forced to endure a monotonous existence that focuses solely on survival (Islam 2008).

Although urban poverty is a growing problem, most of the world's poorest people are rural dwellers and depend on subsistence agriculture (Fan et al. 2005). Strategies to address extreme poverty in rural areas should therefore focus on improving agricultural productivity to allow the poor to produce enough food to survive, the remainder being marketed and generating income. Short-term solutions such as providing food aid will not provide long-term and sustainable progress towards MDG1. Instead, there needs to be a drastic shift in socioeconomic policy focusing on agricultural and commercial development, with modern seed varieties playing an important role because they generate the most vigorous crops (Sanchez 2009). Most subsistence calories are obtained from cereal crops, particularly rice and maize. These two crops are the staple diet of more than 75% of the human population (FAO 2009a). Maize also provides much of the fodder for livestock in the countries where it is grown, including both developed countries such as the US, and many countries in Africa. The short-term objective should therefore be to reduce the yield gap in cereal crops (the gap between potential yields and actual yields) to reduce hunger, improve health and create economic prosperity. In the longer term, it will be necessary to apply the same solutions to diverse fruit and vegetable crops as well as cash crops such as cotton, tobacco and coffee.

The role of plant biotechnology

Plant biotechnology can help to achieve MDG1 through the deployment of high-yielding genetically engineered

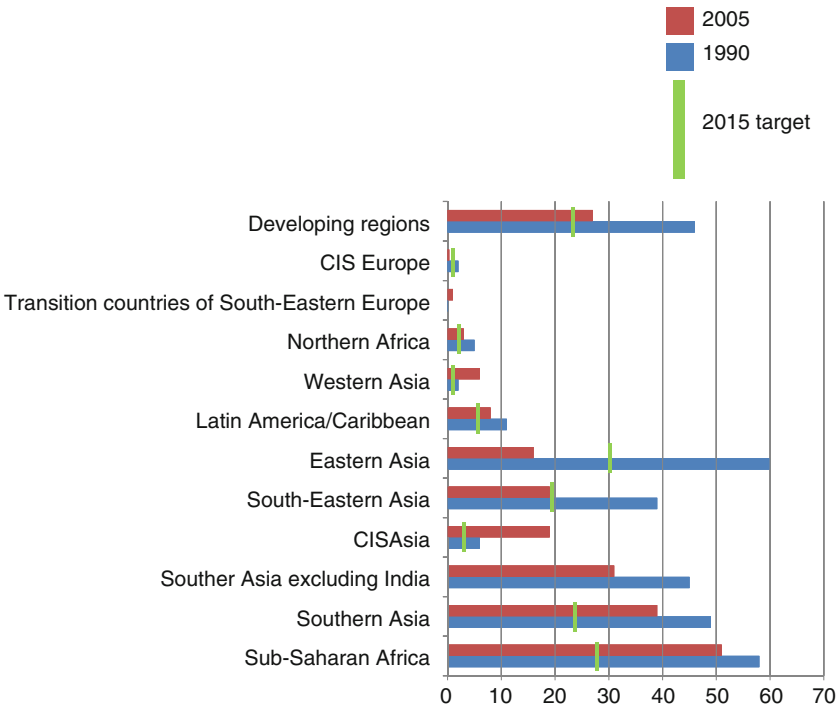

Fig. 1 Proportion of people living on less than US $1.25/day by region, 1990 and 2005, compared to 2015 MDG targets. Source: UN (2010a)

varieties that are resistant to weeds, insect pests and diseases caused by viruses, bacteria and fungi, and that are able to withstand harsh environmental constraints such as drought (Farre et al. 2009). Weeds, insect pests and pathogens can reduce yields either by adversely affecting plant growth and development, or by consuming and/or spoiling the products of food crops in the field or in storage. Globally, this reduces crop yields by up to 30%, but the impact in developing countries can be much higher because the climatic conditions favor the survival and breeding of insect pests and disease vectors. After pests and diseases, unfavorable environmental conditions such as drought, poor soil quality and (in Asia) flooding also have a devastating effect. The development of crops with an inbuilt capacity to withstand these effects could help to stabilize crop production and hence significantly contribute to food security and economic prosperity (Christou and Twyman 2004).

Weeds

Weed management is the largest single input into agriculture in both industrialized and developing countries. However, whereas weed management in the developed world is highly mechanized and has benefited extensively from the technological advantages provided by genetically engineered herbicide-resistant crops and broad-spectrum herbicides, developing country agriculture currently relies on an army of laborers, mostly women, who tend the land and spend long hours removing weeds manually (Akobundu 1991).

Two issues compound the impact of weeds in developing countries—the lack of resources to adopt technological solutions that are taken for granted in the developed world, and the disinterest shown by research organizations in the west to tackle weed species that are specific to Africa and Asia (Gressel et al. 2004). In Africa, maize and sorghum crops are often infested by *Striga*, a genus of parasitic flowering plants that is very difficult to control once established because it builds up a resilient seed bank in the soil (Parker 2009). Striga represents such a severe constraint to maize production that controlling this weed is seen as the key to resolving Africa's dependence on subsistence agriculture (Hearne 2009). There has been some recent success in the conventional breeding of resistant sorghum varieties by combining traits that make the sorghum plants poor inducers of Striga germination and poor hosts for colonization (Ejeta et al. 2007), but it has not been possible to achieve the same goals in maize. Progress towards the selective control of Striga in maize has been made through mutation and conventional breeding for imazapyr resistance (Kanampiu et al. 2002), which has been implemented as StrigAway technology co-developed by CIMMYT, BASF and the Weizmann Institute (Mataruka et al. 2010). Although this requires the application of herbicides, it is not necessary for farmers to spray their crops because the herbicide can be applied directly to the seed. A complementary biotechnology solution is to introduce herbicide resistance directly into maize. Glyphosate-resistant transgenic maize has been adopted in South Africa, which allows one worker with a backpack sprayer to control weeds over several

hectares. Although South Africa does not suffer from Striga infestations to the same extent as other countries in the region, the use of glyphosate resistance for general weed control shows that it could also be applied to tackle *Striga* infestations (Gressel and Valverde 2009).

The industrialization of rice cultivation in Asia has also generated an emerging problem with weeds. The switch from transplanting rice plantlets into flooded paddies (weed control by water) to direct seeding (weed control by herbicides) has led to the emergence of herbicide-resistant *Echinochloa* species that were formerly quite easy to control with selective herbicides (Valverde and Itoh 2001) and feral rice species (Valverde 2005). Here, transgenic strategies need to be applied with care because of the rapid evolution of herbicide resistance that has already been documented, and the likelihood that transgenes conferring herbicide resistance could introgress into weedy rice species and eliminate the selective difference between weedy and cultivated rice (Gealy 2005).

Insect pests, insect-borne diseases and the consequences of pest infestations

Many of our crop plants are attacked by insect pests, and devastating losses occur throughout the world due to pest infestations either in the field or in stored products. In the developing world, about half of all crop production is thought to be lost to insects, 15% of these losses occurring due to post-harvest consumption and spoilage (Christou et al. 2006). Insects not only cause direct yield losses by damaging and consuming plants but also act as vectors for many viral diseases, and the damage they inflict encourages bacterial and fungal infections, the latter resulting in contamination with mycotoxins.

