



Targeting metabolic pathways for genetic engineering abiotic stress-tolerance in crops[☆]

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ABSTRACT

Abiotic stress conditions are the major limitations in modern agriculture. Although many genes associated with plant response(s) to abiotic stresses have been identified and used to generate stress tolerant plants, the success in producing stress-tolerant crops is limited. New technologies are providing opportunities to generate stress tolerant crops. Biotechnological approaches that emphasize the development of transgenic crops under conditions that mimic the field situation and focus on the plant reproductive stage will significantly improve the opportunities of producing stress tolerant crops. Here, we highlight recent advances and discuss the limitations that hinder the fast integration of transgenic crops into agriculture and suggest possible research directions. This article is part of a Special Issue entitled: Plant gene regulation in response to abiotic stress.

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1. Introduction

The ever-increasing human population, together with the loss of agricultural land (due to urbanization, industrialization, desertification, and climatic changes) and the diminishing resources availability pose serious challenges to world agriculture. Since plant were first domesticated ~10,000 years ago and up to the present days, breeding crop plants to increase yield and feed the expanding population has been very efficient. Nevertheless, in order to feed the 9 billion people expected by 2050 (<http://www.fao.org/wsfs/world-summit/en/>), a significant grain yield increase of approximately 44 million metric tons per year will be needed (reviewed by [1]). These yields goals are even more challenging in light of the projected scenarios of global warming.

Water deficit, extreme temperatures (high or low) and ion imbalance (toxicity and/or deficiency) are the major abiotic stress conditions that reduce plant growth and result in significant yield losses. Although plants have evolved a wide spectrum of programs for sensing, responding and adapting to changing environment [2–8], the current understanding of the mechanisms associated with the ability of crops to maintain yield under abiotic stress are poorly understood. New advances in ‘omic’ technologies are providing opportunities leading to the identification of transcriptional, translational and post-translational mechanisms and signaling pathways that regulate the plant response(s)

to stress [9]. The use of model plants, such as *Arabidopsis thaliana*, *Brachypodium distachyon*, and *Medicago truncatula* provided fundamental tools for understanding the genetic and biochemical basis of abiotic stress adaptations [10,11]. Currently, numerous genes related to plant response to abiotic stress have been identified and characterized. However, a limited success in producing abiotic-stress tolerant cultivars through genetic engineering has been achieved, taking into consideration the low number of transgenic crops released to the market so far [12]. An important aspect to consider when breeding for abiotic stress tolerant crops is how to determine the success of the transgenic plants. While from a physiological perspective, survival (or recovery) is the major trait representing plant stress tolerance, from an agronomical point of view crop yield is the key determinant of successful stress-tolerant crops. Thus, while there are many studies reporting abiotic stress resistance, the majority of this work used model plants (reviewed by [13–15], tested under artificial extreme conditions (i.e. very high salinity, severe dehydration, osmotic shock, etc.) with plant recovery after a stress episode as an indication of tolerance. However, under natural field conditions, crops have to cope with multiple environmental stress which varied in time, duration and intensity (reviewed by [16]).

Currently, about 30 genetically engineered crops, occupying almost 300 million acres, are being grown in 25 countries [17]. It is expected that by 2015 more than 120 transgenic crops will be cultivated worldwide [18]. Several reviews regarding genetic engineering for improving plant tolerance to abiotic stress have been published in recent years [14,15,19,20], focusing mainly on model plants. In this review we highlight recent advances in the generation of abiotic stress-tolerant crops, and discuss the limitations that hindered the fast integration of transgenic crops into agriculture and suggest some possible research directions.

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2. Manipulating single traits: The target-gene approach

Manipulation of single genes that affect specific targets (metabolites or proteins) has been the most common strategy for improving abiotic stress tolerance in plants (reviewed by [12,14,15,20–22]). Overexpression of genes encoding enzymes associated with the accumulation of osmolytes, proteins and enzymes that function scavenging oxygen radicals (ROS), molecular chaperones and ion transporters, provided insights on the role of these genes in key physiological and biochemical processes (reviewed by [14,15,23]).

