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REVIEW ARTICLE

Transplastomic plants for innovations in agriculture. A review

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Abstract Food production has to be significantly increased in order to feed the fast growing global population estimated to be 9.1 billion by 2050. The Green Revolution and the development of advanced plant breeding tools have led to a significant increase in agricultural production since the 1960s. However, hundreds of millions of humans are still undernourished, while the area of total arable land is close to its maximum utilization and may even decrease due to climate change, urbanization, and pollution. All these issues necessitate a second Green Revolution, in which biotechnological engineering of economically and nutritionally important traits should be critically and carefully considered. Since the early 1990s, possible applications of plastid transformation in higher plants have been constantly developed. These represent viable alternatives to existing nuclear transgenic technologies, especially due to the better transgene containment of transplastomic plants. Here, we present an overview of plastid engineering techniques and their applications to improve crop quality and productivity under adverse growth conditions. These applications include (1) transplastomic plants producing insecticidal, antibacterial, and antifungal compounds. These plants are therefore resistant to pests and require less pesticides. (2) Transplastomic plants resistant to cold, drought, salt, chemical, and oxidative stress. Some pollution tolerant plants could even be used for phytoremediation. (3) Transplastomic plants having higher productivity as a result of improved photosynthesis. (4) Transplastomic plants with enhanced mineral, micronutrient, and macronutrient contents. We also evaluate field trials, biosafety issues, and public concerns on transplastomic plants. Nevertheless, the transplastomic technology is still unavailable for most staple crops, including cereals. Transplastomic plants have not been commercialized so far, but if this crop limitation were overcome, they could contribute to sustainable development in agriculture.

László Sági and Katalin Solymosi contributed equally to this work.

This paper is dedicated to the memory of Professor István Gyurján (1935–2009), Eötvös Loránd University, Budapest.

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Contents

1 Introduction

Almost 805 million people, i.e., one out of nine people, were chronically undernourished in 2012–2014; the vast majority of them are living in less developed countries (FAO, IFAD, WFP 2014). In addition to starvation and insufficient macronutrient (protein, carbohydrate, lipid, oil, and fiber) and calorie uptake, micronutrient (vitamin, mineral, and phytochemical) deficiency, often termed as hidden hunger, also leads to compromised health and economic losses. Micronutrient deficiency is prevalent in poor populations worldwide where daily calorie intake is largely restrained to non-diversified plant-based diets including staple cereals (Bhullar and Gruissem 2013; Joy et al. 2014) cultivated in regions where soil mineral imbalances occur (White and Broadley 2009). Polishing, milling, and pearling of cereals make them even poorer in micronutrients (Welch and Graham 2004; Borg et al. 2009).

Climate change creates an additional challenge to food security (FAO 2009). Regional decrease in available arable soils and their quality represents a huge global concern. Exploitation, improper land use, and heavy chemical inputs, e.g., from pesticides and fertilizers, by the modern, intensive agriculture in order to achieve high crop yields in monocultures (Zuo and Zhang 2009) often result in pollution and nutrient imbalance in the soil. These perturbances lead to the accumulation of harmful compounds or nutrient deficiency, respectively, in edible plant parts (Khoshgoftarmanesh et al. 2010; Solymosi and Bertrand 2012). However, farmers and companies do not necessarily take into account the long-term consequences of their land use and strive, instead, to maximize their profit on a short term.

Therefore, it is generally accepted that a global integration of improved crop cultivars with innovative and sustainable agricultural methods, i.e., a second Green Revolution, is needed if we want to feed an expected human population of 9.1 billion in 2050 (FAO 2009). In order to adapt farming systems that ensure crop productivity and food security worldwide, alteration of important crops and elevating their yields may be applied in an environment-friendly and well-controlled manner by simultaneously conserving natural habitats and resources (Martino-Catt and Sachs 2008; Clarke and Daniell 2011). Conventional plant breeding is often considered as a relatively time-consuming and lengthy process, while genetic modification of crop plants is frequently referred to as a rapid and promising (although not the sole) solution for several of the aforementioned global problems. Since the commercialization of the first genetically modified (GM) crops in 1994, agricultural biotechnology has delivered several GM plants with improved agronomic traits, such as food functionality, resistance to biotic and abiotic stress factors, decreased allergenicity, etc.

However, there is a heated debate about the use of such organisms. Opponents of genetically modified organisms (GMO) often use emotional or obsolete arguments based sometimes on questionable scientific data and experiments to ban GM products from the market. At the same time, many proponents of genetic modification are alleged to be associated, directly or indirectly, with companies involved in the production of transgenic crop cultivars or are personally too much involved in transgenic research to give an unbiased scientific opinion on this issue. As plant biologists working among others with GM plants and on plastid biology, we attempt to give an objective overview and a general outline about the possible role plastid engineering might play in improving food quantity and quality in the future. This technology seems to be especially promising for GM crops because it can further extend their cultivation as plastids are not transmitted via the pollen in most crops, thus the transgenes will remain better contained (Section 5).

In this review, we refer to GM plants obtained by nuclear or plastid transformation as transgenic or transplastomic plants, respectively. This overview discusses data related to genetic modification of the plastid genome of higher plants with emphasis on crops. For details about plastid transformation protocols developed for eukaryotic (micro)algae, the readers are kindly directed to recent reviews (Koop et al. 2007; Day and Goldschmidt-Clermont 2011; Purton et al. 2013).

2 Genetic transformation of plastids

The best known plastid type is the chloroplast, which is characterized by its capacity to assimilate carbon, nitrogen, and sulfur. In addition to carbohydrates, plastids are also involved in the synthesis and/or the storage of amino acids, lipids and fatty acids, starch, oil, and some secondary metabolites including carotenoids, terpenoids, alkaloids, lignanes, essential vitamins such as vitamin A, B1, B2, B3, B9, E, and K (Fitzpatrick et al. 2012) or polyphenolic compounds like condensed

tannins (Brillouet et al. 2013) (reviewed in Verma and Daniell 2007; Solymosi and Keresztes 2012).

Plastids are semi-autonomous, endosymbiotic organelles of prokaryotic origin. They contain circular double-stranded DNA and have retained their own nucleic acid and protein synthesis machinery. Several genes of the originally engulfed prokaryotes have been either lost or more typically transferred to the nucleus resulting in a highly reduced plastid genome size of 120–220 kb carrying approximately 120 genes. In addition, nearly 10 % of the nuclear gene products are also plastid targeted (Maliga and Bock 2011; Bock 2014) and have a basic role in regulating and determining the function of this organelle.

The plastid genome—also termed plastome, plastid DNA, or ptDNA—is highly polyploid, i.e., it is present in several identical copies in each plastid. This results in 500 to 10,000 plastome copies in a mesophyll cell (Bendich 1987; Koop et al. 2007; Zoschke et al. 2007) and fewer copies in other cell types containing less plastids.

To modify the plastid genome of higher plants, there are four major "technical" steps to accomplish: (1) to deliver foreign DNA through the cell wall, the plasma membrane, and then the double envelope membrane of the plastid; (2) to direct the stable insertion of the foreign DNA into the plastid genome via site-specific recombination; (3) selective enrichment of transferred DNA within plastids and of transformed plastids in cells to reach the high-copy homoplasmic state; and (4) regeneration of homoplasmic cells carrying the transgene into fertile transplastomic plants (Fig. 1).

DNA delivery to plastids can be carried out by a number of alternative methods, two of which are currently available and generally used to stably introduce foreign DNA into plastids: (1) biolistic approach, i.e., bombardment of tissues with a particle gun (Boynton et al. 1988; Ye et al. 1990; reviewed in Altpeter et al. 2005), or (2) treatment of protoplasts with polyethylene glycol (PEG) (Golds et al. 1993; O'Neill et al. 1993; reviewed in Kofer et al. 1998) (Table 1).

Stable insertion of foreign DNA into plastids is obtained using *Escherichia coli* plasmid derivatives as transformation vectors. These contain a selectable marker gene and usually also one or several transgenes of interest, which are introduced to the plastid genome at a carefully chosen specific insertion site by two homologous recombination events of flanking sequences. Although several selection markers and protocols have been developed, those based on *aadA* (encoding aminoglycoside 3″-adenylyltransferase, EC 2.7.7.47, and conferring spectinomycin and streptomycin resistance to bacteria by detoxification) are the most frequent ones (Table 1), most likely because they require low expression levels to confer phenotypic resistance. Detailed description and historical overview of the used vectors including promoters, effective selection markers, reporter genes, their insertion, and eventual removal are provided by recent reviews (Maliga 2003, 2004; Koop et al. 2007; Verma and Daniell 2007; Ruhlman et al. 2010; Day and Goldschmidt-Clermont 2011; Maliga and Bock 2011; Ahmad and Mukhtar 2013; Hanson et al. 2013; Vafaee et al. 2014).

The available methods for the physical delivery of DNA to plastids have relatively low transformation efficiency, i.e., very few plastids and cells get in adequate contact with the introduced DNA to enable homologous recombination and stable integration of the transgene into the plastid genome (Fig. 1). Thus, homologous recombination occurs in the plastid DNA, but only in one or few plastid DNA copies, and the vast majority of plastids still carry non-transformed DNA copies in the cells, i.e., the plastids and cells are heteroplasmic (Fig. 1). In one of the most frequently used species, tobacco, 5–15 plastid transformation events per leaf are in general achieved by bombardment, but in other crops and/or with other methods, this number is drastically lower (Daniell et al. 2001, 2005; Koop et al. 2007).

In order to obtain new and genetically uniform transplastomic crops, the transformed plastid DNA copies have to be maintained, while the plastids carrying non-transformed DNA have to be gradually eliminated on a selective medium (reviewed in Maliga 2003, 2004; Day and Goldschmidt-Clermont 2011; Maliga and Bock 2011). Selective amplification of the transgenic DNA copies and elimination of the non-transformed ones first within the highly polyploid plastids, then in the plant cells, followed by subsequent identification and amplification of these so-called homoplasmic cells, and especially the reproducible regeneration protocol of genetically uniform and fertile plants containing a uniform population of transformed plastid genomes from these cells (and/or tissue cultures) (Fig. 1) represent the major bottlenecks for the extension of plastid transformation technology to new crops (Maliga and Bock 2011).

The most important chloroplast transformation protocols and selection conditions developed for major crops are listed in Table 1.

Plastids have become attractive targets for genetic engineering efforts as compared with nuclear transgenic technologies (reviewed in Meyers et al. 2010) due to several potential advantages. These include (1) absence of gene silencing, epigenetic, and/or position effects, which eliminates the high variation in gene expression and thus in protein accumulation levels among independent transgenic lines; (2) high protein expression levels due to very high plastid DNA copy number per chloroplasts/cells/organ resulting in the accumulation of large amounts of the transgene's product in the chloroplast/cell/organ; (3) possibility of multigene engineering (including cDNAs instead of full genes) through the use of transgene stacking in operons in a single transformation event; and (4) almost complete absence of pleiotropic effects due to subcellular compartmentalization of the transgene products (e.g.,

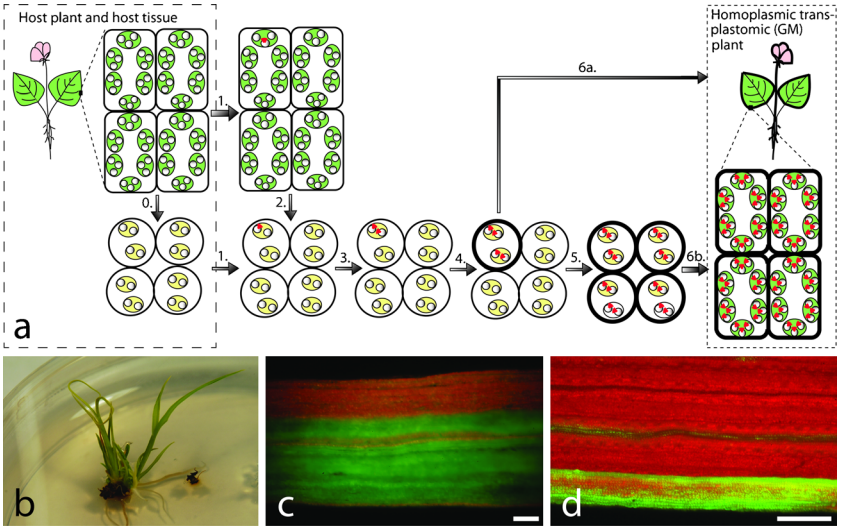

Fig. 1 Production of transplastomic plants. **a** Simplified schematic representation of genetic transformation and subsequent sorting of transformed plastomes at the organelle and cellular levels to yield genetically stable homoplasmic transformed plants. During transformation (*1*), the targets are most often leaf explants, i.e., the sterilized leaf mesophyll cells (harboring several chloroplasts, represented as *light gray/green oval bodies*) of excised leaves are directly bombarded (*1*, *upper row*); however, rarely protoplasts (e.g., in case of polyethylene glycol transformation) or embryogenic cells, calli can be also the targets of transformation. In the latter case (*1*, *lower row*), before transformation, mesophyll cells (or other differentiated plant cells) and their plastids must first undergo dedifferentiation (*0*). During dedifferentiation, cell size, the number, and size of plastids decrease in parallel with chloroplast to proplastid dedifferentiation (proplastids are represented as *light gray/yellow circles*), resulting in lower transformation efficiency. However, during transformation (*1*), only a few plastid genome copies (plastomes, represented as *white circles* within the plastids) of a few plastids in a few leaf cells of the recipient (host) plant are transformed (because the transformation efficiency of the available methods is relatively low also in case of biolistic bombardment of recipient leaves or tissues with the transformation vector, and also in case of polyethylene glycol treatment). Transformed plastomes are represented with a dark (*black/red*) inserted transgene. Repeated rounds of propagation and selection (*3–5*) of the plastids (e.g., in tissue culture medium, sometimes also by regeneration of shoots like in case of tobacco—not shown in this figure) can yield first a homoplasmic plastid carrying only the transformed plastome in a cell that may also contain non-transformed plastids, then a cell with only homoplasmic plastids (carrying only the transformed plastome, represented with thickened shapes) in a chimeric tissue. Regeneration from a single transplastomic cell (containing only homoplasmic plastids), for example by somatic embryogenesis (*6a*), or from several homoplasmic cells, for example by organogenesis (*6b*), facilitates the recovery of homoplasmic plants containing only the transformed plastid DNA (transplastomic plants). Selection and propagation are conveniently carried out on tissue culture medium, and, in addition, the dedifferentiation of mesophyll cell chloroplasts into proplastids of callus cells (*2*) facilitates the selective amplification of the transformed plastid DNA molecules and plastids/cells carrying such molecules also in case of bombarded leaves used as transformation targets (*1*, *upper row*). (Please note that nucleoids—i.e., the clusters of several plastome copies characteristic for plastids—are not shown in this simplified scheme.) **b** Heteroplasmic GFP-expressing transplastomic rice plant in vitro. **c** Leaf from the plant shown in (**b**) under fluorescence microscope with GFP-expressing transformed sectors (*green/light gray*) and untransformed sectors with dominating red autofluorescence of chlorophylls. **d** A different leaf from the same heteroplasmic rice plant at a higher magnification. (Source of (**b**)–(**d**)—Barnabás Jenes and Alfora Stella Gonzalez-Coronel, NARIC Agricultural Biotechnology Institute, Gödöllő, Hungary.) The *bar* indicates 1 mm

Staub et al. 2000; Bock 2001, 2013; De Cosa et al. 2001; Daniell et al. 2002; Quesada-Vargas et al. 2005; Verma and Daniell 2007; Oey et al. 2009; Ruhlman et al. 2010; Meyers et al. 2010). From the biosafety point of view, the plastid technology (5) significantly increases transgene containment because plastids are maternally inherited in most crops, and therefore, the transgenes are not transmitted by pollen (Section 5) and outcrossing with weeds and other plants is not possible (Daniell et al. 1998, 2002; Daniell 2002; Hagemann 2004, 2010).