A good example of the positive impact of plant biotechnology is the development of pest-resistant crops expressing insecticidal toxin genes from the soil bacterium *Bacillus thuringiensis* (Bt). Different strains of Bt produce different toxins which are both potent and highly specific against narrow taxonomic groups of insects, making them harmless to mammals and to beneficial insects (Sanahuja et al. 2011). In developing countries, Bt crops have been extraordinarily successful and beneficial, increasing yields, reducing the use of pesticides and the fuel needed for spraying, and improving the economic status of farmers while at the same time preserving biodiversity (James 2010; Brookes and Barfoot 2010).

The adoption of Bt crops in India provides strong support for the role of plant biotechnology in progress towards MDG1. In 2009, more than 5.5 million small-scale farmers planted a total of 8.4 million hectares of Bt cotton, representing nearly 90% of the national total (James 2010). More than half of these crops contained multiple Bt genes providing resistance against different pests, and for the first time locally developed varieties were planted instead of varieties developed in the US, therefore keeping all the agricultural profits within India's economy rather than servicing foreign royalty payments. India is now the world's largest cotton exporter (having been a net importer at the beginning of the decade), and it is estimated that rural farmers have benefitted from the technology through yield improvements to a total amount exceeding US $5 billion. Net yields per hectare have doubled in 10 years while agrochemical inputs have halved (APCoAB 2006; Manjunath 2008). The widespread adoption of Bt cotton in India has also helped to address the concerns of critics, who highlight the potential for resistant pests to evolve under intense selection pressure. Against these expectations, the first generation of Bt crops has maintained efficacy against nearly all targeted pest populations for more than a decade (Bourguet 2004). The scarcity of resistant populations despite the lack of integrated pest management suggests that resistance may attract a fitness penalty in the absence of the Bt toxins (Sanchis and Bourguet 2008). Resistant populations have appeared for a small number of pests, such as pink bollworm (*Pectinophora gossypiella*) which has evolved resistance to Bollgard I cotton (expressing the Cry1Ac toxin) in the Amreli, Bhavnagar, Junagarh and Rajkot areas of Gujarat. Resistance is anticipated because each toxin binds to a specific receptor in the brush border of midgut epithelial cells, and point mutations affecting toxin/receptor interactions would be strongly favored under selection. However, no resistance has been observed in fields growing the Bollgard II variety, which expresses the Cry1Ac and Cry2Ab toxins simultaneously (Monsanto 2010). These toxins bind different receptors, and the likelihood of mutations occurring in genes for both receptors is much lower than the likelihood of a single mutation, so this strategy of 'pyramiding' resistance genes (i.e. expressing multiple toxins with different targets in the pest) is a very powerful approach to prevent the evolution of resistant pest populations.

In the last year, Indian regulatory authorities also approved Bt brinjal (eggplant), India's first biotechnology-derived major food crop. Eggplant is a profitable crop but is extremely susceptible to pests, which cause up to 70% yield losses. Pest control normally requires repeated generous pesticide applications, up to 40 applications in 120 days, which many farmers cannot afford resulting in less intense treatments that are ineffective (Jayaraman 2010). The Bt variety has the potential to increase net yields by 33% while reducing pesticide use by up to 80%, thus lifting another 1.4 million farmers out of poverty (James 2010), but the regulatory approval was overruled by the government after lobbying by activists, and Bt brinjal is now subject to an indefinite moratorium pending additional

safety data (Balga 2010). The technology behind Bt eggplant was freely donated by Maharashtra Hybrid Seeds Company Ltd. (MAHYCO), who co-developed the product with Monsanto, to public sector institutions in India, Bangladesh and the Philippines for use by small resource-poor farmers, with 18 varieties awaiting final approval. These farmers will now be deprived of an opportunity to increase their economic prosperity for the foreseeable future (Jayaraman 2010).

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

Drought

Agriculture is highly dependent on water, and therefore, access to fresh water is as important for agricultural productivity as the quality of the seeds and the soil. With fresh water resources dwindling, the impact of drought can be devastating on crops, and the use of biotechnology to develop varieties that require less water and that are tolerant to drought conditions is now becoming as important as pest and disease resistance.

Drought stress in crops induces a number of response pathways including protection against reactive oxygen species, the active export of sodium ions and the synthesis of small molecules called osmoprotectants that increase the osmotic potential of cells causing them to retain water. Efforts focusing on direct responses such as the introduction of transgenes encoding antioxidant enzymes, enzymes that synthesize antioxidant compounds, genes encoding sodium transporters, and enzymes that synthesize osmoprotectants have resulted in many laboratory strains of transgenic plants that survive in concentrated salt solutions (Bhatnagar-Mathur et al. 2008). Other researchers have targeted the genes that regulate stress pathways (receptors, intracellular signaling molecules and transcription factors) which may be more useful because they, in turn, regulate a large number of protective genes (Bhatnagar-Mathur et al. 2008).

A drought-tolerant variety of maize co-developed by Monsanto and BASF is to be launched in the US in 2012 (James 2010). This expresses a stress-responsive transcriptional regulator that increases yields by up to 35% under water limiting conditions (Nelson et al. 2007). Stress-responsive transcription factors are one of three key classes of regulators that have been used to develop drought-tolerant varieties, the others being proteins that control signaling and post-translational modification in stress pathways, and regulators of osmoprotectant synthesis and metabolism such as the *Bacillus subtilis* chaperone CspB which is expressed in another drought-tolerant variety developed by Monsanto (Castiglioni et al. 2008). Although Texas in the US suffered its worst drought for 50 years in 2009 (with estimated losses of US $3.5 billion, approximately one-sixth of the agriculture market value), the situation in Africa and parts of Asia is much worse, with regular harvest failures due to insufficient rainfall and the absence of an irrigation infrastructure. Monsanto is part of WEMA (Water Efficient Maize program for Africa) which also includes the Gates Foundation, the Howard Buffet Foundation, CIMMYT and several stakeholders in sub-Saharan Africa, and it is committed to donating a royalty-free drought-tolerant maize variety for humanitarian use by 2017 (Mataruka et al. 2010). Under moderate drought conditions in Africa the yield expected from the tolerant variety should provide an additional 12 million tons of maize, providing food for over 20 million people who would otherwise depend on food given in aid.

MDG2 and MDG3: Achieve universal primary education, promote gender equality and empower women

Overview

Many developing countries are close to providing universal primary education, with the total number of primary-age children not attending school falling from 115 million in

2002 to 72 million in 2007, even with growing populations. Again, however, the picture is less encouraging in sub-Saharan Africa and South Asia, with 41 and 31.5 million primary-age children out of school, respectively (UN 2010a). In all developing regions, children in rural areas are twice as likely to be out of school as children living in urban areas, and children with disabilities and special needs are the least likely of all to receive a school education (Fig. 2a).