2.1. Genes associated with osmoregulation

The biosynthesis and accumulation of compatible solutes is an important adaptive mechanism that enable protection of cell turgor and restoration of water status of cells by maintaining cellular water potential as well as acting stabilizing membranes and/or scavenge ROS. These compatible solutes include amines (polyamines and glycinebetaine), amino acids (proline), sugars (trehalose, fructan), and sugar alcohols (trehalose, mannitol and galactinol) (reviewed by [24]). Overproduction of such osmoprotectants has been extensively used in several target crops in an attempt to improve tolerance to abiotic stress.

Polyamines (PAs) are low molecular weight aliphatic nitrogen compounds positively charged at physiological pH [25], which were shown to be involved in the response to abiotic stress (reviewed by [26]). The modification of PA levels by the overexpression of genes such as ornithine or arginine decarboxylases (*ODC*, *ADC*), *S*-adenosylmethionine (*SAM*) decarboxylase (*SAMDC*), Spermidine synthase (*SPDS*) in *Arabidopsis* [25] and tobacco (*Nicotiana tabacum*) [27], and in crop plants such as rice (*Oryza sativa*) [28–30], potato (*Solanum tuberosum*) [31] and eggplant (*Solanum melongena*) [30] was reported to result in enhanced tolerance of these species to different abiotic stresses. Glycinebetaine (GB), a fully N-methyl-substituted derivative of glycine, accumulates in the chloroplasts and other plastids of many species in response to abiotic stress and is considered the major osmolyte involved in cell membrane protection [7]. The overproduction of GB was shown to be a promising approach in developing abiotic stress tolerant plants tolerance [32]. Transgenic bread wheat (*Triticum aestivum*) plants overexpressing a betaine aldehyde dehydrogenase (*BADH*) gene, showed improved osmotic adjustment and antioxidative defense capacity which support higher photosynthetic rates leading to increased tolerance to drought and heat [33], although no yield was reported. Introducing the *betA* (encoding choline dehydrogenase) gene to maize (*Zea mays*) [34] and wheat [35] resulted in improved yield under stressful conditions in the field. Also, the expression of gene encoding choline monoxygenase (CMO, involved in GB biosynthesis) in cotton (*Gossypium hirsutum*) plants supported higher yield production under saline field condition [36]. However, when grown under control conditions, the transgenic plants showed reduced yield production.

Proline accumulation play adaptive role(s) in plant adaptation to osmotic stress, acts acting as a store of carbon and nitrogen and function as a molecular chaperone stabilizing the structure of proteins, (reviewed by [37]). The expression of the mothbean Δ^1 -pyrroline-5-carboxylate synthetase (*P5CS*) induced increased tolerance to stress in rice [38] and wheat [39]. On the other hand, transgenic chickpea (*Cicer arietinum*) expressing *P5CSF129A* constitutively only displayed a modest increase in transpiration efficiency, suggesting that enhanced proline had little bearing on yield in chickpea [40]. Soybean plants expressing Δ^1 -pyrroline-5-carboxylate reductase (*P5CR*) under the control of an inducible heat shock promoter were found to accumulate higher amounts of proline without deleterious effects in growth being able to retain higher relative water content (RWC) and higher glucose and fructose levels than the antisense and control plants, conferring drought stress tolerance [41]. The contrasting results obtained for manipulating *P5CS*

gene in various crops could result from either different metabolomic pathways involved in stress tolerance in various species, epigenetics, and or the experimental design.

Overproducing mannitol in wheat, which does not synthesize mannitol normally, by constitutively expressing the mannitol-1-phosphate dehydrogenase (*mt1D*) gene resulted in improved tolerance to drought and salinity in terms of growth. However, under control conditions, growth was accompanied with sterility [42]. The ameliorative effect of mannitol was likely to be exerted through the scavenging of hydroxyl radicals and stabilization of macromolecular structures ([42] and references therein). Trehalose (α -D-glucopyranosyl-(1 \rightarrow 1)- α -D-glucopyranoside) which is specially accumulated in desiccation-tolerant “resurrection plants” [43], was engineered in plants either by regulation of trehalase activity [44] or by expression of trehalose synthesis-related genes [45]. Overexpression of two *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB*) was shown to improve tolerance to abiotic stresses in rice [46] and alfalfa (*Medicago sativa*) [47]. In general, stunted growth of the transgenic plants was avoided when an ABA-inducible (*rd29A*) promoter was used [45,47]. The results reported above illustrate the potential of manipulating osmolyte accumulation to genetically engineer abiotic stress tolerant crop plants.