However, another challenge of this technique is to introduce and stably express foreign DNA in(to) non-green tissues containing several kinds of non-green plastids (typically proplastids in dedifferentiated cells), in which gene expression and gene regulation systems are quite different from mature green chloroplasts (Bogorad 2000; Daniell et al. 2002; Valkov et al. 2009). For instance, the transcript levels of photosynthesis genes and tRNA genes are much lower than those of genes encoding other complexes in amyloplasts (Valkov et al. 2009) and chromoplasts (Kahlau and Bock 2008). The latter reflects the reduced need for translation in these plastids. On the other hand, *accD*, a plastid gene involved in fatty acid biosynthesis, showed relatively high levels of total and ribosome-associated transcripts in amyloplasts (Valkov et al. 2009) and chromoplasts (Kahlau and Bock 2008). However, the genome-wide alterations in expression patterns and their exact regulation mechanisms are largely unknown in non-green plastids. This problem is especially persistent in crops that are regenerated in vitro through somatic embryogenesis, which represent a large portion of important crops (Daniell et al. 2005). In these plants, plastid transformation is hindered by the lack of (1) selectable markers, (2) the ability to express transgenes in

Table 1 Important milestones in the development of chloroplast transformation methods and selection conditions for different plant species

Species	Methods	Selection	Expressed marker and target genes	Reference
Nicotiana tabacum (tobacco)	Bombardment	Spectinomycin, streptomycin	*rrnS* (*rrn16*)	Svab et al. 1990
	Bombardment	Kanamycin	*nptII* (*kan*)	Carrer et al. 1993
	PEG	Spectinomycin, streptomycin	*rrnS*	Golds et al. 1993
	Bombardment	Spectinomycin, streptomycin	*aadA*	Svab and Maliga 1993
	Bombardment	Spectinomycin	*aadA*, *uidA*	Staub and Maliga 1995
	Bombardment	5-Fluorocytosine	*aadA*, *codA*	Serino and Maliga 1997
	Bombardment	Spectinomycin	*aadA*::*gfp*	Khan and Maliga 1999
	Bombardment	Spectinomycin, streptomycin, PPT[a]	*aadA*, *bar*, *uidA*	Iamthan and Day 2000
	Bombardment	Spectinomycin	*aadA*, *hST*	Staub et al. 2000
	Bombardment and PEG	Spectinomycin, betaine aldehyde	*aadA*, *badh* (*betB*)	Daniell et al. 2001
	Bombardment	Spectinomycin	*aadA*, Bt *cry2Aa2* operon	De Cosa et al. 2001
	Bombardment	Spectinomycin, glyphosate[a], PPT[a]	*aadA*, *epsps*, *bar*	Ye et al. 2001, 2003
	Bombardment and PEG	Kanamycin	*aphA-6*	Huang et al. 2002
	Bombardment	Photoautotrophy, kanamycin	*petA*, *ycf3*, *rpoA*, and *aphA-6*	Klaus et al. 2003
	Bombardment	Photoautotrophy, spectinomycin	*rbcL*, *aadA*, *uidA*	Kode et al. 2006
	Bombardment	Spectinomycin, PPT[a]	*aadA*, *bar*	Lutz et al. 2006
	Bombardment	Spectinomycin, diketonitrile[a]	*aadA*, *hppd*	Dufourmantel et al. 2007
	PEG	Spectinomycin, streptomycin	*rps12*, *rrnS*, *des*	Craig et al. 2008
	Bombardment	Spectinomycin, leaf color	*aadA*, *bar*^{au}	Lutz and Maliga 2008
	Bombardment	7-Methyl-tryptophan, 4-methylindole analogs	*ASA2*	Barone et al. 2009
	Bombardment	Glyphosate[a]	*epsps* (*aroA*)	Roudsari et al. 2009[c]
	Bombardment	Spectinomycin, autoluminescence	*aadA*, *lux* operon	Krichevsky et al. 2010
	Bombardment	Spectinomycin	*aadA*, *PDF1B*	Fernández-San Millán et al. 2011
	Bombardment	Chloramphenicol, spectinomycin	*cat*, *aadA*	Li et al. 2011
	Bombardment	Spectinomycin, leaf color	*aadA*^{au}	Tungsuchat-Huang et al. 2011
	Bombardment	Spectinomycin, streptomycin, D-valine or D-alanine	*aadA*, *dao*	Gisby et al. 2012
	Bombardment	None (cytokinin-free)	*ipt*	Dunne et al. 2014
Arabidopsis thaliana (thale cress)	Bombardment	Spectinomycin	*aadA*	Sikdar et al. 1998[c]
Solanum tuberosum (potato)	Bombardment	Spectinomycin	*aadA*, *gfp*	Sidorov et al. 1999
	Bombardment	Spectinomycin, streptomycin	*aadA*, *uidA*	Segretin et al. 2012
Oryza sativa (rice)	Bombardment	Spectinomycin	*aadA*::*gfp*	Khan and Maliga 1999[b,c]
	Bombardment	Streptomycin	*aadA*, *gfp*	Lee et al. 2006[b]
Solanum lycopersicum (tomato)	Bombardment	Spectinomycin, streptomycin	*aadA*	Ruf et al. 2001

Table 1 (continued)

Species	Methods	Selection	Expressed marker and target genes	Reference
	PEG	Spectinomycin, streptomycin	*rrnS*	Nugent et al. 2005
Brassica napus (oilseed rape)	Bombardment	Spectinomycin	*aadA*, *cry1Aa10*	Hou et al. 2003[b,c]
	Bombardment	Spectinomycin	*aadA*, *HSA*	Cheng et al. 2010[b]
Lesquerella (*Physaria*) *fendleri* (bladderpod)	Bombardment	Spectinomycin, streptomycin	*aadA*::*gfp*	Skarjinskaia et al. 2003
Daucus carota (carrot)	Bombardment	Spectinomycin	*aadA*, *badh* (*betB*)	Kumar et al. 2004a
Glycine max (soybean)	Bombardment	Spectinomycin	*aadA*	Dufourmantel et al. 2004
	Bombardment	Spectinomycin, diketonitrile[a]	*aadA*, *hppd*	Dufourmantel et al. 2007
Gossypium hirsutum (cotton)	Bombardment	Kanamycin	*aphA-6*, *nptII*	Kumar et al. 2004b
Petunia× *hybrida* (*P.* × *atkinsiana*) (petunia)	Bombardment	Spectinomycin, streptomycin	*aadA*, *gusA* (*uidA*)	Zubko et al. 2004
Lactuca sativa (lettuce)	PEG	Spectinomycin	*aadA*, *gfp* or *aadA*, *HA*	Lelivelt et al. 2005
	Bombardment	Spectinomycin	*aadA*, *gfp*	Kanamoto et al. 2006
	Bombardment	Spectinomycin	*aadA*, *pagA* or *aadA*, *CTB-Pins*	Ruhlman et al. 2010
Brassica oleracea var. *botrytis* (cauliflower)	PEG	Spectinomycin	*aadA*	Nugent et al. 2006
Populus alba (white poplar)	Bombardment	Spectinomycin	*aadA*, *gfp*	Okumura et al. 2006
Brassica oleracea var. *capitata* (cabbage)	Bombardment	Spectinomycin, streptomycin	*aadA*, *uidA*	Liu et al. 2007
Beta vulgaris (sugar beet)	Bombardment	Spectinomycin	*aadA*, *gfp*	De Marchis et al. 2009
Solanum melongena (eggplant, brinjal)	Bombardment	Spectinomycin, streptomycin	*aadA*	Singh et al. 2010
Medicago sativa (alfalfa)	Bombardment	Spectinomycin	*aadA*::*gfp*	Wei et al. 2011[c]

In some cases, synonyms of genes are indicated between parentheses for consistency

PEG polyethylene glycol, *PPT* phosphinothricin/glufosinate

[a] Indicates herbicides involved in selection

[b] Indicates when only heteroplasmic plants were reported

[c] Indicates sterility of the transformed plants or no transmission of the transgenes to the progeny

non-green plastids and (3) adequate tissue culture, and (4) regeneration protocols to obtain homoplasmic plants.

Taken together, these data underline that the lack of (1) proper transformation and especially selection and regeneration protocols to obtain fertile homoplasmic crops, and (2) transgene expression in non-green tissues represent still the bottleneck of the use of this method in several major crops. At present, nuclear transformation has higher efficiency and significantly lower costs. It is also less laborious because the same binary vector can be used in all plants within the *Agrobacterium* host range, and nuclear transformation is also faster than plastid transformation (Meyers et al. 2010). There are fundamental differences between the nuclear and chloroplast genomes, e.g., proteins are not known to be exported from plastids, so the two methods are not always interchangeably applicable. Since plastid transformation has its own advantages as discussed above, the pros and cons of nuclear versus plastid genetic engineering have to be carefully examined for various biotechnology applications (reviewed in Daniell et al. 2002; Meyers et al. 2010).

3 Various uses of plastid transformation

Plastid engineering is considered as a promising technology for crop improvement as well as an emerging approach for the production of recombinant proteins in plants (Meyers et al. 2010). Depending on the construction of the transformation vector, the insertion can be directed at a number of places in the plastid DNA resulting in distinct expression levels and various potential applications. These include (1) a better understanding of plastid biology, metabolism, and evolution in basic science, (2) the optimization of plant performance or quality in natural or artificial environments, and (3) the introduction of new physiological traits or metabolic processes by recombinant protein expression for applied biotechnology and agronomy research. Resistance engineering and molecular pharming are areas that typically require high levels of gene expression that is characteristic of plastid transformation, while metabolic pathway engineering usually requires lower expression levels (Bock 2007).

3.1 Applications in basic science

By replacing a mutant chloroplast gene with a wild-type gene to restore its function, plastid transformation was first achieved in 1988 in the unicellular alga, *Chlamydomonas reinhardtii* (Boynton et al. 1988). Since then, chloroplast transformation technologies have been used to study plastid metabolic processes and the function of plastid genes in different areas of functional genomics (Bock 2001; Daniell et al. 2002; Maliga 2004). Studies using targeted inactivation, i.e., site-directed mutagenesis, gene knockouts, gene replacement, deletion, excision as well as overexpression of countless genes, have greatly contributed to our understanding of basic plastid physiology and biochemistry including among others bioenergetic processes, transcription, RNA editing, translation regulation, etc. They also contributed to the improvement of plastid transformation technology, but are discussed elsewhere in detail (Maliga 2004; Bock 2007; Koop et al. 2007).

3.2 Molecular pharming—accumulation of recombinant proteins

There is significant interest in plant-based production of antibodies, human therapeutical proteins, protein antibiotics, (oral/edible) vaccines, industrial enzymes, and biomaterials. Alterations of the plastid genome represent a promising possibility for high-level, clean, and safe expression of proteins (and other products) in cost-effective commercial applications. The advantages and challenges of plant molecular pharming are extensively reviewed elsewhere (Daniell et al. 2002, 2009; Daniell 2006; Bock 2007, 2014; Verma and Daniell 2007; Chebolu and Daniell 2009; Rybicki 2009; Bock and Warzecha 2010; Cardi et al. 2010; Meyers et al. 2010; Wani et al. 2010; Lössl and Waheed 2011; Maliga and Bock 2011; Obembe et al. 2011; Scotti et al. 2012; Ahmad and Mukhtar 2013). Although several plastid-derived vaccine antigenes have already been tested in animal models (Lössl and Waheed 2011), to our knowledge no transplastomic plants have been licensed for biopharmaceutical use (Section 4).

Bioplastic and biofuel synthesis by plants is also a developing field of transplastomic research (Lössl et al. 2003, 2005; reviewed in Maliga and Bock 2011; Ahmad and Mukhtar 2013; Bock 2014). In the latter case, chloroplast metabolic engineering is used to synthesize low-cost enzyme cocktails for biomass hydrolysis and especially for the digestion of lignocellulosic biomass in order to generate fermentable sugars for ethanol production. However, the stability of the recombinant enzymes under variable field conditions, e.g., abiotic stressors, such as light, temperature, etc., as well as during extraction and storage also has to be maintained (Pantaleoni et al. 2014). An example for bioplastic production is the introduction of complex metabolic pathways such as polyhydroxybutyrate (PHB) synthesis into the plastids (Nakashita et al. 2001; Lössl et al. 2003, 2005). However, it has to be noted that similar extent of PHB accumulation has already been reported in several plant species via nuclear transformation and eventual chloroplast targeting of PHB synthesis genes (Somleva et al. 2008 and references therein). In addition, growth defects and male sterility was associated to PHB synthesis and accumulation (Lössl et al. 2003), necessitating the development of a sophisticated method to obtain inducible gene expression by ethanol spraying of the leaves (Lössl et al. 2005). Taken together, further optimization and improvement of the plastid transformation technique is

necessary to reach higher accumulation levels so that transplastomic plants may represent an economically competitive production platform for biopharmaceuticals, biopolymers, and biofuels.

3.3 Applications in agriculture

Below, we discuss the different potential agricultural applications of transplastomic plants developed so far. The provided categories are sometimes overlapping. For example, transplastomic plants with altered fatty acid unsaturation pattern have increased cold tolerance (Craig et al. 2008) but could have at the same time an improved nutritional value. So in such cases, resistance engineering and increased crop quality may go hand in hand. However, most of the studies discussed below have been conducted in tobacco, a non-food, non-feed crop. This calls for further development in other crop species in order to offer real alternatives in sustainable agriculture and to cope with hidden and conspicuous hunger in the world.

3.3.1 Engineering resistance to biotic and abiotic stress

Improving tolerance towards biotic stressors Decreased pesticide use is not only a cost-effective and labor-saving practice but an important goal of sustainable agriculture and healthy food production (reviewed in Popp et al. 2013). Therefore, efforts to develop and commercialize transplastomic plants with increased pest resistance may one day significantly contribute to a second Green Revolution.

Transplastomic plants with increased insect resistance The crystal proteins of *Bacillus thuringiensis* (Bt) are considered as safe biological insecticides that are not very persistent in nature and have been used in agriculture for more than 60 years (Romeis et al. 2006; Roh et al. 2007; Kumar et al. 2008). Since 1994, several transgenic crops expressing Bt crystal proteins, e.g., Cry1Ab in maize and Cry1Ac in cotton, have been commercialized and grown worldwide on millions of hectares and have significantly decreased insecticide use globally. However, the potential application of transplastomic plants in this field may contribute to overcoming limitations and problems raised in connection with these Bt proteins, e.g., toxicity of transgenic pollen to non-target insects, leakage to the soil, and especially the development of insect resistance to the protein (Tabashnik et al. 2003; Kumar et al. 2008; Jabeen et al. 2010). Several recommendations exist to reduce Bt resistance development (Bravo and Soberón 2008; Tabashnik et al. 2013). These include (1) tissue-specific expression, which may also be beneficial for non-target insects; (2) the high-dose strategy, i.e., increasing the expression levels of the toxin to leave less room for insects to develop resistance; and (3) gene pyramiding, i.e., expression of multiple Bt genes, all of which may be relatively easily realized in transplastomic plants (Kota et al. 1999). In some nuclear transformants, the insecticidal crystal protein is targeted towards the chloroplast to obtain expression levels of up to 2 % of total soluble proteins (e.g., Kim et al. 2009; Lee et al. 2009; Rawat et al. 2011; Kiani et al. 2013) and to circumvent the detrimental effect of the protein accumulation in the cytoplasm or on the in vitro regeneration of the plants (Rawat et al. 2011). However, in contrast with nuclear transformants, the transplastomic plants are able to synthesize much higher amounts of the Bt protein or also the larger, inactive protoxins (instead of the active mature protein), which further limits the damage to non-target insects (De Cosa et al. 2001; Jabeen et al. 2010).

The expression of the Bt (pro)toxins, as in the case of Cry1a(c), in tobacco chloroplasts resulted in significant protein accumulation (3–5 % of the soluble proteins in the leaves) and, thus, in high toxicity of the transplastomic plants against different Bt-susceptible insect larvae (McBride et al. 1995) (Table 2). These figures were much higher than those obtained in nuclear transformants even after synthetic modification of the coding region, and underline the potential of plastid transformation in pest control and also in the management of Bt-resistant insect populations. Subsequently, the method has been extended to other Bt proteins and/or plant species (Table 2). The (pro)toxin accumulated to high levels in all reported studies and formed irregular or cuboidal crystal inclusions within the chloroplast stroma in young or mature leaves, respectively. The morphology of these inclusions was similar to that observed in *E. coli* cells overexpressing the toxin and was maintained throughout senescence (De Cosa et al. 2001). Crystal formation occurred only when the transgene was inserted together with a putative chaperon naturally present upstream on the same operon resulting in very high (45–46 % of leaf soluble proteins) accumulation. Crystals were absent in case of the introduction of a single transgene, i.e., not the complete operon, and resulted in lower transgenic protein levels accounting to 0.4 % of leaf soluble proteins (De Cosa et al. 2001). The strong insecticide effect of the plastid-expressed (pro)toxins was successfully tested in several bioassays against various moths, caterpillars, larvae, etc. (Table 2). In spite of the advantages of the technique, its commercial application is most likely hindered by higher costs and lower yields of producing transplastomic plants, and the efficient protein expression by plastid targeting of nuclear transgenes as an alternative. In addition, a delicate balance has to be defined between the positive effect of the efficient synthesis of insecticidal crystals and their yield penalty (Reddy et al. 2002) as well as the delayed plant development frequently observed in transplastomic plants (Chakrabarti et al. 2006).

