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Novel perspectives for the engineering of abiotic stress tolerance in plants

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Adverse environmental conditions pose serious limitations to agricultural production. Classical biotechnological approaches towards increasing abiotic stress tolerance focus on boosting plant endogenous defence mechanisms. However, overexpression of regulatory elements or effectors is usually accompanied by growth handicap and yield penalties due to crosstalk between developmental and stress-response networks. Herein we offer an overview on novel strategies with the potential to overcome these limitations based on the engineering of regulatory systems involved in the fine-tuning of the plant response to environmental hardships, including post-translational modifications, small RNAs, epigenetic control of gene expression and hormonal networks. The development and application of plant synthetic biology tools and approaches will add new functionalities and perspectives to genetic engineering programs for enhancing abiotic stress tolerance.

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Introduction

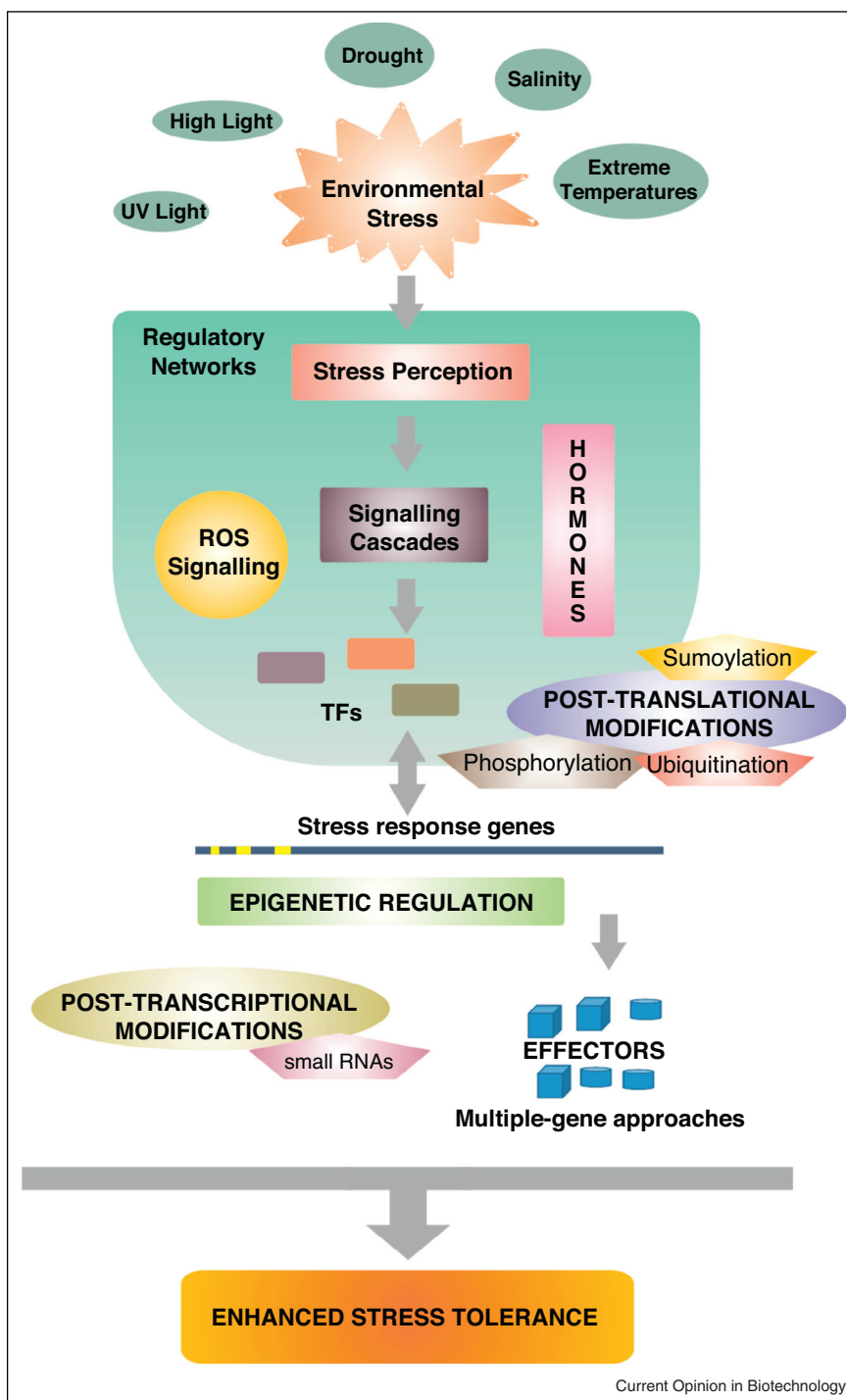
One of the most active fields of research in plant science focuses on the understanding of plant molecular, physiological and genetic responses to environmental stress conditions, and the development of approaches towards improving tolerance and acclimation. Drought, salinity, extreme temperatures and high irradiation among a

plethora of sources of abiotic stress, are perceived by sensor systems leading to the activation of complex regulatory networks controlling the expression of effector genes to counteract the detrimental effects and re-establish cellular homeostasis [1,2*].