2.2. Detoxification of reactive oxygen species

Abiotic stresses induce the generation of reactive oxygen species (ROS) such as $^1\text{O}_2$, H_2O_2 , $\text{O}_2^{\bullet-}$ and $\text{HO}\cdot$ [48]. ROS are toxic molecules that cause oxidative damage to proteins, DNA and lipids [49]. Enzymatic scavenging of ROS involves proteins of the aldehyde dehydrogenases (ALDHs) family, which catalyzes the conversion of aldehydes to the corresponding acids playing an important role in detoxification of acetaldehydes [50]. Overexpression of Mn-superoxide dismutase (*Mn SOD3.1*), that mediates the conversion of $\text{O}_2^{\bullet-}$ to H_2O_2 , in alfalfa [51], wheat [52] and potato [53] resulted in higher tolerance to abiotic stress and improved yields under field conditions. Ascorbate peroxidases (*APX*) and catalases (*CAT*) are two important enzymes that participate in ROS detoxification. Expression of *cAPX* gene in tomato (*Solanum lycopersicum*) improved tolerance to exposure to direct sunlight, under field conditions [54] and expression of the *katE* gene in rice, resulted in improved growth and yield under salt stress [55]. The expression of a combination of antioxidant enzymes was shown to be a promising strategy to enhance abiotic stress tolerance. Transgenic rice plants, constitutively co-expressing Glutathione S-transferase (*GST*) and *CAT* genes showed enhanced tolerance to salinity and oxidative stresses at the vegetative stage [56]. In tobacco, co-expression of three antioxidant enzymes, copper zinc superoxide dismutase (*CuZnSOD*), *APX*, and dehydroascorbate reductase (*DHAR*) resulted in a higher tolerance to salt stress [57]. While many studies have demonstrated that increasing the antioxidant capacity of a plant improves abiotic stress tolerance, testing how these transgenic plants perform under field conditions is needed to confirm the beneficial effect on yield.

2.3. Late embryogenesis abundant proteins

The manipulation the expression of genes encoding for chaperones (CSPs), heat-shock proteins (HSP) and late embryogenesis abundant (LEA) proteins have been widely used for improving stress tolerance in plants (reviewed by [15]). LEA proteins are low molecular weight proteins that play crucial roles in cellular dehydration tolerance preventing protein aggregation during desiccation or water-stress, having antioxidant capacity together with a possible role as chaperones [58–60]. Overexpression of *OsLEA3-1* in rice, resulted in improved yields under drought stress, without yield penalties under control conditions [61]. The barley (*Hordeum vulgare*) LEA protein *HVA1*, was shown to improve yields under drought stress in transgenic wheat [62] and rice [63]. Dehydrins are a subfamily of group 2 LEA proteins [64] that accumulate in vegetative tissues subjected to drought, salinity and cold

stress. Strawberry (*Fragaria × ananassa*) overexpressing a wheat dehydrin *WCOR410* gene showed improved leaf freezing tolerance [65]. Recently, it has been demonstrated that the dehydrin gene *Lti30* is involved in cold stress tolerance by interacting electrostatically with vesicles of both zwitterionic (phosphatidyl choline) and negatively charged phospholipids (phosphatidyl glycerol, phosphatidyl serine, and phosphatidic acid) [66]. This strategy still needs to be tested in crops under field conditions. The expression of two members of a family of bacterial RNA chaperones, *E. coli CspA* and *B. subtilis CspB*, resulted in enhance tolerance to abiotic stress, by maintaining growth, photosynthesis and development in rice, maize and *Arabidopsis* [67]. Multiple locations and years field trials with the transgenic maize expressing *CspA* and *CspB* showed improved yields (11–21%) under water-stress conditions when tested in multiple field locations. Importantly, the improvements in water-limited field trials were not associated with a yield penalty in high-yielding environments [67]. Overexpressing the rice small heat-shock protein gene, *sHSP17.7*, which shown to act as molecular chaperones resulted in improved drought and osmotic stress tolerance (as seedling survival rate) [68]. While LEA, CSPs, and HSP proteins have been repeatedly shown to be involved in abiotic stress response (reviewed by [59]), only limited experiments have used this strategy for engineering abiotic stress tolerant crops.