Another possibility to develop insect-resistant transplastomic tobacco plants is the upregulation of their own pathogen defense mechanisms. This may be achieved

Table 2 Most important reports about transplastomic plants developed to improve resistance towards different biotic stressors (pests and pathogens)

Plant species	Transgene(s)	Expression level (% of total soluble proteins or on fresh weight basis)	Pests/pathogens affected (bioassays)	Reference
Insect resistance				
Nicotiana tabacum (tobacco)	Bt *cry1A(c)*	3–5 %	*Helicoverpa zea*, *Heliothis virescens*, *Spodoptera exigua*	McBride et al. 1995
	Bt *cry2Aa2*	2–3 %	*Helicoverpa zea*, *Heliothis virescens*, *Spodoptera exigua*	Kota et al. 1999
	Bt *cry1A(c)*	[a]	*Helicoverpa armigera*	Zhang et al. 2000
	Bt *cry2Aa2* operon	45.3–46.1 %	*Helicoverpa zea*, *Heliothis virescens*, *Spodoptera exigua*	De Cosa et al. 2001
	Bt *cry1Ia5*	Approximately 3 %	*Helicoverpa armigera*	Reddy et al. 2002
	Bt *cry1C*	1.1–4 %	*Spodoptera litura*	Lin et al. 2003
	Bt *cry9Aa2*	Approximately 10 %	*Phthorimaea operculella*	Chakrabarti et al. 2006
	Bt *cry1Ab*	[a]	Not tested	Jabeen et al. 2010
	bgl-1 (β-glucosidase)	[a]	*Bemisia tabaci*, *Myzus persicae*	Jin et al. 2011
	pta (agglutinin)	5.2–9.3 %	*Bemisia tabaci*, *Helicoverpa zea*, *Heliothis virescens*, *Myzus persicae*, *Spodoptera exigua*	Jin et al. 2012
	Bt *cry1Ab*	45–46 ng mg^{-1} fresh weight	Not tested	Mirza and Khan 2013
Brassica napus (oilseed rape)	Bt *cry1Aa10*	[a]	*Plutella xylostella*	Hou et al. 2003
Glycine max (soybean)	Bt *cry1Ab*	[a]	*Anticarsia gemmatalis*	Dufourmantel et al. 2005
Brassica oleracea var. *capitata* (cabbage)	Bt *cry1Ab*	4.8–11.1 %	*Plutella xylostella*	Liu et al. 2008
Nicotiana benthamiana	*sporamin*, *CeCPI*, *chitinase*	0.85–1 % for each protein	*Spodoptera exigua*, *Spodoptera litura*	Chen et al. 2014
Solanum tuberosum (potato)	Hairpin dsRNA to β-actin gene of Colorado potato beetle	Approximately 0.4 % of total cellular RNA	*Leptinotarsa decemlineata*	Zhang et al. 2015
Disease (pathogen) resistance				
Nicotiana tabacum (tobacco)	*msi-99* (magainin 2 analog)	[a]	*Aspergillus flavus*, *Colletotrichum destructivum*, *Fusarium moniliforme*, *Pseudomonas syringae* pv. *tabaci*, *Verticillium dahliae*	DeGray et al. 2001
	pelB, *pelD* (pectate lyase)	26 and 32 units g^{-1} fresh weight	*Erwinia carotovora*[b]	Verma et al. 2010
	RC101 (retrocyclin)	32–38 %	*Erwinia carotovora*[b], tobacco mosaic virus	Lee et al. 2011
	PG1 (protegrin)	17–26 %	*Erwinia carotovora*[b]	Lee et al. 2011
	pta (agglutinin)	5.2–9.3 %	*Erwinia carotovora*[b], tobacco mosaic virus	Jin et al. 2012
	cpo (chloroperoxidase)	[a]	*Alternaria alternata*	Ruhlman et al. 2014
Nicotiana benthamiana	*sporamin*, *CeCPI*, *chitinase*	0.85–1 % for each protein	*Alternaria alternata*, *Pectobacterium carotovorum* subsp. *carotovorum*	Chen et al. 2014

Bt—Bacillus thuringiensis

[a] Indicates observed adequate gene expression, but no quantification

[b] *Erwinia carotovora* is the former name (synonym) of *Pectobacterium carotovorum* subsp. *carotovorum* used in the original publications

by introducing for example a fungal β-glucosidase (*bgl-1*), an enzyme potentially involved in the release of plant hormones from their conjugates, in their plastome that stimulates the growth and the sugar ester excretion in the glandular trichomes (Jin et al. 2011).

A novel and non-Bt-type insect resistance strategy has recently been demonstrated by expressing long double-stranded RNA (dsRNA) targeting an essential insect gene in transplastomic plants in order to activate RNA interference that disrupts expression of the target gene in the insects (Zhang et al. 2015). As proof of the concept, a hairpin-type dsRNA to the β-actin gene of the Colorado potato beetle was expressed to 0.4 % of total cellular RNA in transplastomic potato leaves, which caused 100 % mortality both in adult beetles as well as larvae within 5 days of feeding (but no effects in case of feeding on respective transgenic plants). The above data emphasize the great potential of transplastomic plants in insect control.

Transplastomic plants with increased disease (pathogen) resistance Plastid transformation also represents a potential tool to increase disease resistance to phytopathogenic bacteria and fungi due to high concentrations of the target protein accumulating in a single compartment and released only locally during hypersensitive reaction. To this end, the *msi-99* transgene, which encodes a magainin 2 analog antimicrobial peptide, was first successfully introduced to the plastid genome and was proven to be efficient against different bacteria and fungi (DeGray et al. 2001) (Table 2).

Similarly, the introduction of a single agglutinin gene (*pta*) of the *Pinellia ternata* herb resulted in high levels of protein expression in leaf chloroplasts and a very promising, broad spectrum resistance against various pests including aphids, flies, lepidopteran insects, and bacterial and viral pathogens (Jin et al. 2012).

The expression of the chloroperoxidase encoding gene of *Pseudomonas pyrrocinia* in transplastomic plants conferred a similar level of fungal resistance in vitro and in planta as seen in nuclear transformants for the same gene (Ruhlman et al. 2014). However, codon optimization and enhanced translation in plastids is needed to further improve the potential of transplastomic plants in this field.

Although we did not discuss work related to the development of transplastomic plants for biofuel production, it is noteworthy to mention that some transplastomic plants originally developed to produce plant-derived enzyme cocktails, especially the pectate lyases PelB and PelD, for releasing fermentable sugars from lignocellulosic biomass also had improved tolerance to *Pectobacterium carotovorum* (Verma et al. 2010).

Similarly, transplastomic plants expressing the antimicrobial and antiviral proteins Retrocyclin-101 (RC101) and Protegrin-1 (PG1), which have important possible applications in human medicine, exhibited antiviral (tobacco mosaic virus) and antibacterial (*Pectobacterium*) activities (Lee et al. 2011). However, in this and other cases (Oey et al. 2009), the plants and plastids are considered as bioreactors producing the compounds in sufficient amount for pharmaceutical applications rather than crops with increased disease resistance.

Recently, in order to generate plants with multiresistance against phytopathogens as well as insects, a construct with *aadA* as marker gene and a gene stack harboring sweet potato sporamin, taro cystatin (*CeCPI*), and chitinase from *Paecilomyces javanicus* has been introduced into *Nicotiana benthamiana* (Chen et al. 2014). Surprisingly, the transplastomic plants conferred a broad spectrum of resistance not only against different pests and diseases (Table 2) but also against abiotic (salt, osmotic and oxidative) stressors (Table 3), and the transgenes were effectively expressed both in leaf and root plastids. These results underline the huge and still unexplored potential of transplastomic plants in molecular breeding and lead us towards discussing the examples of enhanced abiotic stress tolerance mechanisms obtained in transplastomic plants.

Improving tolerance towards abiotic stressors Agronomic productivity affects food quantity and quality and is substantially affected by human exploitation, pollution, and global warming. The latter results in unpredictable water and weather conditions leading to drought, heat, and cold stress in several areas under cultivation. Examples of transplastomic plants with enhanced abiotic stress resistance follow below. For the sake of simplicity, data about plants potentially used for phytoremediation purposes are also discussed here (Table 3). As oxidative stress is often associated to and triggered by different stressors (including both biotic and abiotic stressors), we separately discuss the data available on increased tolerance towards oxidative stress.

Temperature (cold, chilling, or heat) stress Increasing fatty acid desaturation results not only in improved nutritional value but also often confers tolerance for the membranes towards cold and/or chilling stress. A proof-of-concept study demonstrated elevated fatty acid unsaturation levels both in the leaves and the seeds of transplastomic tobacco plants expressing desaturase genes such as Δ9-stearoyl-ACP desaturase from *Solanum commersonii* and Δ9-acyl-lipid desaturase from *Anacystis nidulans* (Craig et al. 2008).

β-Alanine is an essential non-protein amino acid serving as precursor for β-alanine betaine or homoglutathione in some plant species, and being involved in abiotic stress tolerance. The *E. coli* gene (*panD*) encoding L-aspartate-α-decarboxylase is responsible for the decarboxylation of L-aspartate to β-alanine and carbon dioxide. Transplastomic plants overexpressing *panD* had increased heat stress tolerance and 30–

Table 3 Most important reports about transplastomic plants developed to confer improved tolerance towards different abiotic stressors

Plant species	Transgene(s)	Type of stress	Tolerated stress treatment	Reference
Nicotiana tabacum (tobacco)	*TPS1* (trehalose phosphate synthase)	Drought and osmotic stress	24 days drought, 6 % PEG	Lee et al. 2003
	merAB operon	Heavy metal stress (phytoremediation)	400 μM phenyl-mercuric acetate, 300 μM $HgCl_2$	Ruiz et al. 2003; Hussein et al. 2007
	des (fatty acid desaturase)	Chilling and cold stress	Leaf discs at 4 °C for 72 h, seedlings at 2 °C for 9 days	Craig et al. 2008
	CMO (choline monooxygenase)	Toxic levels of choline, salt and drought stress	30 mM choline, 150 mM NaCl, 300 mM mannitol	Zhang et al. 2008
	panD (aspartate decarboxylase)	High-temperature stress	40 °C for 10 h/day during 1 week, or 45 °C for 4 h	Fouad and Altpeter 2009
	DHAR, *GST*, *DHAR*::*gor*, and *GST*::*gor* (dehydroascorbate reductase, glutathione *S*-transferase, glutathione reductase)	Salt, chilling, and oxidative stress	All lines: 200 mM NaCl, 12 days of germination at 15 °C, leaf discs at 8 °C for 8 h; only *DHAR*::*gor* and *GST*::*gor*: 1 μM paraquat	Le Martret et al. 2011
	MnSOD (mitochondrial superoxide dismutase), *gor* (glutathione reductase)	Oxidative, heavy metal and UV-B stress	*MnSOD*: 100 μM paraquat, 253 kJ m^{-2} UV-B light; *gor*: 0.5 mM Cd, 253 kJ m^{-2} UV-B	Poage et al. 2011
	mt1 (metallothionein)	Heavy metal stress (phytoremediation)	Leaf discs up to 20 μM $HgCl_2$ for 10 days	Ruiz et al. 2011
	otsBA operon (trehalose phosphate synthase/phosphatase)	Salt stress	300 mM NaCl	Bansal et al. 2012
	fld (flavodoxin)	Oxidative stress	100 μM paraquat up to 24 h	Ceccoli et al. 2012
	HPT, *TCY*, *TMT* (tocopherol biosynthesis pathway)	Oxidative and cold stress	1 month at 4 °C	Lu et al. 2013
	DHAR, *GST*, *gor*, and *MnSOD* (singly and two pairwise combinations)	Chilling/cold and oxidative stress	All lines: 4 °C at high PAR; *gor* or *GST*::*gor*: 10 °C	Grant et al. 2014
Daucus carota (carrot)	*badh* (betaine aldehyde dehydrogenase)	Salt stress	Maximum 400 mM NaCl for 4 weeks	Kumar et al. 2004a
Nicotiana benthamiana	*sporamin*, *CeCPI*, *chitinase*	Salt, osmotic, and oxidative stress	Maximum 400 mM NaCl, 3 % PEG, 10 μM paraquat	Chen et al. 2014

Data about herbicide resistant transplastomic plants are not included (for details, see Table 1 and Section 3.3.1)

PEG polyethylene glycol, *PAR* photosynthetically active radiation

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40 % higher biomass than non-transformed plants under stress conditions (Fouad and Altpeter 2009). The increased biomass may be—at least partly—the result of increased CO_2 concentration within the plastids, stressing the limiting role of the availability of this compound to RuBisCO in photosynthetic productivity (see later in Section 3.3.2).

Plants with enhanced antioxidant defense system and/or improved content of the antioxidant and reactive oxygen scavenger, vitamin E (see later in Sections 3.3.1 and 3.3.3, respectively, Tables 3 and 4), had better temperature stress resistance than non-transformed plants. This can probably be explained by the increased level of the antioxidants, which could alleviate the deleterious effects of reactive oxygen species (ROS) formed during the stress.

Drought and salt stress In addition to drought stress, due to the excessive use of fertilizers and improper irrigation practices, salt stress represents another major and still increasingly threatening abiotic stress in agriculture. High soil salinity affects 7 % of all land area and 5 % of cultivated lands, respectively (Munns and Tester 2008), which also poses an important economical problem due to retarded growth, development, and low yield of cultivated plants on such soils. Clearly, the breeding of drought- and salt-tolerant crops is of crucial importance in order to feed the world in 2050.

Several osmoprotectants, e.g., the sugar trehalose (Karim et al. 2007; Iordachescu and Imai 2008) and betaines (Giri 2011), are known to confer resistance towards drought, cold, and/or salt stress presumably via macromolecule protection and ROS detoxification in the cell. Therefore, increasing the accumulation of such compounds is an important goal during development of novel crop cultivars tolerant to abiotic stressors.

Targeting and successfully expressing a yeast trehalose phosphate synthase (*tps1*) in tobacco plastids resulted in a 20-fold increase in trehalose accumulation when compared to non-transformed plants, and conferred drought and osmotic stress tolerance to the transplastomic plants (Lee et al. 2003).

Similarly, plastid transformation protocols have been developed to produce tobacco plants with increased salt stress tolerance by expressing the *otsBA* operon encoding the *E. coli* trehalose-6-phosphate phosphatase (OtsB) and trehalose-6-phosphate synthase (OtsA) enzymes (Bansal et al. 2012).

Certain plants, especially those belonging to Chenopodiaceae and Amaranthaceae, have the capacity to convert choline in a two-step oxidation reaction to glycine betaine in their plastids. First, choline-monooxygenase (CMO) converts choline to betaine aldehyde, then betaine aldehyde dehydrogenase (BADH, identical to ω-aminoaldehyde dehydrogenase) catalyzes the transformation of betaine aldehyde to glycine betaine. Introducing glycine betaine accumulation capacity into other crops improves the osmotic stress tolerance of these species.

Transplastomic tobacco plants containing the *Beta vulgaris CMO* gene accumulated the osmoprotectant glycine betaine in the leaves, roots, and seeds. In addition, they had improved tolerance to toxic levels of choline, salt and drought stress, and better photosynthetic performance under salt stress conditions than the non-transformed plants (Zhang et al. 2008).

Carrot (*Daucus carota* L.) root is an excellent source of sugars, provitamin A, fibers, and vitamin C in the diet. The plant is, therefore, one of the most important non-cereal vegetable crops for human and animal consumption. Carrot is a salt-sensitive plant exhibiting 7 % growth reduction for every 10 mM increment in salinity above 20 mM (Gibberd et al. 2002; Daniell et al. 2005). Successful plastid transformation of non-green embryogenic carrot cells and regeneration of transplastomic plants by somatic embryogenesis was first reported by Kumar et al. (2004a). Due to the inserted transgene, *BADH*, the transplastomic cell cultures accumulated approximately 50-fold more betaines (glycine betaine and β-alanine betaine) than non-transformed cells in the presence of 100 mM NaCl. In addition to leaf chloroplasts, elevated transgene expression levels were observed in carrot root chromoplasts and the proplastids of cultured cells as well (Kumar et al. 2004a). The plants developed high level of salinity tolerance to up to 400 mM NaCl, the highest level of salt tolerance reported so far among GM crops, clearly showing the advantage of the transplastomic technology.

Anthropogenic pollutants, phytoremediation Anthropogenic pollutants represent one important segment of abiotic stressors. For instance, several transgenic crops with herbicide resistance encoded in the nucleus are commercialized. Herbicides can also be used as selective agents (Table 1); therefore, much effort has been devoted to develop transplastomic plants with resistance to different herbicides such as glyphosate (Daniell et al. 1998; Ye et al. 2001, 2003; Chin et al. 2003; Roudsari et al. 2009), phosphinothricin/glufosinate ammonium (Iamtham and Day 2000; Lutz et al. 2001; Kang et al. 2003; Ye et al. 2003), sulcotrione (Falk et al. 2005), isoxaflutole/diketonitrile (Dufourmantel et al. 2007), chlorophenylthio-triethylamine (CPTA) (Wurbs et al. 2007), pyrimidinylcarboxylate, imidazolinone and sulfonylurea (Shimizu et al. 2008), and paraquat (methyl-viologen) (Le Martret et al. 2011; Poage et al. 2011; Chen et al. 2014). Herbicide resistance is achieved (1) by the insertion of a bacterial marker gene (such as *bar*) encoding an enzyme that inactivates the herbicide (phosphinothricin/glufosinate ammonium—Iamthan and Day 2000; Lutz et al. 2001, 2006), (2) by overexpression of the genes of plastidial metabolic enzymes that are the targets of herbicides (e.g., *EPSPS*: glyphosate—Daniell et al. 1998; *hppd*: sulcotrione and the isoxaflutole derivative, diketonitrile—Falk et al. 2005 and Dufourmantel et al. 2007, respectively), (3) by expression of the genes of mutant, herbicide-resistant plant enzymes (*CP4*: glyphosate—

Ye et al. 2001, 2003; Roudsari et al. 2009; *mALS*: pyrimidinylcarboxylate, imidazolinone, and sulfonylurea—Shimizu et al. 2008), or (4) by expression of enzyme genes involved in antioxidant defense, minimizing this way the metabolic impact of the herbicides via the generation of ROS (*DHAR*, *GST*, *gor*: paraquat/methyl-viologen—Le Martret et al. 2011; *MnSOD*: paraquat/methyl-viologen—Poage et al. 2011). For instance, glyphosate is a competitive inhibitor of one enzyme of the plastid aromatic amino acid biosynthesis pathway, namely 5-enolpyruvylshikimate-3-phosphate (EPSPS). This enzyme is nuclear-encoded, but plastid targeted. The transplastomic plants overexpressing a mutant *epsps* gene in the chloroplast accumulated 250-fold EPSPS proteins than transgenic plants overexpressing the nuclear gene (Ye et al. 2001). This underlines again the potential and major advantage of plastid transformation in some cases. For more information about herbicide tolerance mechanisms, the readers are kindly directed to Venkatesh and Park (2012).