The underlying reasons for the trends discussed above reflect the direct costs of sending children to school, as well as the impact of losing potential workers on family farms. Achieving universal education therefore requires a shift in attitudes as well as the provision of educational opportunities, and also requires that children are healthy, adequately fed and well nourished. Abolishing school fees and subsidizing costs (e.g. for textbooks, uniforms and transportation) will make primary education more affordable for parents. Programs that link education, health and nutrition, such as school meal programs and social protection measures are necessary to achieve these aims, ultimately leading back to effective governance (Sachs and McArthur 2005). It is also important to encourage parental involvement in achieving MDG2.

Girls are less likely to be educated than boys throughout the developing world, and the prevailing culture is male dominated, a trend exacerbated in rural areas (Fig. 2b, c). Therefore, the level of illiteracy is higher in women; they are less likely to be employed; they tend to fill low-paid positions if they are employed and are often excluded from positions of authority (UN 2010a). Women overall suffer more from poverty and are often completely dependent on men financially. Furthermore, women are more likely to suffer from poor health and malnutrition, and more women than men in developing countries are HIV positive (UNAIDS 2008).

As with MDG2, a change of attitude is important to achieve MDG3, focusing on the rights of women to play an equal role to men in society. Overlapping with MDG2, one of the objectives of MDG3 is to strengthen opportunities for the education of girls and women, while meeting the above-mentioned commitments to universal primary education. Other objectives are to guarantee women's sexual and reproductive health rights, their property and inheritance rights, and their access to infrastructure, strive for gender equality in employment, increase women's influence in local and national governance, and combat domestic violence.

The role of plant biotechnology

Plant biotechnology cannot directly contribute to progress in either MDG2 or MDG3, but it can help by making

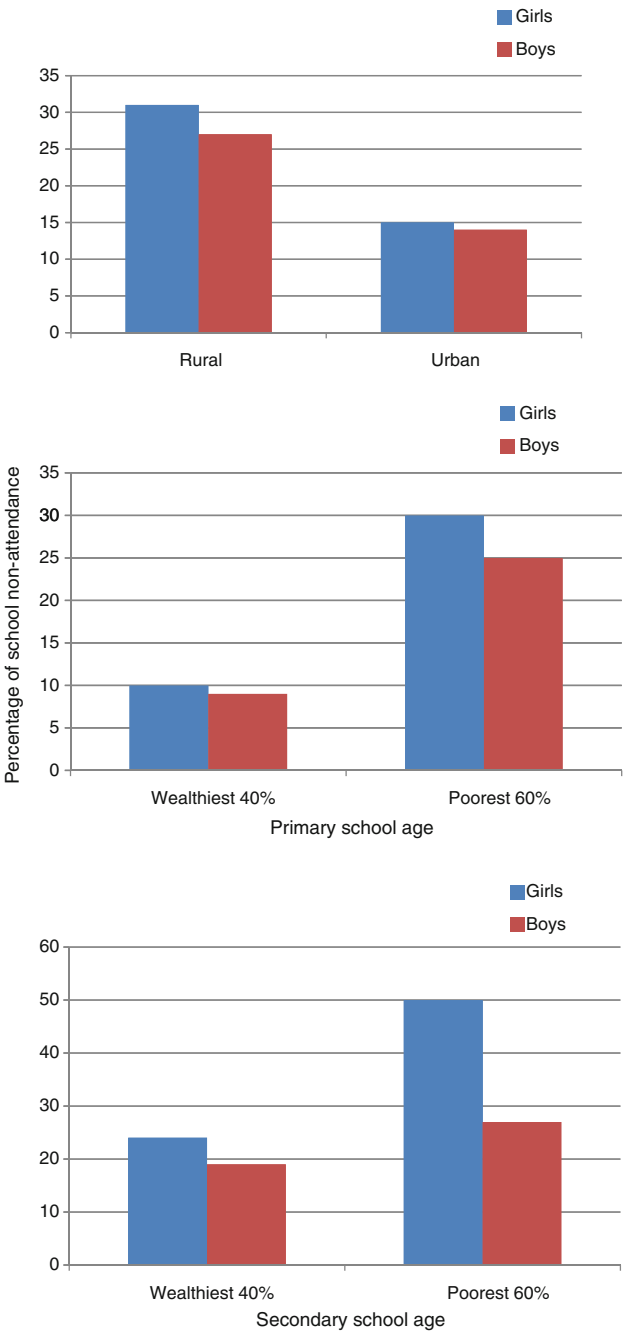

Fig. 2 Percentage of out-of-school children by gender, in 42 countries, up to 2008. **a** All children, by area of residence (rural or urban). **b** Primary-age children, by household wealth. **c** Secondary-age children, by household wealth. Source: UN (2010a)

numerous indirect impacts to improve health, wealth and wellbeing, and by providing educational opportunities. The role of plant biotechnology in the achievement of MDG1 as discussed above is pertinent because this reduces hunger and poverty. Many children from rural communities do not attend school because their parents cannot afford to send them, but increasing the wealth-generating potential of rural farmers by providing them with better crops is one way to

increase the proportion of children going to school. Furthermore, transgenic crops make tillage, pesticide spraying and weeding unnecessary and release women and children who would otherwise be forced to work on the land, allowing them the opportunity for education (Gressel 2009). It is often women that carry out the laborious agricultural work such as soil preparation, planting, weeding and harvesting, either for subsistence farming (as unpaid family workers) or as a service without financial security or social benefits, so the reduction in labor requirements has a disproportionately positive impact on women and girls, simultaneously addressing MDGs 2 and 3.

The widespread adoption of Bt cotton in India is one of the primary reasons for the dramatic increase in school attendance by primary-age children over the last decade, but its impact on girls and women has been even more remarkable (Subramanian et al. 2010; Subramaniam and Qaim 2010). Comparing Bt and conventional cotton, the average wage per hectare increased by US $40, with women experiencing a greater income gain (55% average), equivalent to 424 million additional days of employment for women (Fig. 3). The potential role of plant biotechnology in reducing the nutritional and health burden on women is discussed under MDGs 4 and 5 below.

MDGs 4 and 5: Reduce child mortality and improve maternal health

Overview

Nearly 9 million children under the age of 5 years die every year, 40% during their first month of life, and most of these deaths are concentrated in the world's poorest countries in sub-Saharan Africa and South Asia (UN 2010a). The deaths are predominantly caused by diseases that could be prevented or treated, and the mortality rate is exacerbated by poor maternal health usually reflecting underlying chronic malnutrition. MDG4 aspires to reduce the infant mortality rate in developing countries by two-thirds based on the number of deaths before the age of 5 years per 1,000 live births, specifically targeting the number of deaths before first birthday and specifically mentioning the fight against measles (Fig. 4).