2.4. Regulation of water and ion homeostasis

The ability to maintain water content under stress conditions is critical for plant survival. Aquaporins are intrinsic membrane proteins that mediate the transport of water, small neutral solutes and CO₂ [69,70]. These membrane proteins implicated in water diffusion, are regulated in response to environmental cues and particularly in ABA dependent stomatal conductance pathway [71]. The use of aquaporins for developing transgenic plants with improved tolerance to abiotic stress resulted in contrasting results. *Arabidopsis* plants expressing the wild soybean (*Glycine soja*) tonoplast intrinsic protein (TIP), *GsTIP2;1*, showed more sensitivity to salt and dehydration presumably due to enhanced water loss of the transgenic plants [72]. Transgenic tobacco plants constitutively expressing the *Arabidopsis* plasma membrane aquaporin (PIP), *PIP1b*, wilted rapidly during water-stress [73]. Similarly, transgenic rice plants constitutively overexpressing a barley *HvPIP2;1*, showed more sensitivity (reduction growth rate) to salinity stress [74]. In contrast, heterologous overexpression of rice *OsPIP-1* and *OsPIP2-2* in *Arabidopsis* resulted in improved salinity and dehydration tolerance [75]. Overexpression of wheat nodulin 26-like intrinsic proteins (NIP) gene, *TaNIP*, in *Arabidopsis* enhanced plants tolerance to abiotic stresses. Recently, a tobacco gene encoding aquaporin (*NtAQPI*) was shown to provide protection against salinity stress in transgenic tomatoes [76]. *NtAQPI* plays a key role in increasing mesophyll CO₂ permeability (supporting increased photosynthetic rate), increasing stomata aperture and preventing hydraulic failure under high xylem tensions. The higher transpiration rate and higher CO₂ assimilation rate of the transgenic plants resulted in significant improved productivity under control and salt stress [76]. Tomato plants constitutively overexpressing the TIP aquaporin gene *SITIP2;2* showed increased cell water permeability and whole-plant transpiration, which resulted in improved salt and drought tolerance under field conditions [77].

Under saline conditions, Na⁺ and Cl⁻ are the predominant toxic ions for cell metabolism affecting plant growth and development. Maintaining a high cytosolic K⁺/Na⁺ ratio is essential for plant salt tolerance [78]. Ion transporters can limit Na⁺ accumulation in the cytosol by restricting Na⁺ uptake, by accumulating Na⁺ in the vacuole, and/or by extrusion of Na⁺ out of the cells. Sodium entry into the root cells is mediated by uniporter or ion channel type transporters, like *HKT*, *LCT1*, and *NSCC* (reviewed by [79]). Reduction in Na⁺ uptake by antisense suppression of *TaHKT2;1* gene in wheat resulted in lower net Na⁺ uptake of transgenic roots under salinity stress [80]. However, this strategy was not tested in the field yet. Sodium efflux from the roots is an active

process, which is presumed to be mediated by plasma membrane Na⁺/H⁺ antiporters. The Na⁺/H⁺ antiporter salt overly sensitive 1 (*SOS1*), is the only Na⁺ efflux protein on the plasma membrane characterized so far in plants involved in Na⁺ extrusion and long-distance Na⁺ transport [81]. Transgenic rice plants constitutively expressing the yeast (*Schizosaccharomyces pombe*) Na⁺/H⁺ antiporter *sodium2* (*SOD2*) gene, showed higher accumulation of K⁺, Ca²⁺, Mg²⁺ and less Na⁺ in the shoots as compared to wild type plants [82]. The transgenic rice plants were able to maintain higher photosynthetic levels and root proton transport capacity, whereas ROS generation was reduced. Accumulation of Na⁺ ions into vacuoles through the operation of vacuolar Na⁺/H⁺ antiporters is an efficient strategy to avert the deleterious effect of Na⁺ in the cytosol [83,84]. Overexpression of an *Arabidopsis* vacuolar Na⁺/H⁺ antiporter, *AtNHX1*, resulted in improved salt tolerance in canola [85], tomato [22], cotton [86], wheat [87], beet (*Beta vulgaris*) [88] and tall fescue (*Festuca arundinacea*) [89]. Likewise, expression of the rice ortolog, *OsNHX1*, in rice [90] and maize [91] showed improved salt stress tolerance. Moreover, under field conditions, the transgenic maize plants produced higher grain yields than the wild-type plants. Transformation of another Na⁺/H⁺ antiporter family member, *AtNHX3*, in sugar beet resulted in increased salt accumulation in leaves, but not in the storage roots, with enhanced constituent soluble sugar contents under salt stress conditions [92]. Recently, overexpression of the *Arabidopsis* intracellular Na⁺/H⁺ antiporter *AtNHX5* [93] resulted in enhanced salt and drought tolerance in rice seedlings [94] and paper mulberry (*Broussonetia papyrifera* L. Vent) [95].