Most works developed transplastomic herbicide-tolerant tobacco plants, and only one work involves a major crop, soybean (Dufourmantel et al. 2007). Basic research work on developing new non-antibiotic marker genes resulted in the development of transplastomic plants resistant to D-alanine (Gisby et al. 2012). This opens up the new and relatively environment friendly possibility to develop D-alanine-based herbicides. Similarly, the choline tolerance of transplastomic tobacco lines with increased salt tolerance (Zhang et al. 2008, see previous section) may lead to the development of choline-based herbicides.

Taken together, in our opinion plastid transformation may be useful to grow plants for remediation or recultivation in herbicide-polluted areas, where sensitive cultivars would not be able to be grown. However, this would require (1) detailed knowledge and very rigorous control of possible herbicide accumulation by crop plants and of their effects on humans in case of ingestion, and/or (2) the use of non-food and non-feed crops carefully processed after harvest.

In addition to herbicides, several anthropogenic pollutants, including different metal compounds, represent a challenge to sustainable agriculture. Therefore, the development of transplastomic plants resistant to such pollutants, e.g., organomercurials (like phenyl mercuric acetate) and mercury salts ($HgCl_2$), by successfully integrating a native operon containing the genes of bacterial mercuric ion reductase (*mer*A) and organomercurial lyase (*mer*B) represents an important example for phytoremediation by transplastomic plants (Ruiz et al. 2003; Hussein et al. 2007). Again, it should be emphasized that in this case, the transplastomic tobacco plants represent a useful tool for phytoremediation only and accumulate very high levels of Hg in their tissues even in the aerial part of the plants (Hussein et al. 2007). Therefore, this method is suitable to clean polluted agronomic fields, but such genetic modifications are useful probably only in non-feed and non-food crops, especially when they confer in planta tolerance and would result in the accumulation of these harmful compounds in edible plant parts. In addition, a public concern regarding the use of the *mer*AB system for phytoremediation is the release of Hg^0 to the atmosphere, a problem to be solved with complementary methods (Ruiz and Daniell 2009). For instance, transplastomic tobacco plants expressing the mouse metallothionein gene (*mt1*) were resistant to mercury and accumulated the metal within their tissues (including the leaves) and seemed, therefore, to be ideal objects for phytoremediation purposes (Ruiz et al. 2011).

Oxidative stress The formation of ROS and, thus, oxidative stress can be induced by various biotic (such as pathogen infection) and abiotic stressors (e.g., excess light or UV-B radiation, drought, high salinity, cold, excess of metal ions, pollutants, xenobiotics). It also occurs during aging and strongly impacts the structure and function of plastids (Solymosi and Bertrand 2012). Therefore, fine tuning the ROS-scavenging antioxidant defense system of transplastomic plants may have a strong effect on their yield and adaptation to various stress conditions, and also contributes to a better understanding of its role in general plant stress tolerance.

Monodehydroascorbate reductase (*mdar*) is one of the antioxidative enzymes of the ascorbate-glutathione cycle. Overexpression of *mdar* transgene in tobacco plastids and the fusion of such chloroplasts to *Petunia* cells (Sigeno et al. 2009) was suggested (but experimentally not tested) to possibly protect the plants against oxidative stress.

The inclusion of other transgenes involved in the antioxidant defense system like the *Nicotiana tabacum* mitochondrial superoxide dismutase (*MnSOD*) and the *E. coli* glutathione oxidoreductase (*gor*) led to tolerance of paraquat-induced oxidative damage in transplastomic *MnSOD* tobacco plants, heavy metal tolerance in transplastomic *gor* plants, and UV-B tolerance in both transplastomic lines (Poage et al. 2011). Similarly, the overexpression of rice dehydroascorbate reductase (*DHAR*) and *E. coli* glutathione *S*-transferase (*GST*) in the chloroplasts resulted in increased salt and chilling tolerance (Le Martret et al. 2011). At the same time, paraquat-induced oxidative stress tolerance could be only achieved in *DHAR*::*gor* and *GST*::*gor* plants expressing simultaneously *E. coli* glutathione reductase (*gor*) (Le Martret et al. 2011). In continuation of these works (Le Martret et al. 2011; Poage et al. 2011), transplastomic tobacco lines overexpressing *gor*, *DHAR*, *GST*, and *MnSOD* singly or in pairwise combinations were exposed to low temperatures (Grant et al. 2014). Only *gor* and *GST*::*gor* plants had decreased photoinhibition at 10 °C when compared to non-transformed plants, while *DHAR* and *DHAR*::*gor* plants had increased photoinhibition. All transplastomic lines exerted improved chilling tolerance at 4 °C and simultaneous high light stress compared to the non-

transformed plants; however, transplastomic lines showed stunted growth under transient cool air temperatures. These data indicate the complexity of the interaction between the enhanced ROS scavenging activity and chilling stress tolerance.

Oxidative stress tolerance was also enhanced in transplastomic *N. benthamiana* (Chen et al. 2014, see above). Cyanobacterial flavodoxin (*fld*) is an isofunctional flavoprotein involved in stress tolerance. The leaves of transplastomic tobacco plants expressing *fld* increased the tolerance towards paraquat-induced oxidative stress compared to non-transformed plants (Ceccoli et al. 2012). It is noteworthy that the authors demonstrated a window in the effect of the recombinant protein on photosynthesis and stress tolerance: plants with low and high flavodoxin concentrations had lower photosynthetic parameters and stress tolerance than plants accumulating moderate amounts of flavodoxin. This example clearly shows the importance of tailoring the expression level of the given compounds in transplastomic plants because low expression levels may not be sufficient to confer resistance/tolerance, while high expression levels may represent metabolic burden or toxicity of the transgenic product for the plant.

3.3.2 Attempts to influence crop productivity

Most efforts to improve crop productivity in transplastomic plants aimed at increasing photosynthetic efficiency via engineering the photosynthetic machinery. RuBisCO is the most abundant enzyme in the world and is involved in CO_2 fixation, primary productivity, and thus yield. It is composed of two subunits, one of which (the large subunit, rbcL) is chloroplast encoded, while the small subunit (rbcS) is encoded by a nuclear gene and targeted into the chloroplast. The catalytic activity of RuBisCO is slow and inefficient because it is limited by several factors including different CO_2 and O_2 concentrations, high light intensity, etc., and it also considerably varies among the different organisms. Therefore, successful manipulation of the kinetics and regulation of RuBisCO offers a promising target for genetic engineering to improve crop photosynthesis, productivity, and yield, especially under changing environmental conditions such as increasing CO_2 levels and temperature, and limited water and nitrogen supply (Andrews and Whitney 2003; Whitney et al. 2011a; Hanson et al. 2013; Parry et al. 2013).

Incorporating the nuclear-encoded small subunit gene (*rbcS*) into the chloroplast genome was in general not really efficient (Whitney and Andrews 2001a; Zhang et al. 2002) unless the level of cytosolic production of the nuclear-encoded small subunit was simultaneously reduced by 90 % (Dhingra et al. 2004). Similarly, nuclear transformation had limited influence on RuBisCO activity (Parry et al. 2013).

Several methods have been developed to produce transplastomic plants with altered *rbcL* gene (Whitney et al. 1999; Kode et al. 2006; Whitney and Sharwood 2008; Chen and Melis 2013). However, the attempts to replace the plastid-located tobacco RuBisCO large subunit with the same gene from cyanobacteria (*Synechococcus*—Kanevski et al. 1999), proteobacteria (*Rhodospirillum rubrum*—Whitney and Andrews 2001b, 2003), archaebacteria (*Methanococcoides burtonii*—Alonso et al. 2009), non-green algae (the rhodophyte *Galdieria sulphuraria* and the diatom *Phaeodactylum tricornutum*—Whitney et al. 2001), sunflower (Kanevski et al. 1999), tomato (Zhang et al. 2011), and *Flaveria* (Whitney et al. 2011b; Galmés et al. 2013) resulted in general in non-autotrophic transformants. These produced either no RuBisCO, like in case of the *Synechococcus* gene (Kanevski et al. 1999), or had no properly folded proteins with no assembly of the RuBisCO subunits as a result (Whitney et al. 2001) or assembled into hybrid RuBisCO hexadecamers which were, however, usually less functional than the enzyme of the non-transformed plants (Kanevski et al. 1999; Alonso et al. 2009; Zhang et al. 2011). In some cases, the plants were able to grow also autotrophically under normal (Zhang et al. 2011) or special atmospheric conditions (elevated CO_2 content) (e.g., Kanevski et al. 1999; Alonso et al. 2009; Sharwood et al. 2008; reviewed in Hanson et al. 2013). The low mRNA level, translation, and/or macrodomain assembly of the foreign *rbcL* gene is probably the reason of the observed decreased functionality in the transplastomic plants (Sharwood et al. 2008). However, specific arrangement of the transgenes (*rbcL* and *aadA*) in the vectors used to produce transplastomic plants could result in RuBisCO levels and photosynthetic properties similar to those of non-transformed tobacco (Whitney and Andrews 2003).

In other studies, the introduction of large subunit transgenes into the plastome was used to trigger changes between C_3 and C_4 photosynthesis offering great promise in improving crop productivity under changing environmental conditions (Whitney et al. 2011b; Galmés et al. 2013). Similarly, screening the RuBisCO specificity of several species to find the most efficient enzyme (under given environmental conditions) and transferring it into important crops may lead to an improved net photosynthetic rate by as much as 29 %, at least on the basis of some prediction models and considering unaltered carboxylation rate (Raines 2006).

These data clearly show that (1) a better understanding of factors regulating RuBisCO assembly and activity, (2) potential co-integration with other biotechnological strategies to improve photosynthetic carbon assimilation, and (3) novel methods and further efforts are needed to achieve beneficial changes in RuBisCO activity via genetic engineering which has been restricted until now by the very complex catalytic chemistry and high level expression of the enzyme (Whitney et al. 2011a; Parry et al. 2013).

In a more recent study, Lin et al. (2014) attempted to express the full RuBisCO protein from *Synechococcus elongatus* (together with an internal carboxysomal protein, CcmM35) in tobacco by the simultaneous disruption of the host's native enzyme. Immunoelectron microscopy and autotrophic growth of transplastomic plants demonstrated the correct assembly of active cyanobacterial RuBisCO. In addition, CO_2 fixation rates and specific carboxylase activity of this RuBisCO enzyme was increased, especially at higher CO_2 concentrations, indicating the complete dependence of these plants on the introduced cyanobacterial enzyme for carbon fixation. This is a significant step towards the functional introduction of a complete photosynthetic system from cyanobacteria to plant chloroplasts.

Raising the CO_2 concentration within the plastids is another possibility to improve photosynthetic carbon fixation efficiency and, thus, crop productivity. Transplastomic technology may be useful in the future to implement the highly effective CO_2 concentration mechanisms of cyanobacteria (such as inorganic carbon transporters or maybe even the functional carboxysomes) into the plastids of important C3 crops (Price et al. 2013). However, the cyanobacterial bicarbonate transporter gene (*bic*A) successfully introduced into the tobacco plastid genome showed expression and localization to thylakoids and the plastid envelope, but did not result in discernible changes in ultrastructure and photosynthesis due to low activity of the transporter (Pengelly et al. 2014). This indicates that a better understanding of the CO_2 concentration mechanisms such as enzyme structure and function and its interactions with the host plastid's metabolism is necessary to achieve breakthrough in the field. Similarly, integrating C4 photosynthesis to C3 crops is also a possibility to be considered to increase productivity (Covshoff and Hibberd 2012).

Works with transgenic plants have shown that sedoheptulose-1,7-bisphosphatase (SBPase) is the most important factor for RuBisCO regeneration in the Calvin cycle, and that fructose-1,6-bisphosphatase (FBPase) contributes to the partitioning of fixed carbon for RuBisCO regeneration or starch synthesis (Lefebvre et al. 2005; Tamoi et al. 2006; Rosenthal et al. 2011). On the basis of these results, transplastomic tobacco (Yabuta et al. 2008) and lettuce plants (Ichikawa et al. 2010) expressing cyanobacterial fructose-1,6/sedoheptulose-1,7-bisphosphatase (FBP/SBPase) in the chloroplast have recently been generated, and both exhibited increased productivity. In tobacco plants, expression by three different plastid promoters caused a minimum of 2- or 3-fold increase in the enzyme activity, which resulted in up to 1.8-fold increase of the dry matter when compared to the non-transformed plants. This increase was sufficient to improve productivity and photosynthesis without representing a strong metabolic burden to the plastid and potentially enabled the simultaneous high-level expression of other foreign genes in the plastids (Yabuta et al. 2008). In addition, the hexose, sucrose, and starch contents of the stem, root, and different leaves were in general higher in transplastomic plants than in non-transformed plants (at least during distinct stages of the diurnal cycle) (Yabuta et al. 2008). In lettuce, photosynthetic capacity and productivity of the transplastomic plants increased 1.3- and 1.6-fold, respectively, when compared to non-transformed plants (Ichikawa et al. 2010). These data support that plastid metabolic engineering of enzymes limiting photosynthesis may result in improved yield and productivity. To our knowledge, no transplastomic plants have been produced with the aim to engineer the light reactions of photosynthesis.

Starch is one of the most important macronutrients supplying energy and carbohydrates to humans, but may also be used for biofuel production via fermentation. Its synthesis (during the day in chloroplasts) and degradation (during the night phase in chloroplasts), and its storage (in amyloplasts, for example) are regulated by several, still not completely understood mechanisms. Thioredoxin f (Trx f) is a key enzyme of plastid redox regulation (reviewed in Nikkanen and Rintamäki 2014), plastid development (reviewed in Solymosi and Schoefs 2010), and starch metabolism. When grown in the greenhouse, transplastomic tobacco plants ('Petit Havana' SR1) overexpressing Trx f had up to 7-fold leaf starch accumulation, increased sugar content (up to 5.5-fold), leaf weight, and biomass yield (up to 1.7-fold in dry weight) compared to non-transformed plants (Sanz-Barrio et al. 2013). Similar though somewhat lower induction rates were observed in field experiments (see Section 4) with two different tobacco cultivars expressing the same gene construct (Farran et al. 2014). However, such a strong influence on the plastid redox system may have a negative impact on plant development under specific growth conditions.