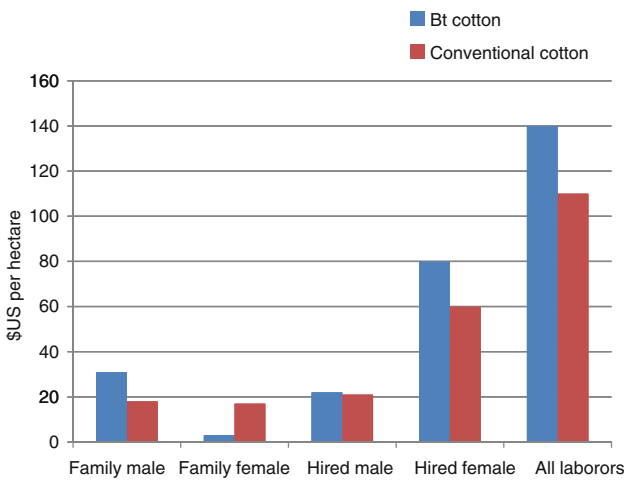

Fig. 3 Remuneration (US $/ha) from labor on farms with Bt and conventional cotton in rural India (Subramanian et al. 2010)

The causes of infant mortality are diverse, but the leading factors are pneumonia, diarrhea, malaria and HIV/AIDS, which together accounted for 43% of all infant deaths worldwide in 2008. We defer the discussion of HIV/AIDS and malaria to MDG6, which specifically focuses on those diseases. Pneumonia and diarrhea together account for a third of all under-five deaths, and most of these lives could be saved through low-cost prevention and treatment measures, including antibiotics for acute respiratory infections, oral rehydration for diarrhea, vaccination against pneumococcal pneumonia and rotavirus, and nutritional supplements. Proper nutrition is essential to fight disease effectively because malnutrition weakens the immune system and reduces resistance to diseases. Iron, zinc and vitamin A deficiencies have the severest impact on child morbidity and mortality, and these are also the most prevalent in developing countries because staple crops such as rice and white maize are naturally deficient in these compounds (Freedman et al. 2005).

There has been strong progress towards MDG4 in some parts of the world, such that the overall infant mortality rate fell from 12.4 million children per year in 1990 to 8.8 million in 2008, a drop of 28% (UN 2009, 2010a). The greatest improvements have been seen in North Africa, Eastern and Western Asia, Latin America and the Caribbean, with substantial progress in some of the world's poorest countries (Bangladesh, Bolivia, Eritrea, Ethiopia, Lao People's Democratic Republic, Malawi, Mongolia, Mozambique, Nepal and Niger). However, the rest of sub-Saharan Africa has fallen well behind and now accounts for 50% of all infant deaths. Also, 1 in 14 children still die before age five in South Asia.

As stated above, neonatal and under-five mortality is influenced by maternal health, i.e. the health of women during pregnancy, childbirth and the postpartum period (especially during breast-feeding). Approximately one in six women die in pregnancy or childbirth in developing countries, compared to 1 in 30,000 in Europe (WHO/UNICEF 2010). Over half of the deaths result from hemorrhage and hypertension, 20% involve comorbidity factors such as malaria and HIV, and 10% result from complications due to the lack of skilled midwives. MDG4 aims to reduce maternal deaths by 75% and increase the availability

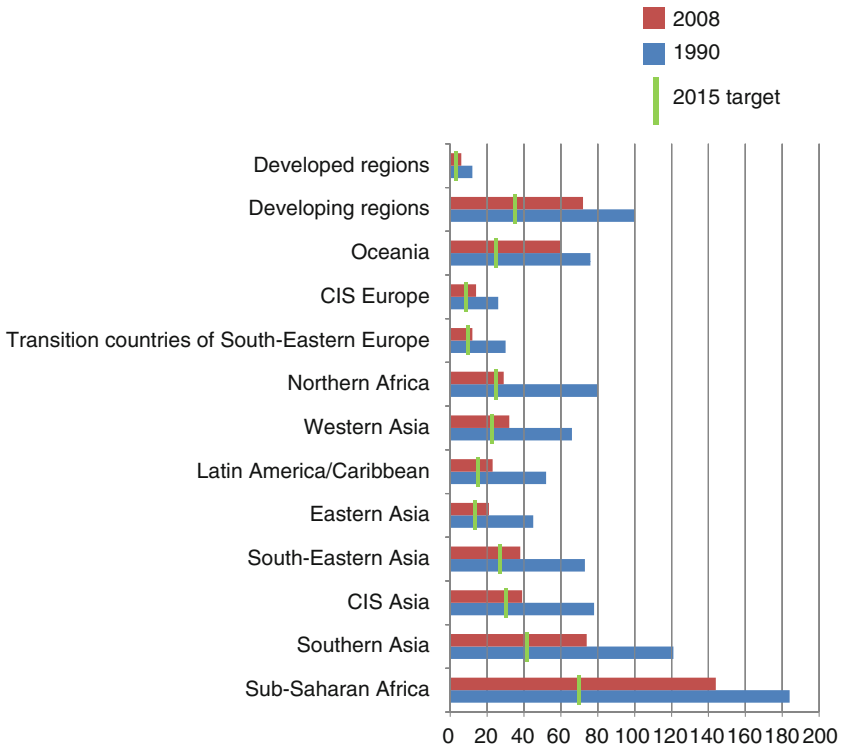

Fig. 4 Under-five mortality rate per 1,000 live births, by region, 1990 and 2008, compared to 2015 MDG targets. Source: UN (2010a)

of skilled medical personnel attending childbirth. Progress towards MDG4 has been rapid in some countries (particularly Bolivia, China, Ecuador and Egypt), but progress in others has been poor, with more than 50% of all maternal deaths now concentrated in six countries (Afghanistan, Democratic Republic of Congo, Ethiopia, India, Nigeria and Pakistan). Global rates are listed in Table 1, with southern sub-Saharan Africa performing worst: the maternal mortality ratio (the ratio of the number of maternal deaths per 100,000 live births) in that region increased from 171 in 1990 to 381 in 2008 (UN 2009).

The role of plant biotechnology: improved nutrition

Malnutrition contributes to poor maternal health and (both directly and indirectly) to poor childhood health. Various strategies have been proposed to deal with micronutrient deficiencies including the provision of mineral supplements, the fortification of processed food, the biofortification of crop plants at source with mineral-rich fertilizers and the implementation of breeding programs to generate mineral-rich varieties of staple crops, and the use of biotechnology for nutritional improvement (Gómez-Galera et al. 2010). Among these approaches, only conventional breeding and genetic engineering provide germplasm as a permanent and sustainable resource, and only genetic engineering allows the introduction of genes from any source directly into local varieties.

Perhaps, the best example of genetic engineering for nutrient enhancement in a developing country context is 'Golden Rice', which is enriched for β-carotene (provitamin A). This compound can be converted into retinal (the major functional form of vitamin A) by humans and other herbivorous/omnivorous mammals. Non-engineered cereal grains including rice and maize are poor sources of β-carotene, and polished rice grains contain no β-carotene at all. Vitamin A is required for vision and the correct functioning of the immune system. Vitamin A deficiency affects 127 million people in developing countries, including 25% of pre-school children, causing more than half a million cases of permanent blindness in children and 2.2 million deaths per year (UNICEF 2006). Therefore, many researchers have attempted to elevate β-carotene levels in staple cereals by introducing the corresponding metabolic pathway. The first significant advance was "Golden Rice 1", where the entire β-carotene biosynthetic pathway was reconstructed in the endosperm by expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase and lycopene β-cyclase, and a bacterial (*Erwinia uredovora*) phytoene desaturase; the resulting grains contained up to 1.6 µg/g of carotenoids by dry weight (Ye et al. 2000). Later, the daffodil phytoene synthase gene was substituted with the equivalent gene from maize, resulting in "Golden Rice 2", in which the total carotenoid content of the endosperm increased to 37 µg/g dry weight (Paine et al. 2005). Both Golden Rice lines were donated to the