3. Targeting pathways: The manipulation of regulatory genes

The approach of manipulating single gene encoding specific metabolic pathway to improve tolerance to abiotic stress in crops had very limited success. The multiple pathways involved in plant adaptation to stress and the complexity of interactions can explain to some extent why such an approach will not work in the field. Moreover, plants always tend to restore the metabolic homeostasis, and therefore can play in contrast to the manipulated enzyme. On the other hand, targeting key regulation genes which affect multiple pathways affecting metabolic fluxes could help to restore plant metabolic homeostasis during stress episodes, increasing the probability of success. Transcription factors (TFs) are involved in almost all biological processes, and therefore likely to be good target candidates for the generation of stress-tolerant crops [96]. Different families of TF such as ERF/AP2, HSF, bZIP, MYB, MYC, NFY, NAC, WRKY, Cys₂His₂, MADS-box and zinc-finger have been shown to regulate the expression of stress-responsive genes [11,96]. Nuclear factor Y (NF-Y) complex is comprised of three subunits; NF-YA (*HAP2*), NF-YB (*HAP3*), and NF-YC (*HAP5*) [97], and was found to confer tolerance to abiotic stress in *Arabidopsis* [98]. Transgenic maize constitutively expressing *ZmNF-YB2* showed enhanced tolerance to severe drought stress in field trials [98]. Under water-limiting conditions, transgenic plants displayed improved grain yield, as well as reduced wilting, lower leaf temperature, etc. The NAC [*NAM* (No Apical Meristem), *ATAF1-2*, and *CUC2* (Cup-Shaped Cotyledon)] TF have been reported to be associated with abiotic stress. Transgenic rice overexpressing *SNAC1* (*STRESS-RESPONSIVE NAC 1*) showed increased yield when grown under drought stress field conditions, throughout the control of stomata movement and maintenance of photosynthetic activity [99–101]. Likewise, the overexpression of two NAC genes, *OsNAC5* and *OsNAC6*, resulted in stress tolerant rice via the up-regulation of the expression of stress-inducible genes such as *OsLEA3* [99]. Recently, expression of *OsNAC10* under control of a root-specific promoter (*RCc3*) yielded more grain in the field under drought conditions [102]. The yield advantage of *P_{RCc3}::OsNAC10* transgenic rice plants was associated with a larger root diameter [102].

Dehydration-responsive element (DRE)/C-repeat (CRT) proteins have been identified to play important roles in drought, cold and salinity response [103]. Overexpression of *CBF1/DREB1B* genes resulted

in improved tolerance to drought, salinity and temperature stress in model plants [104–107] and in crop plants such as rice, wheat and canola [108,109]. At the same time, the transgenic plants showed negative phenological abnormalities such as severe growth retardation under control condition [110]. This problem was reduced when using more specific promoter, such as the ABA-inducible (*rd29a*) promoter [19]. The DRE-binding (*DREB*) and ethylene responsive element binding factors (*ERF*) subfamilies that belong to the large family of TFs APETALA2/ethylene-responsive (*AP2/EREBP*), mediate plant signal transduction pathways in response to environmental cues. The over-expression of *HARDY* (*HRD*), encoding a AP2/ERE-like TF, in rice resulted in reduced transpiration and increase water use efficiency (WUE) under control and drought conditions [111]. Although WUE is a critical parameter associated with improved stress tolerance of plants, it does not necessarily reflect higher productivity under stress conditions (reviewed by [112]) and yield parameters have to be determined.