3.3.3 Biofortification—metabolic engineering in order to enhance nutritional value

Biofortification is the idea to produce and grow edible crops with increased nutritional values. This can be achieved by enhanced agricultural management, conventional breeding or different genetic engineering methods that modify plant metabolism, including plastid transformation. The most important dietary components related to health issues are macronutrients and micronutrients; however, compounds leading to intolerances, having toxicity or allergenicity, or interfering with the bioavailability (absorption) of the nutrients may also be considered and modified to improve food functionality (Newell-McGloughlin 2010). Several transgenic plants with improved compositions and/or levels of crop-based phytonutrients have been produced to alleviate diet-related diseases. These include increased protein levels in potato, increased amino acid (lysine in maize and rice, methionine in alfalfa), choline, folate, flavonoid, anthocyanin, vitamin E,

Table 4 Most important reports about transplastomic plants with increased nutritional value and productivity

Plant species	Transgene(s)	Tested trait	Observations	Reference
Nicotiana tabacum (tobacco)	*ASA2* (anthranilate synthase α)	Trp content	↑ of free Trp in leaves, ↑ of total Trp content in seeds	Zhang et al. 2001
	accD operon (acetyl-CoA carboxylase)	FA content	↑ of FA and galactolipid content, ↑ of 16:3 and 18:3, ↓ of 16:2 and 18:2 FAs in leaves	Madoka et al. 2002
	Polyhistidine-tagged *rbcL*	Zn content	↑ of Zn content in leaves	Rumeau et al. 2004
	hppd (hydroxy-phenylpyruvate dehydrogenase)	Vitamin E content	↑ of vitamin E content and quality in leaves and seeds	Falk et al. 2005
	des (fatty acid desaturase)	Lipid desaturation level	↑ of 16:3 and 18:3, ↓ of 18:2 FAs in leaves, ↑ of 18:2, ↓ of 16:0, 18:0, 18:1, and 18:1*t* FAs in seeds	Craig et al. 2008
	DXR (deoxy-xylulose phosphate reducto-isomerase)	Isoprenoid production	↑ of chlorophyll *a*, phytol, α- and β-carotene, lutein, antheraxanthin, solanesol, and β-sitosterol contents in leaves	Hasunuma et al. 2008a
	crtW and/or *crtZ* (carotene ketolase and hydroxylase)	Astaxanthin production	↑ of astaxanthin, 4-ketoantheraxanthin, and total carotenoid contents in leaves	Hasunuma et al. 2008b
	fbp/*sbp* (fructose-/sedoheptulose-*bis*-phosphatase)	Productivity	↑ of photosynthesis and dry mass	Yabuta et al. 2008
	Operon of mevalonate pathway (*AACT*, *HMGS*, *HMGRt*, *MVK*, *PMK*, *MDD)*	Isoprenoid production	↑ of provitamin A, sterol, squalene, triacylglycerol, mevalonate contents in leaves	Kumar et al. 2012
	Trx f (thioredoxin f)	Starch metabolism	↑ starch and sugar contents in leaves, leaf weight, and biomass	Sanz-Barrio et al. 2013
	tc and/or *tmt* (tocopherol cyclase, methyltransferase)	Vitamin E content	↑ of vitamin E content and quality (γ-tocopherols) in leaves	Yabuta et al. 2013
	HPT, *TCY*, *TMT*, synthetic operon	Vitamin E content	↑ of vitamin E content and quality in leaves	Lu et al. 2013
	Trx f (thioredoxin f)	Starch metabolism	↑ starch and soluble sugar contents in leaves, leaf weight	Farran et al. 2014
	rbcL+*rbcS*, and *rbcX* (chaperone) or *ccmM35* from *Synechococcus*	Productivity	↑ of specific RuBisCO carboxylase activity and CO_2 fixation, especially at elevated CO_2 concentrations	Lin et al. 2014
	kasIII (acyl carrier pr. synthase)	FA content	↑ of 16:0 and ↓ of 18:0 and 18:1 FAs in leaves	Dunne et al. 2014
Solanum lycopersicum (tomato)	*crtY* (bacterial lycopene cyclase)	Provitamin A content	↑ of provitamin A due to lycopene conversion to β-carotene in fruits	Wurbs et al. 2007
	Lyc (plant lycopene cyclase)	Provitamin A content	↑ of provitamin A and total carotenoids in fruits	Apel and Bock 2009
	HPT, *TCY*, *TMT*, synthetic operon	Vitamin E content	↑ of vitamin E content and quality in leaves and fruits	Lu et al. 2013
Lactuca sativa (lettuce)	*fbp*/*sbp* (fructose/sedoheptulose *bis*-phosphatase)	Productivity	↑ of photosynthesis, leaf size, leaf number, and yield	Ichikawa et al. 2010
	tc (tocopherol cyclase)	Vitamin E content	↑ of vitamin E content and quality (γ-tocopherols) in leaves	Yabuta et al. 2013
	crtW, *crtZ* (see above), *idi*	Astaxanthin production	↑ of astaxanthin, especially astaxanthin FA esters in leaves	Harada et al. 2014

For enzymes of the carotenoid biosynthetic pathway, see Fig. 2

↑ and ↓ refer to increase and decrease, respectively. *FA* fatty acid, *Trp* tryptophan

carotenoid, iron, and zinc contents in several crops (Mattoo et al. 2010).

Similar achievements may be reached by transplastomic technology. Ascorbic acid, for example, at an estimated concentration of 300 mM in the chloroplast stroma, is involved in protection against oxidative stress (reviewed in Smirnoff 2000; Valpuesta and Botella 2004). In addition to increased tolerance towards stress conditions, transplastomic plants with altered ascorbic acid metabolism may also have enhanced vitamin C content (see Section 3.3.1).

Monellin is a sweet-tasting protein considered as a prospective sweetener. Therefore, overexpression and production of monellin primarily by molecular pharming may improve taste in transplastomic plants (Roh et al. 2006). Below, we only discuss data about transplastomic plants with increased food functionality (Table 4). Most available data on biofortification (reviewed in Hirschi 2008; Solymosi and Bertrand 2012) merely demonstrate the improved nutritional content of the new cultivars, but the bioavailability and pharmacological studies by controlled animal and human experiments are often missing, which are crucial for the public acceptance of genetic engineering and also for decision makers to assess the real significance of this molecular approach (Newell-McGloughlin 2010).

Mineral content Micronutrient deficiency is a major health concern, especially in developing countries where plants represent the main component in the diet. More than 60 % of the world population suffers from iron deficiency, and over 30 % of the global population suffers from zinc deficiency (Rawat et al. 2013). Iron and/or zinc deficiencies lead to severe health problems such as poor growth, reduced immunity, fatigue, irritability, weakness, hair loss, wasting of muscles, sterility, morbidity, and even death in acute cases (Prasad and Halsted 1961; Haas and Brownlie 2001; Pfeiffer and McClafferty 2007; Wintergerst et al. 2007; Stein 2010). The risk for many serious illnesses like cardiovascular disease, Alzheimer's disease, cancer, and aging can be reduced by proper intake of macronutrients, micronutrients (Ca, Mg, Cu, I, or Se), and/or vitamins.

Therefore, breeding of biofortified crops containing increased levels of essential minerals represent an important goal in agriculture (Palmgren et al. 2008; White and Broadley 2009; Gómez-Galera et al. 2010; Solymosi and Bertrand 2012). Excess metals are mostly sequestered in the vacuole or the cell wall. Metal uptake, transportation, and accumulation in different organs or compartments are very complex processes. They include important competition/interactions between different metals, and involve also problems about the bioavailability of metals in the soil, processes that are still not very well understood. However, plastids harbor several metalloenzymes and also play a role for example in iron homeostasis by storing excess iron in the form of ferritin inclusions (reviewed in Solymosi and Bertrand 2012; Solymosi and Keresztes 2012). In addition, they are able to express different proteins at very high levels. Therefore, they may represent a possibility to produce plants with improved mineral content, as it has been demonstrated via nuclear transformation in case of rice seed endosperm expressing soybean phytoferritin, which led to a 3-fold increase in iron content when compared to non-transformed seeds (Goto et al. 1999). In addition, the overaccumulation of ferritin also provided resistance towards cold stress in transgenic tobacco (Hegedűs et al. 2008).

Due to its extremely high abundance, RuBisCO is an interesting target to express peptides or small proteins as fusion products at high levels. Transplastomic tobacco plants with polyhistidine-tagged *rbcL* transgene had normal RuBisCO levels and activity, but their leaves accumulated more (maximum two or three times) zinc than non-transformed plants when grown on zinc-enriched medium (Rumeau et al. 2004). This represents a proof-of-concept study to produce biofortified crops by plastid transformation. However, further research has to be performed due to the complexity of the metal uptake and the binding process including competition for the essential metals between metal-binding recombinant peptides and metalloenzymes, and competition between different metals for the binding sites of the same peptides. This way, synthetic peptides designed to bind specifically heavy metals or radionuclides can be used either for biofortification or phytoremediation purposes. It is noteworthy to mention that a molecular pharming application, such as the expression of an anticancer therapeutic agent, the single-copper protein azurin, resulted in 2-fold copper accumulation in transplastomic chloroplasts compared to wild-type plants (Roh et al. 2014). Unfortunately, the copper content of whole plants was not reported in this work; therefore, the potential of these plants in biofortification remains uncertain.

However, simpler methods raising no public concern and regulatory issues, such as intercropping (growing two crop species on the same plot of land simultaneously) can also lead to a significant increase in crop yield and also in micronutrients, as another way of biofortification (reviewed in Zuo and Zhang 2009).

Carotenoid composition Carotenoids are tetraterpenes involved—among others—in plant photosynthesis and exert in humans protective effects against cardiovascular diseases, certain cancers, and aging-related diseases due to their antioxidant activity (Hammerling 2013). The link between carotenoid intake and health was first established after the discovery that assimilated β-carotene (also termed provitamin A) serves as precursor of vitamin A, an important molecule for vision, skin protection, and cell growth (reviewed in Mayer et al.

2008; Hammerling 2013). In contrast to photosynthetic organisms and some non-photosynthetic bacteria (e.g., *Erwinia herbicola*) and fungi (e.g., *Phycomyces blakesleeanus*), most animals and humans are unable to synthesize carotenoids or vitamin A de novo. Therefore, these compounds have to be obtained in their diet. Unfortunately, vitamin A deficiency is prevalent in one third of the countries of the world and may cause severe diseases such as increased susceptibility to respiratory, gastrointestinal, and childhood diseases, and may even lead to blindness (Ye et al. 2000). Transgenic rice plants, the so-called Golden Rice varieties, expressing carotenoid biosynthesis genes with plastid-targeting signals in the nucleus of endosperm cells have been developed to fight vitamin A deficiency in poor countries (Ye et al. 2000; Beyer et al. 2002; Al-Babili and Beyer 2005). Attempts to produce transplastomic plants with increased or enhanced carotenoid pattern include (1) increasing the availability of the biosynthetic precursors, (2) modifying the enzymes/regulatory mechanisms of the biosynthetic pathway, and (3) shifting the pathway towards the synthesis of novel compounds.

The precursors for carotenoid biosynthesis and several other plant metabolites are isopentenyl diphosphate and dimethylallyl diphosphate molecules synthesized by two independent pathways, i.e., the so-called mevalonate pathway located in the cytoplasm and the peroxisome (for simplicity referred to as cytoplasmic pathway), and the plastid located 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (also termed non-mevalonate or MEP/DOXP pathway) (Fig. 2, reviewed in Champenoy et al. 1999; Kribii et al. 1999; Laule et al. 2003; Clotault et al. 2012; Heydarizadeh et al. 2013; Vickers et al. 2014). Carotenoid biosynthesis normally makes use of precursors from the non-mevalonate pathway. Recently, Kumar et al. (2012) successfully introduced the entire cytoplasmic mevalonate pathway (six genes from yeast on a synthetic operon) into the tobacco chloroplast genome (Fig. 2). This resulted in an increased accumulation of carotenoids (β-carotene), but also other compounds such as mevalonate, sterols, squalene, and triacyl-glycerides in the transplastomic plants, indicating the complexity of isoprenoid biosynthesis and its central role in plant metabolism.

Other works aimed at engineering distinct steps of the existing plastid non-mevalonate isoprenoid and/or carotenoid biosynthesis pathway to alter the carotenoid composition of some crops. Similarly to Kumar et al. (2012), a general increment in isoprenoid content (chlorophyll *a*, phytol, β-carotene, lutein, antheraxanthin, solanesol, and β-sitosterol) was observed when the enzyme responsible for the first committed step of the plastidial non-mevalonate pathway, i.e., 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) from *Synechocystis* was overexpressed in transplastomic tobacco plants (Hasunuma et al. 2008a).

More specifically, distinct enzymes of the carotenoid biosynthesis pathway were also modified to alter carotenoid composition of important crops (Fig. 2). Tomato (*Solanum lycopersicum*) is a widely cultivated, popular edible crop well known for accumulating high levels of lycopene (a carotenoid with no provitamin-A activity) in its fruit chromoplasts. After the development of successful plastid transformation protocol in tomato (Ruf et al. 2001), metabolic engineering of the carotenoid biosynthesis has also been performed in this plant to induce lycopene-to-provitamin A conversion and thus increase the nutritional value of the fruits (Wurbs et al. 2007; Apel and Bock 2009). For this purpose, first the lycopene β-cyclase genes from the carotenoid-producing eubacterium *Erwinia herbicola* (*crtY*) and the carotenoid-producing zygomycete fungus *Phycomyces blakesleeanus* (*carRA*) were introduced to the plastid genome, but only the bacterial gene was stably expressed in the plastids (Wurbs et al. 2007). In addition to increased tolerance towards a herbicide, namely 2-(4-chlorophenylthio)-triethylamine (CPTA) that inhibits lycopene β-cyclase, the transplastomic plants containing the *crtY* gene had 4-fold higher β-carotene content (286 μg/g dry weight), but 10–15 % lower lycopene, and 10 % lower total carotenoid content in the fruits than non-transformed plants. Not surprisingly, the carotenoid pattern of transplastomic tobacco and tomato leaves (which do not accumulate lycopene) remained unchanged, indicating that the success of metabolic engineering also depends on the concentration of the substrate of the enzyme encoded by the transgene.

As a next step, Apel and Bock (2009) introduced to tomato plastids the same lycopene β-cyclase gene (*crtY*) from *Erwinia herbicola* with a different promoter having higher activity in the chromoplasts than the one used earlier by Wurbs et al. (2007) and combined with the strongest known ribosome binding site. Similarly, in another set of experiments, the lycopene β-cyclase gene (*Lyc*) of higher plant daffodil (*Narcissus pseudonarcissus*) was also introduced into the tomato plastid genome (Apel and Bock 2009). The expression of the bacterial enzyme did not strongly alter carotenoid composition of the fruits and the leaves in this experiment, while the expression of the plant enzyme efficiently converted lycopene, the major storage carotenoid of the tomato fruit, into β-carotene (provitamin A, accumulating up to 1 mg g^{-1} dry weight) in the fruit. Unexpectedly, transplastomic tomatoes also showed a greater than 50 % increase in total carotenoid accumulation in their fruits, indicating that lycopene β-cyclase expression enhanced the flux through the pathway in chromoplasts, and that this enzyme from daffodil is probably less susceptible to negative feedback inhibition of β-carotene than the bacterial gene. In green leaves of the transplastomic tomato plants, more lycopene was channeled into the β-branch of carotenoid biosynthesis, resulting in increased accumulation of xanthophyll cycle

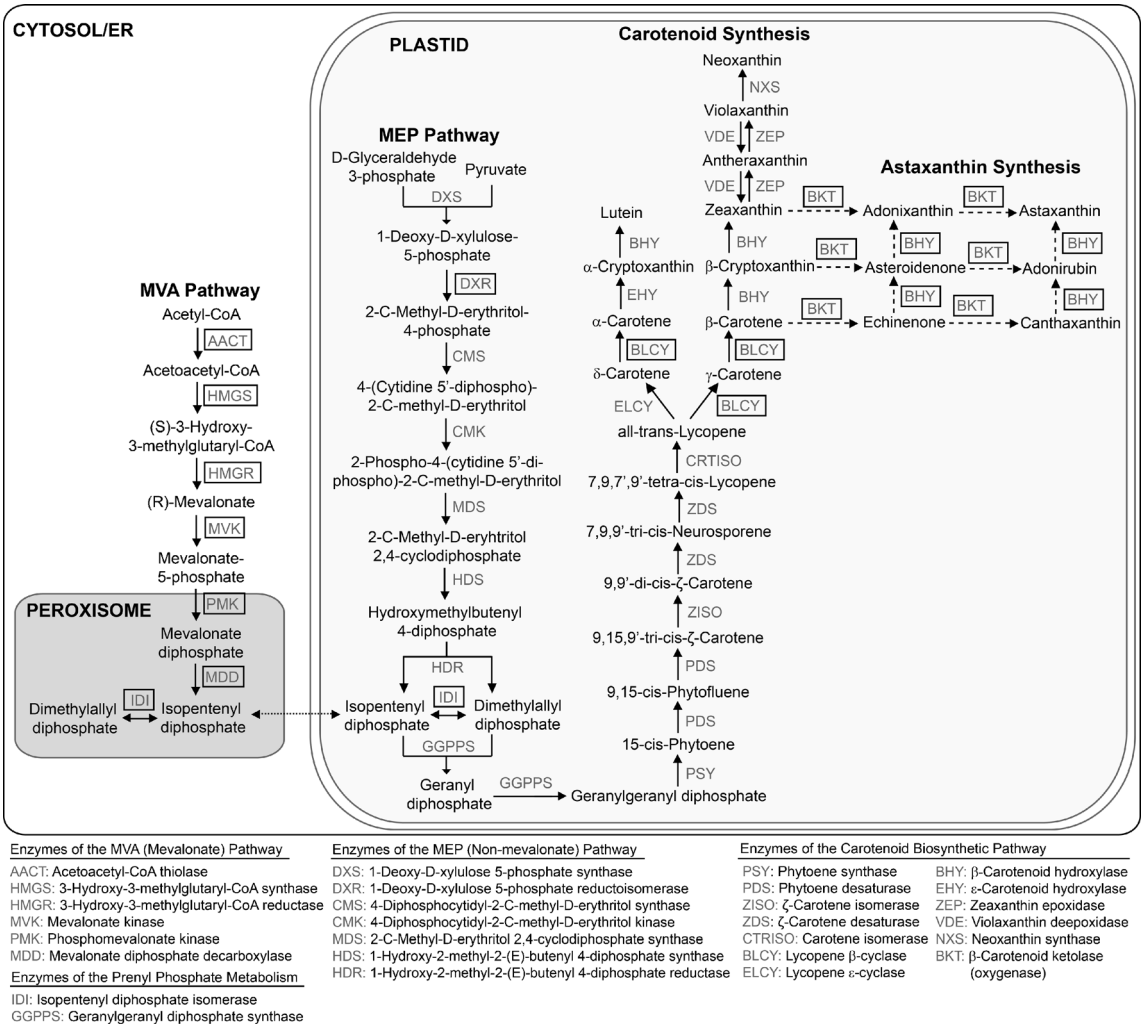

Fig. 2 Outline of the carotenoid biosynthetic processes engineered in transplastomic plants. Enzymes affected are in *squares* (for references and more details, see Section 3.3.3 and Table 4), *dotted line* indicates transport processes, and *broken line* indicates newly introduced biosynthetic pathways (i.e., astaxanthin synthesis) in the transplastomic plants. HMGR is the only enzyme located in the endoplasmic reticulum (ER). (In fact, GGPPS requires geranyl diphosphate and two isopentenyl diphosphate molecules to produce geranylgeranyl diphosphate via farnesyl diphosphate intermediate, that is, GGPPS catalyzes the condensation of three isopentenyl diphosphate molecules and one dimethylallyl diphosphate molecule)

pigments and correspondingly reduced accumulation of α-branch xanthophyll lutein, but no alterations in the total carotenoid content (Apel and Bock 2009). These results provide new insights into the regulation of carotenoid biosynthesis in different organs and demonstrate the potential of plastid genome engineering for the nutritional enhancement of food crops.