Table 1 Maternal mortality ratio (uncertainty interval) per 100,000 live births by region and country (Hogan et al. 2010)

	1990	2000	2008
Asia-Pacific	14 (13–15)	10 (9–11)	8 (8–9)
Asia, central	72 (68–77)	60 (56–64)	48 (45–52)
Asia, east	86 (76–98)	55 (48–62)	40 (35–46)
Asia, south	560 (391–794)	402 (293–555)	323 (232–444)
Asia, southeast	248 (187–337)	212 (155–293)	152 (112–212)
Australasia	7 (6–8)	6 (5–7)	6 (5–7)
Caribbean	348 (234–518)	323 (218–483)	254 (168–372)
Europe, central	34 (31–37)	18 (17–20)	13 (12–14)
Europe, eastern	43 (39–48)	41 (37–45)	32 (29–35)
Europe, western	10 (10–11)	8 (8–9)	7 (7–8)
Latin America, Andean	229 (176–295)	156 (116–205)	103 (77–134)
Latin America, central	85 (77–94)	70 (64–78)	57 (51–63)
Latin America, southern	54 (49–60)	44 (39–49)	41 (36–45)
Latin America, tropical	113 (66–184)	71 (47–107)	57 (37–87)
North Africa/Middle East	183 (154–218)	111 (92–135)	76 (61–94)
North America, high income	11 (10–12)	13 (11–15)	16 (14–18)
Oceania	416 (252–649)	329 (202–518)	279 (174–434)
Sub-Saharan Africa, central	732 (488–1,101)	770 (535–1,108)	586 (392–839)
Sub-Saharan Africa, east	690 (574–842)	776 (639–948)	508 (430–610)
Sub-Saharan Africa, southern	171 (132–222)	373 (280–499)	381 (288–496)
Sub-Saharan Africa, west	582 (485–709)	742 (608–915)	629 (508–787)

Golden Rice Humanitarian Board, and up to six events of Golden Rice 2 were developed in the background of the American Kaybonnet variety, with one event selected for regulatory approval and commercialization. This line provides enough β-carotene in a 100-g portion of milled rice to achieve the recommended daily intake (RDI) of vitamin A for a child under five (Virk and Barry 2009) and could therefore prevent vitamin A deficiency (VAD) if consumed on a regular basis. Local popular rice varieties have been selected in several countries with widespread VAD (Bangladesh, India, Indonesia, The Philippines and Vietnam), and it is likely Golden Rice will be commercially available by 2012 in at least The Philippines and Bangladesh, the other countries following later (Zeigler 2009). There has been widespread criticism of the length of time it has taken to achieve regulatory approval and the barriers that have to be overcome to achieve adoption, a subject we discuss in detail below (Potrykus 2010).

Another key nutrient relevant in MDG4 and MDG5 is folic acid. Deficiency for folic acid in pregnancy leads to neural tube defects in the fetus and a greater chance of abortion or complications during delivery. Pregnant women require at least 600 mg of folate per day, but rice and maize provide nowhere near adequate amounts. Whereas processed food is supplemented with folic acid in the west, developing countries have not implemented sustainable folic acid supplementation programs. Folate synthesis in plants involves two separate pathways (the pterin and para-aminobenzoate branches) whose products are eventually conjugated together. Folate biofortification in rice seeds has been achieved by overexpressing two *Arabidopsis thaliana* genes, one from each of the pathways, resulting in a 100-fold enhancement. This means that 100 g of polished grains contains four times the RDI for folate (Storozhenko et al. 2007).

Although plants engineered to accumulate single nutrients are beneficial, they address only individual micronutrient deficiencies and would ultimately serve to displace rather than prevent malnutrition. For example, in the future where individual rice varieties with higher levels of β-carotene, folate, iron, zinc and other micronutrients are approved and widely available, people might have to choose between nutrients because it would be difficult to eat enough rice to cover all requirements. Two solutions offer themselves, i.e. the creation of nutritionally improved varieties that have such high levels of nutrients that only small portions are required (allowing a mixed meal of different varieties to satisfy all nutritional requirements) or the creation of varieties simultaneously enhanced for multiple nutrients. The latter would be simpler and more economical although the technical hurdles would be more difficult to overcome.

In an effort to address this issue, transgenic maize plants simultaneously enhanced for carotenoids, folate and ascorbate provide the first example of a nutritionally enhanced crop targeting three entirely different metabolic

pathways (Naqvi et al. 2009). This was achieved by transferring four genes into a white maize variety resulting in a 407-fold elevation of β-carotene levels (57 μg/g dry weight), 6.1-fold increase in ascorbate (106.94 μg/g dry weight) and a 2-fold increase in folate (200 μg/g dry weight). The decision to engineer three pathways at the same time rather than crossing lines individually engineered to increase the level of single nutrients was taken because the crossing strategy is slow and inefficient (Zhu et al. 2008). The simultaneous transformation strategy results in all the transgenes integrating at a single locus, which therefore remains stable through subsequent generations.

Pregnant women and infants also tend to have higher mineral requirements and particularly fall victim to deficiencies in iron (recommended daily allowance/adequate intake = 8 mg/day for males but 18 mg/day for women of reproductive age and 27 mg/day in pregnancy), zinc (RDA/AI = 8–13 mg/day for all) and calcium (RDA/AI = 1,000–1,300 mg/day for all). Calcium is essential for bone development, iron is needed for the synthesis of hemoglobin and various enzymes, and zinc is a cofactor for numerous enzymes and transcription factors. Mineral biofortification requires different strategies to vitamin biofortification because minerals are not synthesized de novo like organic compounds and must be sequestered from the environment (Gómez-Galera et al. 2010). One notable recent report describes the hyperaccumulation of iron in rice plants transformed with two genes, one encoding nicotianamine synthase (which is required for iron transport through the vascular system) and the other ferritin (which increases the capacity for iron storage) (Wirth et al. 2009). Many, although not all, of the channels and transporters that increase iron uptake also work with zinc often resulting in co-accumulation. Calcium levels in carrot roots and lettuce leaves were increased by 30–100% by overexpressing the H^+/Ca^{2+} transporter sCAX1, and this is another strategy that could be transferred to cereal crops (Morris et al. 2008; Park et al. 2009).

The role of plant biotechnology: using plants to produce inexpensive (oral) vaccines

Plants have been used for medicinal purposes for hundreds of years, but it is only recently that they have been deliberately engineered to produce specific pharmaceutical products (Twyman et al. 2005). Two broad strategies are envisaged. In the first, plants are simply an expression platform like any other (e.g. bacteria, yeast or mammalian cells), and the product is purified and formulated in the standard manner. In the second, plants are used as both the expression platform and the delivery vehicle, and this category includes the use of plants to produce oral vaccines. The principle is that a recombinant subunit vaccine is expressed in an edible plant organ such as potato tubers or cereal seeds and then administered as part processed food (e.g. puree or juice) which would be suitable for the large-scale immunization of adults and children in developing country settings (Yusibov and Rabindran 2008).