The classical approach to engineer plants for enhanced tolerance to abiotic stress consists in strengthening the endogenous systems by intervening at different levels of the response, from sensors and signalling/regulatory elements (e.g. kinases, transcription factors), to direct-action genes or effectors (e.g. antioxidant enzymes, heat-shock proteins, enzymes for the synthesis of osmo-protectants) [2*,3,4]. Even though this strategy shows relative levels of success with an ever-increasing number of tolerant plants being reported [5**], it is not exempt of downsides. In the first place, there is considerable crosstalk between several regulatory, metabolic and developmental pathways. Therefore, while intervening upstream in the signalling network could lead to increased tolerance towards different sources of stress, there is a higher probability of producing undesired pleiotropic effects like growth handicap and developmental alterations. On the contrary, up-regulation of the expression or activity of direct-action genes normally provides enhanced performance only against individual sources of stress [3]. These aspects are particularly relevant as plants growing in natural environments are often simultaneously challenged by a combination of stresses, for example, drought and heat, which lead to synergistic, neutral or even antagonistic effects [6]. A better and more comprehensive knowledge of the complex mechanisms involved in the stress responses provided by the various ‘omics’ platforms has allowed to identify novel points of intervention, dealing in particular with a new layer of control or fine tuning of the main response scheme. Manipulation of processes like post-translational modification of signalling components, regulatory systems based on small RNAs, epigenetic control of gene expression and the intertwined effects of several hormones within these networks, among others, provide ways of achieving a more generalized stress tolerance while keeping a tighter control on the response [2*,4,7].

We describe herein recent reports of successful approaches to obtain enhanced tolerance to environmental stress, especially focusing on further perspectives provided by engineering the above-mentioned novel regulatory targets (Figure 1). We also analyse the limitations and challenges encountered when translating these

Figure 1



Plant abiotic stress response and intervention points for genetic engineering strategies. Environmental stress conditions, such as drought, salinity, extreme temperatures and high irradiation induce the activation of complex regulatory networks leading to the establishment of a defence response. Sensor systems trigger downstream signalling and transcriptional control cascades, involving numerous families of transcription factors (TF), which results in extensive changes in cellular gene-expression programs. In this context hormonal responses integrate plant development and physiology with environmental cues. Reactive oxygen species (ROS) operate as powerful signalling molecules within the regulatory networks. Stress-induced genes encode for regulatory components and effector proteins that activate stress-responsive mechanisms to re-establish cellular homeostasis, eliminate toxic compounds and protect and repair damaged proteins and membranes. Fine-tuning of the response is supported by post-translational modifications to proteins, for example, ubiquitination, sumoylation and phosphorylation, as well as by epigenetic control of gene expression. Post-transcriptional regulation by stress-induced small RNAs (miRNAs and siRNAs) constitutes an alternative regulatory layer amenable for genetic engineering approaches.

strategies ‘from the lab to the field’. We explore the potential of integrating synthetic biology approaches into current genetic engineering programs, opening up new perspectives for the rational design of molecular tools and strategies to improve plant stress tolerance.

Engineering of direct-action genes

Detoxification of ROS – old strategy, new ways.

Osmoprotection. Role of ROS as signalling molecules

Most environmental stress conditions lead to an increased production of toxic reactive oxygen species (ROS), which cause oxidative damage to biomolecules resulting in severe cellular impairment [1,8]. Consequently, one classical approach towards obtaining increased stress tolerance relies on boosting endogenous enzymatic and non-enzymatic ROS scavenging systems (extensively reviewed by [8,9]). Recent progress includes the simultaneous overexpression of several antioxidant enzymes, for example, dehydroascorbate reductase, glutathione reductase, and glutathione-S-transferase [10,11], or increased production of the osmoprotectant and antioxidant metabolite proline, by introducing *Escherichia coli* biosynthetic enzymes in tobacco and Arabidopsis together with an antisense approach for down-regulation of proline dehydrogenase [12]. Further refinements comprise the utilization of stress-inducible promoters for better expression control [13], as well as transplastomics approaches to obtain high levels of antioxidant enzymes in plastids [11] (Table 1).

An alternative strategy involves the expression of cyanobacterial isofunctional counterparts to replace decaying endogenous components under stress. The [2Fe–2S] protein ferredoxin is the final electron acceptor of the photosynthetic electron transport chain delivering electrons for key metabolic and regulatory pathways in chloroplasts. Ferredoxin levels decrease under adverse environmental conditions. Introduction of cyanobacterial flavodoxin in tobacco plants to compensate for ferredoxin decline resulted in an overall antioxidant effect, reestablishment of electron distribution and cellular homeostasis, leading to generalized stress tolerance [3,14–16].