4. Challenging hormone homeostasis

Phytohormones regulate every aspect of plant growth, development and the responses of plants to environmental cues [113–127]. The hormonal response machinery rapidly alters gene expression by inducing, preventing or controlling the degradation of regulators as TFs via the ubiquitin–proteasome pathway [128]. One of the primary plant responses to stress is the accumulation of ABA which results in stomatal closure and reduced water loss via transpiration [129,130]. While a large number of genes associated with abscisic acid (ABA) metabolic pathways have been indentified in *Arabidopsis* using loss and gain of function (reviewed by [120,131]), only a few genes involved in ABA metabolism has been successfully manipulated in crops to attain drought tolerance. Transgenic rice plants overexpressing *LOS5/ABA3*, a key enzyme in the last step of ABA biosynthesis, showed improved yield in the field under drought stress [132]. In tomatoes, overexpression of *LeNCED1* (a drought-inducible gene encoding a rate-limiting enzyme in ABA biosynthesis) resulted in increased ABA accumulation and improved drought tolerance [133]. However, under control conditions the transgenic tomato plants showed negative physiological and morphological changes associated with the constant increase of ABA level, which resulted in the reduction of assimilation rates. *ERA1* encodes the β -subunit of farnesyl transferase, an enzyme associated with ABA signaling. Transgenic canola carrying *era1* antisense (driven by the drought-inducible *rd29a* promoter) displayed enhanced yield under mild drought conditions in the field [134]. These results further highlight the need of specific promoters to control gene expression and to avoid negative effects [133]. Recently, overexpression of a Harpin-encoding (*hrf1*) gene in rice was shown to improve drought tolerance through ABA signaling promoting stomatal closure increasing the levels of free proline [135].

Cytokinin (CK) has been found to be associated with plant responses to various abiotic stresses (reviewed by [136,137]). CK could promote survival under drought stress, inhibiting leaf senescence and increasing levels of proline [138]. Modification of endogenous CK levels was shown to be an effective strategy in delaying senescence processes [139]. *IPT*, a gene encoding isopentenyltransferase, an enzyme mediating the rate-limiting step in CK biosynthesis, has been overexpressed in several plant species [140]. Transgenic plants varied depending on the type of promoter used to drive *IPT* expression [141]. Expression of the *IPT* gene under the control of *SARK* (senescence associated receptor kinase), a maturation- and stress-induced promoter, in both tobacco and rice resulted in increased drought tolerance, without the negative effects of high CK content on plant phenology [142–145]. The transgenic plants displayed enhanced drought tolerance and superior yields compared with wild type plants [142]. Transgenic Cassava (*Manihot esculenta* Crantz), expressing *IPT* under control of a senescence induced promoter, *SAG12*, was tested for drought tolerance under field conditions displaying higher tolerance to the stress due to the inhibition of

stress-induced leaf abscission and fast recovery from stress [146]. Tomato plants grafted on rootstocks constitutively expressing *IPT* resulted in a decrease of root biomass under control conditions while under salinity-stress conditions the transgenic plants yielded 30% more than the wild type plants [147].

5. Targeting pathways: Expressing genes in tandem

Under natural field conditions plants have to cope with different stress combinations at different developmental stages and for varying duration. Tolerance to abiotic stress is a consequence of genetic and environmental interactions through a complex network that implies physiological, molecular and biochemical responses. Modifying the expression of different components simultaneously has the potential to generate responses apt to the complexity of a combination of stresses. There are only few examples where the simultaneous co-expression of different components of the same pathway has been tried. Increase in biosynthesis of proline was achieved by co-expression of *E. coli* P5C biosynthetic enzymes gamma-glutamyl kinase 74 (*GK74*) and gamma-glutamylphosphate reductase (*GPR*) and the antisense transcription of proline dehydrogenase (*ProDH*) in *Arabidopsis* and tobacco [148]. The transgenic plants displayed improved tolerance to heat stress associated with the accumulation of cell wall proline-rich proteins [148]. Simultaneous co-expression of dehydroascorbate reductase (*DHAR*), glutathione reductase (*GR*) or glutathione-S-transferase (*GST*) and glutathione reductase (*GR*) in tobacco plants also resulted in the increased tolerance of the transgenic plants to a variety of abiotic stresses [149]. In tobacco seeds, higher antioxidant enzymes activity driven by the simultaneous overexpression of the *CuZnSOD* and *APX* genes in plastids, allowed the increase of germination rates and longevity of long-term stored seeds under combined stress conditions [150], demonstrating the enormous potential of simultaneous gene expression in plant engineering.