Plants have been used to produce many different vaccine candidates, including oral vaccines to prevent hepatitis B, cholera, rabies and diarrheal diseases in humans that have been successful in phase I clinical trials (Tiwari et al. 2009). The main technical challenge with oral vaccines is to induce a sufficient immunological response through mucosal immunity, which can be achieved by linking the antigen to a mucosal adjuvant such as the labile enterotoxin B subunit (LTB). The LTB protein was the first plant-derived recombinant oral antigen to be tested in phase I trials (Tacket et al. 1998).

Since diarrheal diseases account for a large proportion of under-five deaths in developing countries, the use of plant-derived oral vaccines to prevent sickness and diarrhea is the most relevant application of the technology in the context of MDG4. As proof of this concept, Tacket et al. (2000) developed an oral vaccine against Norwalk virus (which causes travelers' sickness), and the results of the phase I trials were similar to those with LTB, with nearly all of the volunteers who participated in the trial showing significant increases in the numbers of IgA-antibody forming cells (AFCs) and six also showing increases in IgG AFCs. There were also noticeable increases in serum IgG and stool IgA against the virus.

The provision of edible vaccines against common diseases in school children in developing countries could give parents additional encouragement to bring their children to school. For example, an oral vaccine comprising the cholera toxin subunit (CTB) expressed in rice under the control of an endosperm-specific promoter, induced antigen-specific mucosal and systemic immune responses in mice, and would be an excellent candidate to develop for human use in the developing world (Nochi et al. 2007). Advantages of vaccines delivered in cereal grains include the increased stability in storage and after administration, addressing distribution problems and the lack of a cold chain, and also prolonging the window of opportunity to induce an effective immune response after administration. The rice/CTB vaccine could be stored at room temperature for more than 18 months without degradation, and once administered it resisted the harsh environment in the stomach because it accumulated in endosperm storage organelles known as protein bodies which provided shielding (bioencapsulation). Oral immunization induced CTB-specific serum IgG and mucosal IgA, and conferred protection because serum from immunized mice prevented cholera toxin binding to GM1-ganglioside, which causes severe diarrhea.

As well as their use in humans, oral vaccines produced in plants also provide an inexpensive and convenient way to prevent disease in domestic animals, which would also help to increase the productivity and economic prosperity of farmers. Hundreds of vaccines for animal diseases have been expressed in plants, many proving efficacious in challenge studies. One worth particular mention is the recently developed vaccine against Newcastle disease in poultry, which was developed by Dow AgroSciences and became the first plant-derived vaccine to receive USDA approval. This product was developed to test the regulatory pathway and has not yet been marketed, but it has cleared the way for other vaccines produced using the same platform technology.

Despite the demonstrated efficacy of plant-derived vaccines, deployment for human populations seems unlikely at present. The approval process for Golden Rice indicates that there is an unwritten tiered approach to acceptability, with crops engineered to prevent pests and diseases now widely accepted, those with improved nutritional traits receiving guarded approval but still distrust of those with value-added products such as pharmaceuticals in an uncertain regulatory environment (Spok et al. 2008). There is a general regulatory consensus that crops producing pharmaceutical products would need to be segregated from food crops to prevent adventitious exposure to the bioactive substance and reduce the likelihood of outcrossing (Spok et al. 2008). An additional challenge specific to oral vaccines is achieving consistent doses of the antigen when delivering as part-processed food or feed/fodder. Paul and Ma (2010) present a critical review of plant-derived oral vaccines and the challenge of developing effective delivery strategies.

MDG 6: Combat HIV/AIDS, malaria and other diseases

Overview

HIV/AIDS, malaria and tuberculosis represent the major public health challenges in the world's poorest countries (Fig. 5). HIV is a virus often transmitted not only through sexual contact but also by intravenous drug use and from mother to child. The disease has caused more than 25 million deaths since it was first recognized in 1981, and 33.4 million people are currently thought to be HIV positive, 95% of whom live in developing countries (UNAIDS/WHO 2009). AIDS remains the leading cause of adult mortality in Africa today, and the sixth leading cause of death in the world. MDG6 aims to halt and reverse the spread of HIV/AIDS by 2015 and provide wider access to HIV drugs. Malaria is caused by parasites of the genus *Plasmodium*, transmitted by mosquitoes. It affects 350–500 million people each year, and

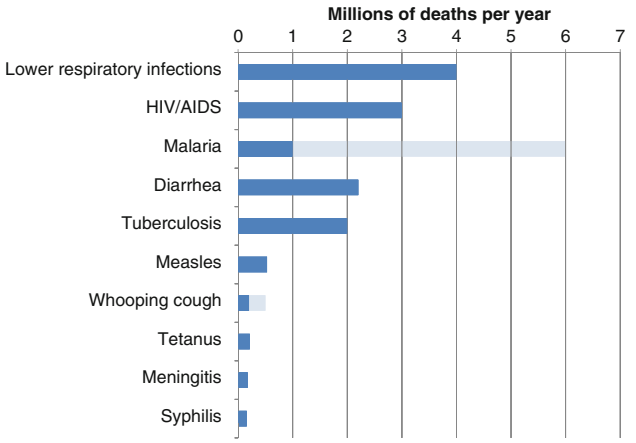

Fig. 5 Ranking of fatal diseases in the developing world (millions of deaths per year). Where accurate figures are not known, the two *bars* represent minimum and maximum estimates. Source: World Health Organization

one million die from the disease, particularly children under five and pregnant women, who are particularly vulnerable. As for HIV/AIDS, the poor are disproportionately affected and make up the vast majority of the 40% of the world's population living in high-risk areas. MDG6 aims to reduce the incidence of malaria globally and provide access to drugs and mosquito nets. Tuberculosis is a respiratory disease transmitted by aerosol, caused by the bacterium *Mycobacterium tuberculosis*. More than one-third of the world's population is thought to be infected, and the disease kills 1.7 million people each year, predominantly in developing countries (Elías-López et al. 2008). An approved tuberculosis vaccine, BCG (Bacille Calmette Guérin), is used worldwide and is administered to approximately 100 million infants per year providing good protection against the most severe childhood forms of the disease, and antibiotics can also be used to treat infections. However, these resources are not easily accessible in developing countries, hence the prevalence of the disease. HIV activates dormant tuberculosis, and more than 10 million people worldwide are infected with both HIV and tuberculosis.

The role of plant biotechnology (HIV)

Barrier methods help to prevent new HIV infections as well as protecting against other diseases and unplanned pregnancies, and one of the objectives under the HIV component of MDG6 is to increase education about the disease and the availability of condoms and other barrier devices. However, gender inequality and cultural preferences (see MDG3) place many women in the position of being unable to negotiate condom use without male cooperation, even if the male is known to be HIV positive (Population Council 2000; Padian et al. 1998).