It may be interesting to recall here the importance of understanding the organ-, plastid-, and/or development-specific transcription regulation processes. Protein expression levels of the carotenoid biosynthesis genes in the chromoplasts of ripe tomato fruits were very high (Ruf et al. 2001) or lower but sufficient to induce metabolic alterations (Wurbs et al. 2007; Apel and Bock 2009), in contrast with no transgene expression observed in the ripe fruits in case of the HIV antigen (Zhou et al. 2008).

In addition to metabolic engineering aiming at increasing the total carotenoid content and/or altering the metabolic fluxes through the existing carotenoid biogenesis pathways, genes enabling the synthesis of novel carotenoids may also be introduced into higher plant chloroplasts (Fig. 2). The natural pigment astaxanthin has attracted much attention because of its (1) beneficial effects on human health and importance in the cosmetic industry, (2) importance as animal feed, especially for fish aquacultures such as salmon cultures where it provides the orange color of salmon meat or as human food coloring in the European Union (E161j), and (3) very high market price (Lemoine and Schoefs 2010; Heydarizadeh et al. 2013; Ambati et al. 2014; Solymosi et al. 2015). Astaxanthin is synthesized only by some marine bacteria (e.g., *Brevundimonas* sp.) and algae (e.g., *Haematococcus pluvialis*) (Lemoine and Schoefs 2010), but not in higher

plants (except *Adonis aestivalis* petals—Cunningham and Gantt 2011; Maoka et al. 2011). Its biosynthesis starts with β-carotene and/or zeaxanthin as precursors, which are converted to astaxanthin by two enzymes, β-carotene ketolase and β-carotene hydroxylase.

The proof-of-concept study of astaxanthin synthesis by transplastomic tobacco has been done by Hasunuma et al. (2008b). In this work, the leaves of transplastomic tobacco plants expressing both β-carotene ketolase gene (*CrtW*) and/or β-carotene hydroxylase gene (*CrtZ*) from the marine bacterium *Brevundimonas* in their plastids accumulated more than 0.5 % (5.44 mg g^{-1} dry weight) astaxanthin (accounting to more than 70 % of total carotenoids) and also synthesized a novel carotenoid, 4-ketoantheraxanthin. The accumulation of astaxanthin resulted in a peculiar reddish brown coloration of the transplastomic plants when compared to the green non-transformed plants. Moreover, the total carotenoid content in the transplastomic tobacco plants was 2-fold higher than that of non-transformed tobacco. No detectable yield penalty or metabolic burden was observed, i.e., the size of the aerial parts of the transplastomic plants was similar to those of non-transformed plants at the final stage of their growth, and the photosynthesis rate of the transformants was also not different from that of non-transformed plants.

In addition to the aforementioned two genes (*CrtW* and *CrtZ*) of *Brevundimonas* (Hasunuma et al. 2008b), an isopentenyl diphosphate isomerase gene (*idi*) from another marine bacterium (*Paracoccus*) has also been introduced into lettuce plastids (Harada et al. 2014). In this case, transplastomic plants accumulated different ketocarotenoids, including free astaxanthin, and different astaxanthin fatty acid esters at the expense of their own native carotenoids.

Vitamin E composition Tocopherols and tocotrienols are methylated phenolic compounds (termed collectively tocochromanols), which have vitamin activity, and are, therefore, correctly referred to as vitamin E. Tocochromanols represent important lipid-soluble antioxidants synthesized by photosynthetic organisms such as cyanobacteria, algae, and plants for protection against lipid peroxidation. In plants, tocopherols and tocotrienols are formed from the condensation of homogentisic acid with isoprenoid chains, i.e., phytyl diphosphate and geranylgeranyl diphosphate, respectively (reviewed in Lushchak and Semchuk 2012; Lu et al. 2013). In plants, the synthesis of the isoprenoid chain as well as the condensation reaction is plastid located. Animals and humans cannot synthesize vitamin E; they must fulfill their requirement of vitamin E by uptaking plant and/or algal tocopherols in their diet. By preventing lipid peroxidation, vitamin E reduces the risk of a number of serious human disorders, including cardiovascular disease (Pryor 2000), cancer (Prasad et al. 1999), Alzheimer's disease (Mangialasche et al. 2010), and other chronic diseases (Traber et al. 2008), and also enhances the function of the immune system (Adachi et al. 1997). Due to the high nutritional value and benefits of vitamin E in human health, increasing the tocochromanol content of major crops has long been in the focus of breeding programs and transgenic engineering approaches (Hirschberg 1999; DellaPenna 2005; Karunanandaa et al. 2005). As the corresponding biosynthesis pathway is primarily located in chloroplasts, plastid transformation of important crops is another alternative to improve vitamin E content or composition in crop plants by metabolic engineering.

Since 4-hydroxyphenylpyruvate dioxygenase (HPPD) is the only enzyme of the biosynthetic pathway of tocochromanols that is localized outside the plastids, the barley *hppd* gene has been inserted into the plastid genome of tobacco to investigate whether a plastid-localized HPPD enzyme could affect vitamin E content (Falk et al. 2005). Only a moderate increase and altered composition was observed in the vitamin E content of the leaves and seeds of transplastomic plants (Falk et al. 2005). Therefore, overexpression of the *hppd* gene in the plastids did not prove to be advantageous when compared to transgenic plants with high HPPD expression levels in the cytoplasm (Tsegaye et al. 2002).

In order to enhance vitamin E content by chloroplast transformation, Yabuta et al. (2013) produced three types of transplastomic tobacco lines carrying either tocopherol cyclase (*ttc*, also termed *tcy*) or γ-tocopherol methyltransferase (*tmt*), and both of these tocopherol biosynthetic genes of *Arabidopsis*. There was a significant increase in total levels of tocopherols in *ttc* plants and the plants expressing both transgenes, with an improved vitamin E composition in case of the latter (Yabuta et al. 2013). The same authors reported overexpression of tocopherol synthase (*ttc*) in lettuce resulting in increased vitamin E content in this edible crop (Yabuta et al. 2013).

Homogentisate phytyltransferase (*hpt*) is the enzyme responsible for the condensation of homogentisic acid and the isoprenoid chain. In other studies, the *hpt* gene—in addition to *tcy* and *tmt*—was also introduced to the plastid genome of tobacco and tomato to engineer the tocopherol metabolic pathway and to assess the impact of these three plastid localized enzymes on tocochromanol biosynthesis in chloroplasts and also in chromoplasts (Lu et al. 2013). In this case, all three genes originated from a cyanobacterium, *Synechocystis*, and *tmt* from *Arabidopsis* has also been additionally tested. Nearly 5-fold higher total tocochromanol level and slightly altered vitamin E composition was found only in transplastomic plants expressing the *hpt* gene. This clearly demonstrated that the HPT enzyme represents a rate-limiting step in the biosynthetic pathway (Lu et al. 2013). In addition, synthetic operons containing all three aforementioned enzymes (in addition to an

intercistronic expression element—Zhou et al. 2007) were also constructed and introduced into the plastid genome, resulting in a 10-fold increase in tocochromanol levels in the transplastomic plants when compared to non-transformed plants. In addition, cold-stress recovery assays revealed that the higher tocochromanol levels of the transplastomic plants conferred strong protection against oxidative stress in them. Studies conducted on the vitamin E content in the leaves of tomato plants transformed with vectors containing the same transgenes were in line with data observed in tobacco leaves, with enhanced biosynthesis and accumulation of tocotrienols, which normally do not accumulate in the leaves of dicots (Lu et al. 2013). Tocochromanol content and composition could also be increased in the fruits of transplastomic plants; however, it strongly depended on the ripening stage and also on the cultivar used, i.e., fruits of a red-fruited cultivar had low transgene expression levels and, thus, lower vitamin E content than the leaves. Taken together, in addition to a better understaning of vitamin E biosynthesis, chloroplast engineering is also useful to enhance the vitamin E content in crops.

Lipid and fatty acid composition α-Linolenic acid (an omega-3 fatty acid) and linoleic acid (an omega-6 fatty acid) are essential for humans. Other fatty acids such as gamma-linolenic acid and very long chain polyunsaturated fatty acids (VLCPUFAs) like arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid are thought to be essential only under certain developmental or disease conditions (Martinez 1992; Ulmann et al. 2014). However, an unbalanced diet in VLCPUFAs has been associated with abnormal cell growth and division, platelet aggregation, inflammatory responses, and hemorrhage (Dnyaneshwar et al. 2006). Commercial oilseed crops represent the main source of oils and fats in the human diet (Gunstone et al. 2001) and contain predominantly four fatty acid species (linoleic acid, palmitic acid, lauric acid, and oleic acid) and lack VLCPUFAs (Murphy 1993; Lands 2005; Singh et al. 2005; Cahoon et al. 2007; Napier 2007; Rogalski and Carrer 2011). As fatty acids are also essential for plant cells' growth and development, and the plastids harbor the fatty acid biosynthesis machinery, nuclear transformation of oilseed plants has been carried out to produce additional fatty acids or lipids of nutritional importance (Wu et al. 2005; Napier 2007; Damude and Kinney 2008; Napier and Graham 2010) or for biodiesel production (Durrett et al. 2008; Graef et al. 2009; Lu et al. 2011). In addition, lipids and fatty acids are considered as antioxidants, and also have role in different stress tolerance mechanisms. For instance, increased unsaturation of fatty acids may result in improved cold stress tolerance of the plants in addition to their improved nutritional value (Craig et al. 2008; see Section 3.3.1).

The first work dealing with the engineering of the lipid pathway by plastid transformation aimed at replacing the promoter of the endogenous plastid *accD* gene encoding the β-carboxyl transferase subunit of the key enzyme in de novo fatty acid biosynthesis, namely acetyl-CoA carboxylase (ACCase), in tobacco (Madoka et al. 2002). The leaves of transplastomic plants overexpressing the endogenous plastidial ACCase gene under a strong rRNA promoter had an increased leaf longevity reflected as delayed senescence, lower starch content, but higher fatty acid and lipid (monogalactosyldiacylglycerol) content, and altered fatty acid composition with increased unsaturation levels (Table 4) than non-transformed plants. The fatty acid content and composition of the seeds remained unchanged in transplastomic plants; however, they had 2-fold higher seed production, resulting in increased seed oil yield per plant (Madoka et al. 2002). Other works later successfully improved seed fatty acid composition of tobacco (Craig et al. 2008, see Section 3.3.1).

The 3-ketoacyl acyl carrier protein synthase III (KASIII) is an enzyme responsible for initiating both straight- and branched-chain fatty acid biosynthesis. During the proof-of-concept study of a new, cytokinin-based selection method for the production of transplastomic tobacco plants, the insertion of *kasIII* into the plastid genome resulted in altered fatty acid composition of the leaves (Dunne et al. 2014).

Essential amino acid composition Tryptophan (Trp) is an essential amino acid in humans, needed for normal growth in infants and for nitrogen balance in adults. In addition to fatty acid biosynthesis, the synthesis of most essential amino acids is also plastid located. However, nuclear genes encoding mRNAs translated on cytosolic ribosomes and targeted to chloroplasts are responsible for Trp biosynthesis, probably as a result of gene transfer from the endosymbiont's genome towards the nucleus during endosymbiogenesis. Anthranilate synthase catalyzes the conversion of chorismate to anthranilate. It is a key enzyme of Trp biosynthesis regulation because (1) it represents the first committed step of Trp synthesis, in competition with the synthesis of several other aromatic compounds, which also rely on chorismate as precursor, and (2) it is feedback inhibited by the presence and accumulation of the product, free Trp that binds the α-subunit of anthranilate-synthase as inhibitor (Zhang et al. 2001). In addition, Trp synthesis in the plastids is also regulated by the abundance of the mRNA of anthranilate synthase (Radwanski and Last 1995). Therefore, metabolic engineering of Trp biosynthesis by insertion and overexpression of a feedback-insensitive α-subunit of anthranilate synthase (*ASA2*) in tobacco plastids resulted in a 10-fold increase in free Trp in the leaves and slight increase in total Trp in the seeds (Zhang et al. 2001). Transplastomic plants had increased resistance to 5-methyl-Trp, an inhibitor of the anthranilate synthase (Zhang et al. 2001), which led to the development of plastid transformation protocols based on toxic Trp and indole analogs as selective agents, using the *ASA2* gene (Barone et al. 2009). These

studies also demonstrated that due to the site-directed insertion of the transgene into the plastid, high and less variable levels of gene expression (and as a consequence more uniform Trp content) could be observed in the different transplastomic lines, when compared with transgenic plants obtained by random transgene insertion during nuclear transformation (Zhang et al. 2001). This is a proof-of-concept study showing that metabolic engineering of essential amino acid biosynthetic pathways in transplastomic plants can lead to biofortification.

4 Field trials and commercialization attempts of transplastomic plants

Transplastomic field trials are not separated from transgenic ones in relevant databases, but careful analysis reveals a few pieces of valuable information in this area. The main conclusion of a worldwide search in the biosafety data of 21 countries that have a track record of publishing original research with generation of transplastomic plants (Argentina, Australia, Brazil, Canada, China, India, Iran, Japan, Mexico, Pakistan, South-Korea, Ukraine, the United States, and eight countries in the European Union) is that verifiable field tests with transplastomic plants have so far been carried out only in the European Union and the United States.

Browsing the European GMO Register (gmoinfo.jrc.ec.europa.eu/gmp_browse.aspx) resulted in only three field tests. Two experiments, one with petunia between 2009 and 2012 (Notification Number B/DE/08/203; Fig. 3) and the other with tobacco ('Petit Havana') between 2012 and 2016 (Notification No. B/DE/10/210), have been aimed at evaluating the spread of model transgenes (*aadA*+*uidA* or *aadA*+*gfp*, respectively) via pollen under field conditions. The experimental plots cover(ed) a surface from 500 to no more than 3000 m^2 (Fig. 3). Final or interim reports and papers on these field experiments are not yet available publicly (Prof. Inge Broer, University of Rostock, Germany, personal communication). The third experiment (Notification No. B/ES/12/16) was carried out on ca. 150 m^2 in 2012 by the Public University of Navarra in Spain with two transplastomic tobacco lines ('Virginia Gold' and 'Havana 503B') that expressed thioredoxin f (see Section 3.3.2) for testing them as alternative stocks for biofuel production. In a published analysis of field data, both transplastomic lines showed 2.3- to 3.6-fold leaf starch accumulation and increased soluble sugar content (up to 74 %) as well as higher specific weight (up to 10–25 % in dry weight) of leaves compared to non-transformed plants (Farran et al. 2014). Importantly, the plants were phenotypically equivalent (with respect to plant size, total plant weight, and relative chlorophyll content) to the wild-type control in the field.

More field tests—in total nine—have been performed in the United States with transplastomic plants (www.isb.vt.edu/search-release-data.aspx). A recent series of four tests between 2013 and 2015 are coordinated by the University of Illinois with tobacco expressing genes for enhanced photosynthetic activity. Among the tested genes are a bicarbonate transporter (*bicA*), a β-carboxysome shell protein subunit gene (*ccmK2*), and the large subunit gene of RuBisCO (*rbcL*). Derived from the cyanobacterium *Synechococcus*, the first two genes are aimed at improving photosynthetic CO_2 fixation (see Section 3.3.2) by making use of the efficient CO_2-concentrating mechanism from this blue-green alga.

Metabolix, Inc., an advanced biomaterials company, run a field trial in Kentucky state in 2009 (Permission No. 08-337-105r) in order to prove the concept of producing polyhydroxybutyrate (PHB), a biodegradable polyester plastic, in transplastomic tobacco. According to published results (Bohmert-Tatarev et al. 2011), high levels of PHB were accumulated in T1 plants, up to an average of 18 % dry weight in leaves and 9 % in the dry biomass of the entire plant.