6. Epigenetic and post-transcriptional control

Epigenetic processes such as DNA methylation, histone modifications, generation of small RNAs (sRNA) molecules and transposable element activity, play essential roles in modulating gene activity in response to environmental stimuli [151–153]. While most mechanisms involved in epigenetic and its heritage have not yet indentified, they play a major role in gene silencing on one hand and as a target for manipulation on the other. Abiotic stress can induce changes in gene expression through hypomethylation or hypermethylation of DNA which are related with stress tolerance. The stress-induced-specific CpHpG-hypermethylation in the halophyte *Mesembryanthemum crystallinum* L. induced the switch in photosynthesis mode from C_3 to CAM, contributing to the adaptation to salt stress [154]. In wheat, the use of the methylation inhibitor 5-azacytidine resulted in the increased tolerance to salt stress at the seedling stage [155]. Decrease levels of histone acetylation levels (antisense) in tomato resulted in higher photosynthetic rates under water-stress [156]. The control of methylation and histone patterns is emerging as a potential tool for improving tolerance to abiotic stress in crops, however, little is known about how to control the effect of post transcriptional manipulation.

Small non-coding RNAs, including small RNAs (sRNAs), short interfering RNAs (siRNAs) and micro RNAs (miRNA), have been to be important regulators of protein-coding gene expression [157,158], controlling mRNA stability and translation, or targeting epigenetic modifications. Abiotic stress can induce both the over- or under-expression of specific sRNAs that are involved in pathways that contribute to re-program complex processes of metabolism and physiology. Several reports have recently indicated the possible use of these sRNAs as targets for the genetic manipulation of crops. The overexpression of *miR398* in *Arabidopsis*, which targets two closely related Cu/Zn superoxide dismutases (cytosolic *CSD1* and chloroplastic *CSD2*) resulted in increased tolerance to oxidative stress [159].

Transgenic tomatoes expressing *Sly-miR169c* displayed decreased stomata opening, a decrease in leaf water loss and enhanced drought tolerance [160]. Transgenic rice constitutively expressing *osa-MIR396c* showed increased sensitivity to salt stress [161]. The identification and characterization of the role(s) of sRNAs in the regulation of gene expression (reviewed by [162]), together with the development of artificial miRNA methodologies [163] open new avenues for the generation of transgenic stress tolerant crops.

7. Modifying function: Engineering C₄ photosynthetic pathway into C₃ crops

Abiotic stress is the major factor limiting photosynthetic activity, resulting in growth and yield reduction. The photosynthesis machinery also affects metabolic processes such as carbon and nitrogen partitioning [164–166] and oxidative stress regulation [167]. The projected effects of climate change in rising ambient temperatures and CO₂ concentrations will have influence plant CO₂ assimilation (and yield), and photorespiration. The ability of the C₄ photosynthetic pathway to suppress ribulose 1,5-bisphosphate (RuBP) oxygenation and photorespiration represents the most efficient form of photosynthesis on Earth [168]. In recent years, efforts have been given to engineer C₄ photosynthesis into C₃ crops [169,170]. The expression of genes encoding enzymes such as phosphoenolpyruvate carboxylase (PEPC), the chloroplastic pyruvate orthophosphate dikinase (PPDK), and NADP-malic enzyme (NADP-ME) into rice [171–174], tobacco [175] and potato [176] improved photosynthetic rate and yield. Although considerable efforts have been made, the overexpression of either single or multiple C₄-enzyme related genes in C₃ plants have resulted in contradictory results. [170,177,178].