Microbicides that are applied well in advance of sexual intercourse would place the means to control HIV infection in the hands of monogamous women. Several candidate products have been developed based on surfactants, HIV-neutralizing antibodies and lectins, alone or in combination with anti-retroviral drugs (Ramessar et al. 2010). One drawback of this approach is that antibodies must be used in very high doses (up to 1 g per application) because of their stoichiometric mechanism of action and to ensure enough of the active ingredient survives the harsh mucosal environment. The microbicide would have to be applied daily, perhaps several times a day, and with the anticipated demand this would require the relevant antibodies to be produced on a multi-ton scale which is several orders of magnitude above current production capacities. Moreover, antibodies are generally produced by fermentation in mammalian cells and are therefore among the most expensive biopharmaceuticals on the market. In order to supply microbicides to impoverished women in the rural communities of sub-Saharan Africa and South Asia, a revolutionary change in production technology would be necessary.

Plant biotechnology has a role to play in this scenario because plants provide a key advantage over animal cells for the production of biopharmaceuticals—the economy of scale. Increasing the scale of production in animal cells requires larger fermenters and facilities, whereas plants can be scaled up much more readily through additional land or greenhouse space (Ma et al. 2003; Twyman et al. 2005; Ramessar et al. 2008a, c). Many promising microbicide compounds have been successfully expressed in transgenic plants, including the antiviral lectins griffithsin (O'Keefe et al. 2009) and cyanovirin-N (Sexton et al. 2006). Plant-derived griffithsin showed broad spectrum activity against HIV at picomolar concentrations, was directly virucidal by binding to HIV envelope glycoproteins, and was capable of blocking cell-to-cell HIV transmission. It was also nonirritating and non-inflammatory in human cervical explants and in vivo in the rabbit vaginal irritation model. Cyanovirin-N was produced using hydroponic cultures and was shown to bind HIV gp120 and protect T cells from HIV infection in vitro.

HIV-neutralizing antibodies have also been produced in plants, including 2G12 (produced in tobacco and maize) (Ramessar et al. 2008b; Rademacher et al. 2008; Strasser et al. 2009), 2F5 (produced in tobacco) (Floss et al. 2009) and 4E10 (produced in tobacco). The HIV-neutralizing activity of tobacco and maize 2G12 was equal to or superior to that of the same antibody produced in CHO cells, and 2G12 has now been produced under GMP conditions in preparation for phase I clinical trials (the first plant-derived antibody to reach clinical development, through a publicly funded initiative). Another interesting example is the production of a combined microbicide candidate to minimize the risk of viral adaptation and the appearance of resistant strains and to provide sufficient cross-clade protection (Ramessar et al. 2010). Sexton et al. (2009) combined the HIV-neutralizing antibody b12 with cyanovirin-N and produced the fusion protein in transgenic tobacco. The fusion protein was more potent against HIV than either individual component.

The role of plant biotechnology (malaria)

Plants have also been used to express malarial antigens in an attempt to develop an inexpensive vaccine candidate, but such products are at a very early stage in development and would not be expected in the clinic for at least 5 years. However, plants are not solely used for the production of recombinant proteins—they are also valuable sources of antimalarial drugs, such as artemisinin. The cost of extracting artemisinin from its source means the drug is too expensive for the poorest people in developing countries, those most in need of it. The cost could be reduced by recreating the metabolic pathway leading to artemisinin in a plant species that is more accessible or easy to culture although there is currently insufficient knowledge of the enzymatic steps in the pathway (Ma et al. 2009).

The role of plant biotechnology (tuberculosis)

Plant-derived vaccine candidates against tuberculosis have been produced in tobacco and Arabidopsis, with some evidence that they generate immune correlates of protection. For example, Rigano et al. (2004) produced transgenic Arabidopsis plants expressing the immuno-dominant tuberculosis antigen ESAT-6 fused to a mucosal adjuvant and fed the oral vaccine to mice. They found that the fusion protein induced an immune response but unfortunately not enough to reduce the bacterial load and to protect mice against disease challenge. More recently, ESAT-6 and Ag85B were expressed in tobacco as fusions with an elastin-like peptide to increase their accumulation (Floss et al. 2010). Purified TBAg-ELP was obtained by inverse transition cycling and tested in mice and piglets for safety and efficacy. Antibodies recognizing mycobacterial antigens were produced in both species. A T-cell immune response recognizing the native mycobacterial antigens was detected in mice.

In a related approach, Elías-López et al. (2008) produced transgenic tomato plants expressing interleukin-12, a key cytokine. Oral delivery studies in which crude fruit extracts (lyophilized preparations) were fed to mice infected with various strains of the tuberculosis agent showed that the animals were more resistant to the disease and suffered less lung tissue damage having ingested the tomato extracts.

MDG 7: Ensure environmental sustainability

Overview

The objectives under MDG7 are to integrate the principles of sustainable development into country policies and programs and reverse the loss of environmental resources, reduce biodiversity loss significantly by 2010, reduce the proportion of the population without sustainable access to safe drinking water and basic sanitation to 50% of initial levels by 2015, and achieve a significant improvement in the lives of at least 100 million slum dwellers by 2020.

Sustainable development requires that natural resources are conserved, and while progress is being made in all areas, the rate of environmental destruction is still alarmingly high. Although urbanization and industrialization play an important role in this process, agriculture also has a major impact. As discussed above, access to safe water is limited in many countries because of pollution with both pathogens and chemical residues, particularly the run-off of agrochemicals. The 2010 target for biodiversity conservation has been missed, and key habitats for threatened species are not being adequately protected (UN 2010a). The rate of deforestation is slowing but even so averaged 5.2 million hectares per year over the last decade. More carbon dioxide is being released into the atmosphere than ever before, 35% more than 10 years ago. This trend needs to be stabilized and reversed if MDG7 is to be achieved.

The role of plant biotechnology

Plant biotechnology has a critical role to play in the improvement of environmental sustainability. Some of the major impacts have already been discussed in the context of other MDGs and will only be summarized here. These are: (1) the development of crops that require less water (drought-tolerant crops), thereby releasing more freshwater resources for drinking and for infrastructure development; (2) the development of high-yielding crops that produce adequate yields on smaller plots, thereby reducing the need for forests to be cut down to provide agricultural land; (3) the development of crops that are resistant to weeds, insect pests and pathogens to reduce chemical use and fuel consumption. The deployment of Bt crops has reduced the use of pesticides, also saving on fossil fuels required for spraying. The deployment of herbicide-tolerant crops has reduced fuel use and CO_2 emissions by limiting the need for plowing, and conserving soil and moisture by encouraging no-tilling agriculture. The cumulative reduction in pesticide use for the period 1996–2008 was approximately 356,000 tons (8.4%), which is equivalent to a 16.1% reduction in the associated net environmental impact as measured by the environmental impact quotient (EIQ). The corresponding data for 2008 alone revealed a reduction of 34,600 tons of pesticides (9.6%) and a reduction of 18.2% in EIQ (Brookes and Barfoot 2010). In countries such as India, China, Argentina and Brazil, which are the most enthusiastic adopters of Bt agriculture after the US and Canada, the greatest impact of Bt has been the reduction in the number of pesticide sprays (Naranjo 2009). In India, for example, the reduction is from 16 down to 2–3 sprays per growing season (Qaim et al. 2006; Karihaloo and Kumar 2009).