Another company, Chlorogen, Inc., was active between 2003 and 2007, and produced transplastomic tobacco containing several, mostly undisclosed genes of interest and the *aadA* selectable marker gene. The company licensed chloroplast transformation technology of tobacco from two pioneers in this field: first, the University of Central Florida (Daniell lab, in 2002) and later also from Rutgers University (Maliga lab, in 2005). The portfolio of the company contained various pharmaceutical innovations including the production of insulin-like growth factor, serum albumin, interleukin, anti-Müllerian hormone, and hantaviral antigens in transplastomic tobacco plants. Eventually, four field experiments were carried out (with another two withdrawn) in Kentucky, Missouri, and South Carolina on a surface typically between 400 and 8000 m^2. Results of only one of these experiments (Permission No. 04-114-01r) were published in a scientific paper (Arlen et al. 2007). In this work, the T1 generation of homoplasmic transplastomic tobacco expressing a type I interferon, IFN-α2b, was planted in a field of about 1000 m^2. A single premature harvest before flowering yielded a biomass that was equivalent to more than 85 g of IFN-α2b, enough for 50,000 years of treatment against hepatitis C for an individual. It is noteworthy to mention that 1 year of treatment may easily cost $10,000. Both the crude extract and the purified form of IFN-α2b possessed in vitro as well as in vivo activity in several virus tests (Arlen et al. 2007).

Due to its aggressive licensing strategy and a successful second round of securing a venture capital of $6 million in 2005, Chlorogen was in a strong position to realize the first ever commercial transplastomic product. What made its apparently unexpected going out of business in 2007 is not clear; it may have had to do with the shaping economical crisis that the investors wanted to minimize their anticipated losses. Other reasons could be related to the principal way transplastomic plants may be utilized commercially, which is

Fig. 3 Field trial in 2009 with pollen donor transplastomic *Petunia* plants ('Pink Wave' line T16, *left insert*), containing *uidA* (*gusA*) and *aadA* transgenes (Zubko et al. 2004), and untransformed recipient plants (white, 'W115', *right insert*) in Sagerheide near Rostock, Germany. In the foreground is an experimental setup with low pollen pressure (one-one row series of 'Pink Wave' T16 and 'W115'); in the background is an experimental setup with high pollen pressure (six rows of 'Pink Wave' T16 next to one row of 'W115'). (Source—Prof. Inge Broer et al., University of Rostock, Germany)

2-fold: (1) directly, as crops with improved agronomical traits for example, or (2) indirectly, by using the plants as bioreactors for the production, extraction, and processing of therapeutic or other valuable proteins. The first direction is still hindered by the fact that few crop plants are accessible for chloroplast transformation technology (Table 1). As to the pharmaceutical utilization of transplastomic plants, refined technologies of transient or viral (i.e., non-integrative) transgene expression have also emerged in the meantime (Yusibov and Rabindran 2008). These plant systems can deliver close to or as high levels of expression as plastid transformation, but considerably faster because they do not require multiple rounds of selection to reach the homoplasmic state.

Bayer CropScience France in Lyon had an active program with transplastomic plants between 2004 and 2009. Major practical achievements were the first generation of transplastomic soybean (Dufourmantel et al. 2004), then the production of insect-resistant (Dufourmantel et al. 2005) and herbicide-tolerant transplastomic soybean (Dufourmantel et al. 2007), but no field trials have been established with these plants.

Finally, Plastid AS has been active in this field since June 2007 in Norway. The company is a spinout from the University of Stavanger, with the main profile to produce foreign proteins such as kinases and proteins from parasites in tobacco chloroplasts, and to develop plastid transformation technologies in maize and wheat. A core asset in the portfolio of Plastid AS is the utilization of plant hormone-based selection and differentiation (Dunne et al. 2014), which addresses a major bottleneck in the generation of transplastomic plants. To our knowledge, the company currently has no field tests in progress.

Though transplastomic plants may present an increased level of environmental safety compared to (nuclear) transgenic plants (see Section 5.1), their hitherto unsuccessful commercialization may be associated with a lack of interest on behalf of the major companies in the agribiotech sector. The latter have already heavily invested in the generation of transgenic products, especially in maize, cotton, canola, and soybean, and may thus have less motivation to invest in the time-consuming development and extension of transplastomic technologies for these and other important crop species. Another reason for the delayed innovation could be that one of the most promising applications for transplastomic plants, i.e., molecular pharming, should make this technology attractive to the biopharma industry rather than to agribusiness.

In summary, numerous field tests have been carried out with transplastomic plants in Europe and the United States, but no products are currently in an advanced stage of commercial development.

5 Public concerns related to the safety of transplastomic plants

Although the production and the possible perspectives of the use of transplastomic plants are extensively reviewed in the literature, data about field trials (see Section 4), biosafety considerations, or public concerns are very rarely discussed in connection with transplastomic plants. Therefore, in this section, we attempt to critically review and discuss data related to these issues.

5.1 Biosafety of transplastomic plants

There are two main aspects to consider about the safety of transplastomic plants (and GM plants, in general). The first one is a possible further flow of the incorporated transgene itself. This can happen (1) vertically, i.e., sexually (predominantly by pollen drift) as introgression to an offspring, and/or (2) horizontally or laterally, by asexual modes (e.g., by grafting) or via DNA transfer from decaying plant tissue into

a competent living organism. The second aspect is the acute or chronic (cumulated) direct or indirect effect caused by the expression of the transgene (e.g., by the RNA transcript, recombinant protein, or as a consequence of its activity) on human or livestock health as well as on any non-target organism that may be present in the surrounding ecosystem. This effect includes but is not restricted to toxicity, allergenicity, fitness, and reproduction rate.

Before setting out to discuss these points briefly, it should be emphasized that a great deal of the available information on the first aspect has been obtained in model or simulated experiments. In these, transplastomic plants were not even included, for instance plants with chloroplast-specific markers were used instead (e.g., Wang et al. 2004; Haider et al. 2009), or if yes, then predominantly tobacco and in a controlled, artificial environment (laboratory or greenhouse). In other words, no controlled field experiments have so far been completed specifically with transplastomic plants in order to evaluate their safety under more real conditions (see Section 4).

Concerning vertical gene transfer, in general, transplastomic plants are regarded as safe, even several magnitudes safer, containers of the introduced transgene than nuclear transgenic plants. This is based on the fact that plastid genes (unlike nuclear ones) universally follow extranuclear (cytoplasmic) inheritance, a particular type of non-Mendelian inheritance. In this case, plastid genes are usually transferred to a progeny uniparentally, i.e., plastid DNA is transmitted (almost) exclusively maternally or paternally (Daniell et al. 1998, 2002; Daniell 2002; Hagemann 2004, 2010). An alternative in higher plants is the biparental mode of transmission when both parents contribute significantly to the progeny's plastome composition, although rarely to an equal extent (Sears 1980; Corriveau and Coleman 1988; Zhang et al. 2003; Bock 2007). There may be a continuous distribution of higher plants in nature with transmission from strictly maternal to strictly paternal (the biparental mode being in the middle section of the distribution), but even in the two extremities a low frequency of transmission via the opposite sex can still occur. However, this is negligible compared to (homozygous) nuclear genes, which are normally carried and transmitted in up to 100 % of the pollen.

Coming back to vertical gene transfer in transplastomic plants, two parallel studies demonstrated experimentally that crossing male sterile tobacco (*N. tabacum*, strict maternal transmission) with transplastomic pollen donors reveals in this otherwise self-fertilizing species some paternal transmission of the marker transgenes (*aadA* and *gfp*) to the germline of the F1 progeny. The frequency of these events did not exceed 0.001 % (10^{-5}) with a prevalence around 0.0002 % (2×10^{-6}) (Ruf et al. 2007; Svab and Maliga 2007), also independently confirmed in *Arabidopsis thaliana* (Azhagiri and Maliga 2007—3.9×10^{-5}). In addition, field conditions are radically different from this experimental setup in the laboratory because self-fertilization rates are much higher (ca. 90 %) with fertile maternal plants and can be enhanced up to ten times with the inclusion of isolation distance and pollen barriers, and no selection pressure is applied for the low-frequency paternal transmission events. Therefore, it is estimated that a more likely frequency of transgene escape via pollen in transplastomic plants may easily be less than 1 in 100 million seeds ($<10^{-8}$) under field conditions (Ruf et al. 2007). It is noteworthy in this context that this frequency is well below the detection limit of routine field sampling, and, for comparison, up to 0.9 % contamination with GM products is allowed for marketing without labeling in the EU. Furthermore, this level of safety can still be elevated with a combination of other gene containment tools such as controlled (cytoplasmic) male sterility (Ruiz and Daniell 2005; Daniell 2007), seed sterility, and transgene mitigation strategies that will decrease—in the absence of selection—the fitness of transgene-containing (hybrid or volunteer) plants when competing with wild relatives or non-GM cultivars (Gressel 2010). These measures will become especially relevant when transplastomic plants would once be developed for contained cultivation of pharmaceutical products.

As a caution, it should also be noted that these assumptions may be valid for tobacco and related species with a similar mechanism of plastid transmission but cannot immediately be extended to other species, including the cereals. As indicated above, empirical confirmation and extension of this evidence is required under real field conditions. Finally, it is needless to point out that transplastomic gene containment will not work in plants with (preferentially) paternal or explicit biparental plastid transmission.

But that is only one side of the vertical gene transfer story. Gene containment in transplastomic plants may efficiently (though not absolutely) restrict transgene flow via pollen spread, but it cannot prevent the same plants from being fertilized by pollen of a sexually compatible species. In other words, via repeated (recurrent) backcrossing with pollen from a wild or weedy relative, it is perfectly possible to generate a cytoplasmic transgene introgression (chloroplast capture) in the wild gene pool (Allainguillaume et al. 2009; Haider et al. 2009). As a result, in specific situations, crop-to-weed transgene flow could still happen via transplastomic plants despite their efficient control of transgenic pollen spread. This scenario, though still theoretical, all the more underlines that the role of transgene mitigation strategies (Gressel 2010) cannot be overestimated.

Finally, a straightforward measure against vertical (and to some extent horizontal) gene transfer, at least in cases like some biopharming applications, is simply harvesting the plants before flowering, which will preclude both maternal and paternal escape of the transgene, unless of course the seeds are needed.

Horizontal gene transfer, in turn, can be anticipated to occur more frequently with transplastomic than with transgenic

plants based on two assumptions: (1) high copy number and thus the possible release of about 1000 times higher amounts of the transgene via the plant tissue, and (2) the prokaryotic nature of the plastid genome, which may provide sequence homology and possible recombination sites for genetic exchange with naturally competent prokaryotic microorganisms.

The overwhelming majority of experimental data on elucidating horizontal gene transfer with transplastomic plants comes from a circle around scientists affiliated with the University of Lyon in France (Kay et al. 2002; Demanèche et al. 2011). In a first series of experiments, transplastomic tobacco plants were colonized by naturally competent *Acinetobacter baylyi* cells that contained a plasmid without or with (pBAB2) tobacco chloroplast sequences incorporated to facilitate homologous recombination (Kay et al. 2002). Spectinomycin-resistant bacterial colonies were isolated and selected from transplastomic plants at a low frequency (in the order of 10^{-8}). These colonies contained the *aadA* marker gene from the transplastomic tobacco, but only in the presence of the homologous sequences. The data demonstrated that under specific conditions, horizontal gene transfer can take place in planta. In a second step, the in situ transformation capacity of transplastomic plant DNA was studied (Ceccherini et al. 2003). Here, the DNA purified from decaying or enzymatically treated leaf tissue was found to degrade within 72 h but was able to transform in vitro the above *Acinetobacter* strain at a low frequency (between 10^{-6} and 10^{-8}). Unfortunately, longer treatments were not tested systematically. Similar results were obtained after visual screening on residues of plant tissue for transformed bacteria expressing an *aadA*::*gfp* fusion gene that is restored only upon recombination with total DNA from the same transplastomic plant line studied before (Pontiroli et al. 2009). A similar magnitude of gene transfer frequency was also found with DNA purified from a different transplastomic tobacco line and used to transform the same bacterial strain in vitro (De Vries et al. 2004). Some of the missing long-term studies were later performed by Pontiroli et al. (2010). In this case, transplastomic plant leaf disks (cut or ground, 0.05 or 0.5 g) were mixed with 10 g "live" soil containing natural microorganisms and incubated for up to 4 years in test tubes. The authors concluded that intact *aadA* gene must have been still present in the soil after 4 years of incubation because DNA extracted from the soil (but only with 0.5 g plant tissue) was capable to transform *Acinetobacter* in vitro at a low frequency (between 10^{-7} and 10^{-8}) as also confirmed independently by PCR. The presence and adsorption of transplastomic DNA in soil was investigated in more details by Poté et al. (2010). These authors showed that purified transplastomic plant DNA can pass through unsaturated soil columns (300 g) and is able to transform in vitro competent *Acinetobacter* cells at a low frequency (typically around 7×10^{-6}) up to 2 days after adsorption. However, it remains to be seen how intact or decaying transplastomic plant organs and tissues would act in the same soil columns in situ or especially under real field conditions.

So far, one naturally competent *Acinetobacter* strain (BD413) was used in all above experiments directed for the determination of horizontal gene transfer from transplastomic plants. More recently, Demanèche et al. (2011) studied 16 bacterial isolates (each possessing partial DNA sequence similarity to chloroplast genes) belonging to seven genera in order to survey the biological range of potential horizontal gene transfer. Only two isolates were able to take up a plasmid containing the transplastomic plant DNA (*aadA* gene) under natural conditions (i.e., without pretreatment for competence) and this at a frequency below 10^{-8}. Moreover, the cloned plant DNA was maintained on the plasmid but never integrated on the bacterial chromosome, and none of the bacterial isolates tested was able to take up intact (uncloned), purified transplastomic plant DNA.

A single study contains data on the side-by-side comparison of horizontal gene transfer with transplastomic and nuclear transgenic plants (Kay et al. 2002). In the highly competent *Acinetobacter* strain, no recombinants that carried the nuclear transgene were identified in planta, which was surpassed by a detectable transfer frequency of 10^{-8} with transplastomic tobacco. However, this dataset was too small and preliminary to support a firm conclusion that the higher number of transgene copies in transplastomic plants could indeed lead to a more elevated horizontal gene transfer than those found in separate studies with only nuclear transgenes.

All the above data and available evidence point to the same conclusion: horizontal gene transfer from transplastomic plants to bacteria might happen but only under very specific, optimized conditions, e.g., in the presence of homologous sequences and highly competent bacteria, and at an extremely low frequency, i.e., around or just above the detection limit of the actually applied methods. This conclusion is supported by ample evidence that under field conditions, though with nuclear transgenic plants, no horizontal gene transfer has been detected so far (Paget et al. 1998; Gebhard and Smalla 1999; Badosa et al. 2004; Demanèche et al. 2008; Wagner et al. 2008; Kim et al. 2010; Isaza et al. 2011; Ma et al. 2011).

As a side mark, chloroplast capture—described above for vertical gene transfer—can also be achieved by the horizontal transfer of plastid DNA via asexual grafting of related species, at least within the *Nicotiana* genus (Stegemann and Bock 2009; Stegemann et al. 2012). The resident plastid genome of the recipient plant is replaced as a whole by the donor plastome without interplastomic recombination (Stegemann et al. 2012). In addition, cell-to-cell movement of entire plastids in interspecific graft tissues has also been demonstrated (Thyssen et al. 2012). Thus, grafting may be used for rapid introgression of transformed plastids into commercial cultivars in some species instead of performing repeated backcrossings (Thyssen et al. 2012). However, due to the

presence of several chimeric multiprotein complexes in the plastids (with one part of the subunits being nuclear encoded and the other one being encoded in the plastid genome), this method will likely result in plastome-genome incompatibilities and mutant phenotypes. This is especially probable between phylogenetically more distant recipient and donor plants, restricting this horizontal plastome transmission method to closely related species or cultivars (Greiner and Bock 2013; Bock 2014). Furthermore, the grafting technique has major limitations: usually it works within a single genus (though there are exceptions) and—due to the lack of a continuous vascular cambium—monocots, including cereals, cannot be grafted.

Considering the second biosafety aspect, only the potential environmental effects of transplastomic plants were studied so far. Brinkmann and Tebbe (2007) amplified 16S ribosomal RNA and DNA sequences from soil bacterial communities in order to test the effect of potted transplastomic tobacco plants (containing the *aadA* gene) on the genetic diversity in these microbial communities. Single-strand conformation polymorphism (SSCP) analysis indicated that transplastomic tobacco caused a reduction of a particular sequence (out of ca. 60 products in the overall SSCP profile) that may be specific for *Flavobacterium* sp., a common species in the rhizosphere, which is important for balancing the soil ecosystem. The authors also stated that it remains unclear whether the observed change was really caused by the transplastomic modification or whether it is in the range of natural variation in bacterial community structures.

Brusetti et al. (2008) designed a similar experiment with primers specific for the 16S–23S intergenic spacer (IGS) region of ribosomal DNA in order to study the effect of rhizosphere exudates from phytotron-grown transplastomic tobacco on the stability and changes of the genetic diversity in soil microcosms (2 g of soil in test tubes). The conclusions suggested that the impacts of transplastomic tobacco on the soil bacterial community structure were transient and the plant genotype rather than its transgenic nature was the most important factor influencing the soil bacterial diversity. The observed slight genetic changes were not attributed to horizontal transfer of transplastomic DNA into soil microorganisms because even the very efficient in vitro transformation of *Acinetobacter* with root exudates did not yield any recombinant bacteria (detection limit at 10^{-8}) (Brusetti et al. 2008).