Research efforts are also focused on obtaining Kranz anatomy [169], especially in rice which have an intermediate anatomical characteristics between C₃ and C₄ plants [179]. While most genes controlling bundle density in C₄ plants are still unknown, it has been postulated that about 20 genes will be required (reviewed by [180]). Thus, in order to obtain C₄ crops, new transformation methods together with additional efforts to better understand the function of C₄ enzymes in a proper leaf anatomy [178] are needed. Thus, in order to obtain C₄ crops, new transformation methods are needed. Another important aspect that has to be addressed is source/sink relationships. From an evolutionary perspective C₃ plants have modified their sink size proportionally to the source size (i.e. photosynthesis organs). Thus, more efficient carbon fixation via C₄ pathway in the transformed plants would require to adapt the sinks to attain efficient harvest index [181].

8. When and how much to express: The key role of promoters

Most of the genes engineered into crops to improve abiotic stress tolerance were driven by constitutive promoters. In general, the most common promoters used for the manipulation of gene expression are the Cauliflower mosaic virus 35S (*CaMV35S*; [182]), ubiquitin (*UBI1*; [183]) or actin [184]. Although these promoters have been effective in the production of transgenic plants with enhanced stress tolerance, the constitutive expression of candidate genes is not always desirable because of negative (pleiotropic) effects on growth and development under control conditions. This appears to be very relevant with the manipulation of key regulatory genes such as transcription factors or enzymes mediating plant hormone synthesis (reviewed by [113]). A solution to this problem is the use of stress-inducible promoters that allow transgene expression during the stress episode. As an example, the constitutive expression (35S) of the *Arabidopsis CBF1* in transgenic tomato plants resulted in improved tolerance to chilling, drought and salt stress, whereas under normal conditions the transgenic plants showed a dwarf phenotype and reduction in fruit set [185]. In contrast, when the same gene was driven by an inducible promoter (barley *HAV22*) the transgenic tomato plants exhibited enhanced tolerance to

the applied stresses with no effect in growth and yield under control conditions. The constitutive expression of *IPT* or *knotted 1* (*kn1*, a homeobox gene) under the control of 35S in tobacco plants resulted in leaf and plant size reduction, altered leaf shape, loss of apical dominance, delay in senescence, and formation of ectopic meristems [186]. In contrast, the use of stress-induced promoters (*SARK*) to drive *IPT* expression did not altered plant phenology and resulted in enhanced drought tolerance of the transgenic plants [142–145]. The use of strong constitutive promoters to control the expression of transgenes could accelerate the process of RNA silencing [187] that can occur at the transcriptional (TGS) and post-transcriptional (PTGS) levels (reviewed by [188]). Besides, gene expression in specific cell types has resulted in the increase of salt tolerance when enhancer trap system was used to drive root stele specific expression of *HKT1*, but not when driven constitutively using 35S [189]. These results support the use of the conditional expression of the gene of interest as a useful strategy to control gene expression, without the negative effects on growth and development, and possibly reducing epigenetic effects of the transgene.

9. Conclusions and perspectives

New technologies are providing opportunities to generate transgenic crops able to maintain high yields under stressful and changing environments. Many genes associated with plant response(s) to abiotic stresses have been identified and used to generate stress tolerant plants. Most of these studies were conducted under laboratory conditions applying artificial stress conditions, using model plants and focusing on recovery from a stress episode as the main trait. However, crops grown in the field face heterogeneous conditions and are exposed to the simultaneous occurrence of different stresses (reviewed by [16]). Thus, more emphasis should be placed on the development of transgenic crops under conditions that mimic the field situation (i.e. combination of environmental stresses) and focus on the plant reproductive stage (during flowering and seed/fruit/grain maturation), the most critical stage determining crop yield. From a biotechnological stand, the interaction of transgene × environment can have significant effects that will depend on the conditions (i.e. greenhouse versus field, vegetative stage versus reproductive stage, etc.) at which plants are phenotyped. For example, Zeller et al. [190] showed, using transgenic wheat expressing the powdery mildew resistance gene, *Pm3*, as model, that while the transgenic lines displayed the desired phenotype across a range of environments in a greenhouse experiment, some of these effects were reversed when the transgenic lines were grown in the field.

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