MDG8: Develop a global partnership for development

The MDGs represent a global partnership for development, and developing countries must take on the primary responsibility to work towards achieving the first seven MDGs. They must do their part to ensure greater accountability and efficient use of resources. But for developing countries to achieve this, it is absolutely critical that developed countries deliver on their end of the bargain with more effective aid, more sustainable debt relief and fairer trade rules, well in advance of 2015.

The objectives in MDG8 are to (a) address the special needs of least developed countries, landlocked countries and small island developing states; (b) develop an open, predictable, nondiscriminatory trading and financial system; (c) deal comprehensively with developing countries' debt; (d) make available the benefits of new technologies in cooperation with the private sector, especially information and communications. In terms of plant biotechnology, the fourth objective is the most relevant, and we already have several examples of how this has been put into practice with the Golden Rice Humanitarian Board and WEMA (see above). These programs form the basis for technology donation for humanitarian purposes, where technology can be used royalty-free for subsistence agriculture or to alleviate poverty, hunger, malnutrition and disease. The two examples cited above focus in one case on nutritional improvement and in the other on the avoidance of starvation during drought, but the same principles apply to pharmaceutical plants. For example, all the partners in the Pharma-Planta consortium (http://www.pharma-planta.org), which has established the regulatory pathway necessary to produce HIV-neutralizing antibodies in plants (Spok et al. 2008), have signed up to a humanitarian use clause which allows all the technology developed in the project (as well as any necessary background IP) to be used royalty-free for humanitarian purposes. The HarvestPlus Challenge Program is a similar concept although focusing on conventional biofortification strategies and mostly eschewing genetic engineering.

Because MDG8 will depend on political cooperation between developed and developing countries, this is the appropriate juncture to discuss the role of politics in plant biotechnology and the barriers to adoption that have been erected (Farre et al. 2009). Plant biotechnology is one of a raft of strategies that can be combined to make progress towards the MDGs, and many of the technological barriers have been overcome. However, the impact of this scientific progress is being neutralized by the unwillingness of politicians to see beyond immediate popular support and to take politically controversial decisions that would in the short to medium term save millions of lives and in the long term would make a significant impact on the health, well-being and economic prosperity of the world's poorest people. The problem is essentially that whereas political decision-making should be based on rational scientific evidence, it is more often dictated by certain NGOs with dubious agendas and the media, which thrives on sensationalism (Farre et al. 2010). Unfortunately, this feeds back in such a way that those charged with regulating biotechnology are pressured into implementing excessive regulation, which extends development times unnecessarily and results in many more lives being put at risk (Farre et al. 2010).

Conclusions and outlook

Each of the MDGs reflects one or more fundamental aspects of socioeconomic development in countries that depend predominantly on subsistence agriculture to feed their populations. Therefore, it seems natural that the improvement of agricultural productivity should form the keystone upon which the framework of progress can be built. In this context, technological solutions to improve agricultural productivity and sustainability can be regarded as a valuable approach to ensure rapid progress towards the MDGs, particularly technologies that improve yield, vigor and nutritional value in staple crops and allow the production of added-value products such as pharmaceuticals.

The prospects of implementation vary considerably, with some products already deployed and having a strong impact, others on the verge of approval, and others unlikely to see large-scale deployment by 2015 if at all. The success of Bt crops in India and China is likely to be repeated in Africa and South Asia as these have reduced hunger and led to economic prosperity within a remarkably short time. Many additional Bt varieties are waiting in the wings, and perhaps, even more exciting is the prospect of multi-trait crops simultaneously protected against a range of pests and viral and microbial diseases, as well as drought and other environmental factors. Within the next 2 years, we should also see the first commercial release of Golden Rice, and this will hopefully open the door for a range of additional nutritionally enhanced crops that will address food insecurity in a sustainable manner.

The prospect of more ambitious technologies such as the use of plants to produce vaccines and drugs is unlikely to have an immediate impact in the developing world because the regulatory burden would be high and the construction of contained facilities would provide no further advantage compared to production in the west. In the short term, it is more likely that plant-derived pharmaceuticals will fill niche markets in the west and then spread to high-volume, low-margin products as yields improve, but the royalty-free donation of technologies and products may lower the cost of goods to the extent required to meet the demands of local health authorities in developing countries.

Most importantly, it is clear that the irrational political handling of plant biotechnology must be resolved so that developing countries are not put in the position of choosing between principles and lives. National and International funding agencies and charitable organizations should encourage collaborative projects with universities and other research organizations in target countries so that capacity-building programs can prepare a generation of local experts to establish their own research facilities, enabling them to operate independently, without political pressure, to develop sustainable solutions for their own populations. Most importantly, there must be leadership from the top—the EU needs to stop pandering to activists and the media, and should take decisions based on rational scientific evidence in order to help the world's most vulnerable people. Only when bold decisions are made in Europe and elsewhere in the industrialized world, can the fruits of our scientific endeavor be used to accelerate progress towards the MDGs.

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Appendix 1: The Millennium Development Goals in full (UN 2010a)

Goal 1: Eradicate extreme poverty and hunger

- Target 1A: Halve the proportion of people living on less than $1 a day
- Target 1B: Achieve decent employment for women, men, and young people
- Target 1C: Halve the proportion of people who suffer from hunger

Goal 2: Achieve universal primary education

Target 2A: By 2015, all children can complete a full course of primary schooling, girls and boys

Goal 3: Promote gender equality and empower women

Target 3A: Eliminate gender disparity in primary and secondary education preferably by 2005 and at all levels by 2015

Goal 4: Reduce child mortality rate

Target 4A: Reduce by two-thirds, between 1990 and 2015, the under-five mortality rate

Goal 5: Improve maternal health

Target 5A: Reduce by three quarters, between 1990 and 2015, the maternal mortality ratio
Target 5B: Achieve, by 2015, universal access to reproductive health

Goal 6: Combat HIV/AIDS, malaria, and other diseases

Target 6A: Have halted by 2015 and begun to reverse the spread of HIV/AIDS
Target 6B: Achieve, by 2010, universal access to treatment for HIV/AIDS for all those who need it
Target 6C: Have halted by 2015 and begun to reverse the incidence of malaria and other major diseases

Goal 7: Ensure environmental sustainability

Target 7A: Integrate the principles of sustainable development into country policies and programs, and reverse loss of environmental resources
Target 7B: Reduce biodiversity loss, achieving, by 2010, a significant reduction in the rate of loss
Target 7C: Halve, by 2015, the proportion of people without sustainable access to safe drinking water and basic sanitation
Target 7D: By 2020, to have achieved a significant improvement in the lives of at least 100 million slum-dwellers

Goal 8: Develop a global partnership for development

Target 8A: Develop further an open, rule-based, predictable, non-discriminatory trading and financial system
Target 8B: Address the special needs of the least developed countries (LDC)
Target 8C: Address the special needs of landlocked developing countries and small island developing states
Target 8D: Deal comprehensively with the debt problems of developing countries through national and international measures in order to make debt sustainable in the long term
Target 8E: In co-operation with pharmaceutical companies, provide access to affordable, essential drugs in developing countries
Target 8F: In co-operation with the private sector, make available the benefits of new technologies, especially information and communications

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