The only study to evaluate the environmental effects of transplastomic plants on non-target organisms in the field has recently been performed in China (Lv et al. 2014). In this experiment, transplastomic tobacco (containing the *aadA* and *gfp* marker genes) was compared to wild-type plants during four developmental stages (seedling, vegetative, flowering, and senescence) for the following parameters in the rhizosphere: colony counts of bacteria, actinomycetes, and fungi; and diversity in microbial utilization of carbon sources and in denaturing gradient gel electrophoretic (DGGE) fingerprints of the 16S rRNA gene region for the total rhizosphere microbiome. A thorough statistical analysis revealed no significant differences for any of the parameters tested in none of the developmental stages, which indicates that these transplastomic plants exerted no detectable effect on the structural diversity of the rhizosphere microbial community during their entire life cycle. The authors also stressed the need for more long-term studies in this field.

Finally, the cumulated health and unintended effects of a recombinant gene product on the consumer and non-target organisms is evaluated on a case-by-case basis and according to strict regulations (Peterson and Arntzen 2004; Sparrow et al. 2013). Though transplastomic plants are, in general, not expected to be essentially different from nuclear-transformed GM plants in this aspect, for pharmaceutical applications non-food and non-feed plants (such as tobacco or camelina) represent an additional layer of safety in order to avoid that drugs could enter the food chain (Breyer et al. 2009).

5.2 Public concerns associated with transplastomic plants

Apart from a few unsubstantiated and theoretical arguments (Ho and Cummins 2005), there are no major concerns articulated about transplastomic plants specifically. Therefore, it may be worth considering which of the general concerns alleged to GM plants may be particularly valid for transplastomic plants. Two issues are discussed below: the horizontal transfer of antibiotic resistance marker genes (ARMGs) and genetic containment of transplastomic plants in association with biopharming.

A common public concern about GM (including transplastomic) plants carrying ARMGs is related to the potential adverse effects of these plants (1) on the health of humans and livestock as a consequence of ingestion, and (2) on the natural environment, in both cases due to horizontal gene transfer to living microorganisms. This may represent a real concern when repeatedly occurring in human pathogenic bacteria, which could this way become multi-resistant "superbugs" in clinical use. Therefore, the European Union Directive 2001/18/EC required by 31 December 2004 the "phasing out antibiotic resistance markers in GMOs, which may have adverse effects on human health and the environment" in the case of GM plants to be placed on the market. With effect on 31 December 2008, this rule was extended to any other deliberate release of GM plants in the environment. However, the Directive did not explicitly and absolutely ban all ARMGs in all GM plants, i.e., those generated for basic research in a contained space remained exempt.

Several methods have been developed to remove the (antibiotic resistance) marker genes in order to facilitate the acceptance of transplastomic crops and also to allow multiple

rounds of plastid transformation with the same marker gene (also called marker recycling) due to the low number of efficient selection systems available for plastid transformation (reviewed in Day et al. 2005; Koop et al. 2007; Day and Goldschmidt-Clermont 2011). In addition, constitutive and high level expression of a marker gene represents a metabolic burden to the transformed plant and may negatively impact its fitness and yield. Therefore, removal of marker genes may also confer a relative agronomical advantage to GM crops in general. The first GM plant (LY038, a lysine overproducing transgenic maize) with an ARMG (*nptII*) excised via site-specific recombination (Cre-*loxP*) was approved for cultivation in 2005 in the United States and subsequently for food and/or feed in seven other countries in Asia and America, but not in the European Union.

With relevance to transplastomic plants, it is important that the European Union's own food safety authority (EFSA, GMO panel) has later nuanced the principles of the Directive 2001/18/EC (EFSA 2004). EFSA divided the ARMGs in three categories according to their potential risks. The *nptII* gene, belonging to the first group, should not be restricted for any field release of GM plants (including commercial cultivation) due to its long, by now more than 20-year history of safe use in GM plants and also to a long track record of widespread natural occurrence of kanamycin-resistant microbes both in the environment as well as in humans (Benveniste and Davies 1973; Smalla et al. 1993; Riesenfeld et al. 2004; Wright 2010; Forsberg et al. 2012). Genes conferring resistance to spectinomycin and streptomycin, the most frequently applied antibiotics for the selection of transplastomic plants, were placed in the second category, which means release for contained field trials only. In other words, transplastomic plants containing the *aadA* marker gene (Table 1) and intended for placing on the market should first have this gene removed from their genome (see above). It should be noted that spectinomycin has a limited importance for veterinary applications and, for instance, in the United States it is not even distributed since the end of 2005 (source—CDC, Centers for Disease Control and Prevention). Thus, spectinomycin is not likely to exert a major selective pressure in the environment and a hypothetical release of resistance to this antibiotic via transplastomic plants would not have a health effect on humans or farm animals. Together with the very low probability of horizontal gene transfer from transplastomic plants (see Section 5.1), the above precautionary measures result in an extremely tight control of ARMGs.

This particular safety issue of transplastomic (and other GM) plants can be more realistically viewed in the context of agricultural ecosystems. In these, there is a complex web of much more important sources and factors that affect the stability, recycling, and horizontal transfer of (at least some) ARMGs (Martinez 2009). Occasional or mass-scale delivery of ARMGs, including the *aadA* gene (Binh et al. 2009), into the ecosystem is very well characterized: DNA-contaminated veterinary antibiotics (Lu et al. 2004), resistant microbial biocontrol agents (Zhang et al. 2006), livestock manure (Heuer et al. 2011; Marti et al. 2013a; Udikovic-Kolic et al. 2014), and waste water or even drinking (including chlorinated) water (Xi et al. 2009; Marti et al. 2013b; Shi et al. 2013) are all identified and proven components of this antibiotic resistance cycle. Through the same channels, also antibiotics are efficiently distributed, which provides an increased pressure for the selection of further antibiotic-resistant microbes. The consideration of these factors will not nullify but certainly and significantly diminish the often overestimated role that transplastomic and GM plants may play in the inadvertent spread of antibiotic resistance in the biosphere.

Obviously, the production of transplastomic (and GM) plants expressing pharmaceutical compounds or veterinary agents must be strictly separated from the production and distribution chains of food. There are a number of biological, chemical, and physical methods and their combinations to provide a high level of containment for such biopharmed plants. These measures include transplastomics itself (see Section 5.1), alternative production in non-food/non-feed crops or even non-crop plants, and inducible or transient expression systems (reviewed in Murphy 2007).

As an example, almost perfect containment can be expected in case of transplastomic carrot, which has predominantly maternal plastid inheritance, and is a biennial plant flowering and producing seeds only in the second year, while harvesting can be done in the first year (Daniell et al. 2005). However, in contrast with some other molecular strategies such as female or male sterility, gene deletor, GeneSafe™ developed to prevent gene flow via pollen and seed from transgenic plants, the seeds of transplastomic plants still carry the transgene (reviewed in Ding et al. 2014; see also Section 5.1). Therefore, culturing transplastomic plant cells (and carrot is ideal for this purpose) under aseptic conditions on synthetic media in bioreactors (Michoux et al. 2013) is another, reasonably cost-effective alternative to field or greenhouse cultivation. This approach provides fully contained conditions desirable for certain biopharmaceutical products, which may be sufficient to manage public concern or to ensure the deregulation of transplastomic plants (Bock 2014).

In our opinion, correct and strictly controlled experiments will always have to be undertaken for testing the biosafety of transplastomic and GM plants if these are really meant to be evaluated case by case. Since the inception of its research framework programs, the European Union has actively promoted this type of experimental approaches: altogether some €300 million were spent during 30 years to support about 150 projects that involved 500 research groups (EC Report 2010), and this trend will certainly continue in the current "Horizon 2020" period. One essential conclusion of these studies was

that biotechnology, and in particular GM plants, are not per se more risky than conventional genetic improvement technologies (European Commission Report 2010) and food derived from or containing GM plants are as safe as food produced from alternative sources. According to many experts, foodborne pathogens represent a greater threat to human and livestock health than GM plants (DeFrancesco 2013).

It is not appropriate to discuss here regulatory questions and controversies around GM plants in detail, yet we would like to emphasize that—as desired since the early days of transgenic technology development (Kim 1992)—the regulatory approaches, risk assessment methods, and food standards of GM crops should ideally be harmonized over large regions of the world (Querci et al. 2007; Ramessar et al. 2009; Adenle et al. 2013). Moreover, the same principles should commonly be applied to the environmental risk assessment of GM and non-GM improved plants (Conner et al. 2003; Nap et al. 2003), which would avoid the existence of double standards that may generate unnecessary conflicts. In a more transparent situation, as a result, all kinds of improved cultivars could be directly compared or assessed according to their specific properties and performance in the market locally as well as globally. This uniform approach could ensure that new cultivars would be deregulated after passing identical general evaluation criteria (besides specific ones) and when they are superior to all their competitors in a specific region and for a particular application no matter that they are GM or non-GM cultivars. In addition, the conservation of agricultural biodiversity including varieties and landraces (Jacobsen et al. 2013) and the genetic improvement of crop plants represent mutually complementary and not competing aspects and are, therefore, both important for sustainable agriculture. Public acceptance may also be improved by regulating the financial interests of private capital in GM plant production for instance by lowering the costs of deregulation and registration or when academic institutions and public and/or governmental funding will gather momentum in the development of GM cultivars. Finally, an increasing number of analysts have cautiously suggested (Breithaupt 2004; Rowe 2004; Lusk and Rozan 2005; González et al. 2009) that a benefit-driven pragmatic communication rather than a confrontative debate will likely be more convincing and motivating for the public to face and balance more rationally its concerns and needs (e.g., vitamin-fortified or other functional food) in this field.

In summary, the risks of transplastomic and GM plants in general cannot be excluded with 100 % certainty, just as much as of about everything from organically cultivated plants to mutagenized ones. The presented and future developments and applications of transplastomic plants can and will have a place in a more sustainable agricultural production with benefits for farmers as well as consumers. However, a careful risk and benefit analysis and its objective communication will be indispensable in helping the public to create an unbiased perception of GM plants and on the general role of modern agriculture in our world.

6 Conclusions and future perspectives

In addition to increasing human population and pollution of arable lands, climate change introduces new challenges to agricultural production in yield (quantity) as well as in its qualitative aspect (nutritious food). To ensure global food security in the long term, adaptation to extreme climate change is a must. This adaptation includes the ability both to mitigate exposure and to cope with the changing climate (Clarke and Daniell 2011). Genetic engineering of crop plants is one possibility to be considered as solution to these challenges. Production of GM plants by *Agrobacterium*-mediated nuclear transformation was first achieved in 1983 (Herrera-Estrella et al. 1983), and GM crops have been commercialized and cultivated since 1994 with continuously expanding crop range and area of cultivation (Jacobsen et al. 2013). Transgenic plants carrying nuclear modifications that result in herbicide or pesticide tolerance are already predominantly used in several countries and in four major crops (soybean, maize, canola, and cotton) (Fernandez-Cornejo and Caswell 2006; Kumar et al. 2008). In agricultural applications, incorporating transgenes in the plastid genome of crops with maternal plastid inheritance provides an efficient tool to control gene flow. Transgene containment in the plastids represents a significant improvement as compared to the present practice of incorporating transgenes in the nuclear genome, when 100 % of shed pollen carries the transgene and can move to non-transgenic crops or wild relatives (Maliga 2004). Thus, the use of transplastomic plants could successfully address public concerns related to gene flow. In addition, plastid genetic engineering is a valuable tool to understand plant metabolism, to engineer complex metabolic pathways into the plastids, and especially to produce high amounts of biopharmaceuticals and/or plastid proteins and plastid-derived compounds in an economic and environment-friendly way. It is therefore quite striking that in spite of clear advantages of chloroplast transformation technology, no transplastomic plants have been commercialized almost 25 years after the first report on these plants (Svab et al. 1990).

Although plastid genetic engineering is promising for plant biotechnology, there is still a long way to go before the technology can reach its full potential. The major problem is that the majority of available protocols for plastid transformation has been performed on tobacco leaf chloroplasts as targets, but is not (yet) directly applicable to other crops. Among the greatest challenges that remain to be addressed is the crop range of stable plastid transformation, which has to be further extended into the main staple food crops, especially to cereals (wheat, maize, fertile homoplasmic rice). The success of

plastid transformation depends on a number of essential factors, including information about the plastome sequence of the crop to be transformed, identification of intergenic spacer regions for transgene integration, optimization of DNA delivery, selection including the development of new selectable marker genes, regeneration, and progression towards homoplasmy in different tissue and/or organ culture conditions (Clarke and Daniell 2011). In addition, often the transformation protocol has to be specifically developed for various cultivars of a species, representing a further great disadvantage for this technique (Lu et al. 2013). Although chloroplast genome sequences of several monocots, including wheat and maize, have been available for several years, the plastome in none of these species has been fully transformed so far due to lack of efficient molecular tools, resistance to selection with common antibiotics, and especially to the lack of highly efficient tissue culture conditions and plant regeneration protocols. The development of tissue-culture independent plastid transformation protocols (like vacuum infiltration or floral dipping for *Agrobacterium*-mediated nuclear transformation of *Arabidopsis*) would make the technology accessible to a wider range of users, but is at present only a very distant goal for primary plastid transformation (Bock 2014). The possibilities of interspecific plastome transmission towards different recalcitrant species and cultivars by protoplast fusion or grafting may open up new possibilities to expand the crop range available for plastid manipulation (Bock 2014). The aforementioned difficulties may explain why no transplastomic plants are commercially grown at the moment and may be the reason why manipulating the plastid metabolism by insertion of plastid-targeted transgenes into the nucleus sometimes represents a more efficient and technically simpler alternative to plastid engineering.

Except green leafy vegetables such as lettuce, cabbage, spinach, etc. or other green vegetables and fruits like broccoli and avocado, the edible parts of the crops often contain non-green plastids. For example, leucoplasts (amyloplasts) are characteristic for edible seeds such as cereals and most leguminous species, for most tubers such as potato, and roots like *Petroselinum*. Chromoplasts occur in some roots like carrot and in red or yellow fruits and vegetables such as mango, orange, tomato, and chili. This clearly shows that in order to apply the technology more widely to food crops in order to improve food quality and nutritional value, a better understanding of the regulation of gene expression and its control including the identification of suitable promoters, 5′ and 3′ untranslated regions in non-green plastids of the above organs is necessary (Bock 2007). In case of cereals, the recovery of transformed cells during the initial selection phase is probably also attenuated by the lower transcription and translation levels present in the proplastids of embryogenic cells than in leaf chloroplasts (Silhavy and Maliga 1998; Clarke and Daniell 2011). In addition to these problems, the development of chemically inducible transgene expression systems would be highly favorable in several cases to avoid (1) deleterious pleiotropic effects, (2) yield penalty and metabolic burden, and (3) accidental contamination of the food chain with the special compounds produced by transformed plastids. Such methods are still in their infancy when compared to transgenic plants obtained with nuclear transformation, and although achieved under laboratory conditions, inducible expression for production-scale application remains a future challenge for plastid transformation as well.

The commercialization of transplastomic plants is further hindered and/or limited by (1) the predominant use of antibiotic resistance markers that have to be eliminated and/or replaced by new and similarly efficient selection methods due to public concern, (2) the lack of economically more rentable plant regeneration protocols, and (3) the lack of thorough (environmental) evaluation of transplastomic plants. Non-antibiotic, native plant genes that offer dominant and portable selectable markers like *ASA2* (Barone et al. 2009) or confer additional agricultural advantage to the transformed crops such as *BADH* conferring salt or drought tolerance to transformed crops (Daniell et al. 2001; Kumar et al. 2004a) may be good alternatives to antibiotic selection markers, and a few examples have also shown improved and in some cases inducible (Mühlbauer and Koop 2005; Lössl et al. 2005; Buhot et al. 2006; Tungsuchat et al. 2006; Verhounig et al. 2010) transgene expression in different organs and plastid types (e.g., Valkov et al. 2011; Zhang et al. 2012; Caroca et al. 2013). A recently developed positive selection protocol utilizes the *Agrobacterium tumefaciens* isopentenyl transferase (*ipt*) gene involved in early stage of cytokinin biosynthesis and allows cell proliferation and differentiation into shoots without the use of exogenous cytokinin in the selection medium (Dunne et al. 2014). However, further basic research and developments towards commercial applications are necessary. Commercialization of transplastomic plants can in the first place be expected to occur in the field of pharmaceutical products (Bock 2014), but several studies discussed in this review have already shown that transplastomic plants with improved yield, nutritional quality, and tolerance/resistance towards different stressors represent a viable alternative to conventional transgenic crops.

Taken together, much technical progress of plastid transformation (i.e., increase in publicly available vectors, development of highly efficient selection, tissue culture and regeneration protocols for major crops, understanding the biology of non-green plastids, etc.), improved public acceptance, and more field tests with approved and economically viable products are still needed to assess the real impact of transplastomic plants on sustainability of agriculture and on their potential in a second Green Revolution aimed at feeding the world by 2050.

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