Despite their harmful nature at high concentrations, there is now compelling evidence that ROS are also powerful signalling molecules involved in the acclimation response to stress stimuli [8,17**]. A departure from cellular homeostasis leads to changes in ROS steady-state levels thus generating a signal. ROS, and several antioxidant molecules, are tightly linked to redox and hormonal networks, involving different cellular compartments and thus, directly integrating the metabolic and physiological status with complex retrograde signalling pathways that will lead to a cellular response [6,17**,18,19]. In addition, ROS were recently proposed to work as rapid long distance auto-propagating signals, ROS waves, transferred throughout the plant and coordinating systemic

plant stress responses [17**]. Therefore, modulation of ROS homeostasis constitutes a novel and exciting potential intervention point for the design of strategies to improve plant stress tolerance by integrating plant development and physiology with environmental cues.

Soluble sugars as antioxidants and signalling molecules

Amino acids, polyamines, and several soluble sugars are well known compatible solutes synthesised in response to stress, which help stabilizing cellular membranes and maintain cell turgor. It was recently described that soluble sugars, especially at high concentrations, act as true ROS scavengers [20], and transgenic approaches to manipulate sugar metabolism have resulted in enhanced tolerance to different sources of oxidative stress [21]. In addition, there is emerging evidence that at low concentrations, sugars like the raffinose family of oligosaccharides and fructans, might function as substrate or signal for hormone and stress-related signalling pathways [20], thereby comprising a new potential target for improving stress tolerance.

Chaperones

A classical approach to obtain increased stress endurance involves the overexpression of aquaporins and ion transporters to achieve ionic and osmotic homeostasis [2*,5**], or heat-shock proteins, late embryogenesis abundant (LEA) proteins, and chaperones for protecting cellular components [2*]. We focus here on recent reports involving chaperones with novel functional and regulatory features.

Overexpression of NADPH-thioredoxin reductase, type C (NTRC), a component of a high-molecular weight complex with redox-dependent holdase-chaperone activity, or a Ca²⁺-dependent molecular chaperone, calnexin, yielded Arabidopsis and tobacco plants with enhanced heat and drought tolerance, respectively [22,23]. These strategies allowed linking cellular redox status and Ca²⁺-dependent signalling under stress conditions with a protective effect on protein stability. Proteins harbouring RNA chaperone activity, RNA-binding proteins (RBPs), have been recently described to prevent RNA misfolding or help resolving non-functional structures, thereby playing an important role in post-transcriptional gene regulation under stress conditions [24]. Ectopic expression of a bacterial RBP, CspB from *Bacillus subtilis*, resulted in improved yield under stress [24].

Engineering of regulatory networks

Intervening at high levels of the hierarchical regulatory networks involved in plant response to environmental hardships constitutes a promising strategy towards achieving broad-range enhanced stress tolerance. Accordingly, overexpression of stress sensor proteins, ion channels, Ca²⁺ binding proteins, and members of kinase

Table 1

Genetic engineering strategies and selected targets for enhancing abiotic stress tolerance in plants.

Strategies	Target	Approach	Phenotype	Selected examples	Reference
Engineering transcriptional regulators	Transcription factors	Overexpression	Abiotic stress tolerance with or without pleiotropic effects	OsNAC5 HaHB1 OsDREB2A GmbZIP1	[5**,26,27,28*]
		Use of tissue specific or inducible promoters Fine-tuning of protein levels by post-translational regulation (ubiquitination, sumoylation and phosphorylation)	Abiotic stress tolerance without pleiotropic effects	pRCc:OsNAC10 pRAB17:TaDREB2 y 3 ICE1 AtHB6 MYB30 OsWRKY30	[28*,31] [33*,35,37**,38]
Manipulating single traits	Genes involved in ROS metabolism	Overexpression of simple genes	Abiotic stress tolerance	MnSOD GOR CuZnSOD+APX DHAR+GR GST+GR	[5**,8,9] [9,11,59]
		Overexpression of multiple genes		P47:ScVTE P80:ScVTE	[13]
	Genes associated with osmoregulation	Expression of cyanobacterial genes		Fld	[3,15]
		Overexpression of enzymes involved in sugars biosynthesis		GalS RS2 HXK	[20,21]
Chaperones	Overexpression of single genes		BiP GRP94 Calnexine CRT	[2**,22]	
Challenging hormone homeostasis	Genes involved in hormone biosynthesis	Overexpression	Abiotic stress tolerance with pleiotropic effects	OsNCED3	[40**,43]
		Inducible expression	Abiotic stress tolerance without pleiotropic effects	pSAG12:IPT pSARK:IPT pWRKY:CKX1	[2**,40**,45,46] [39,47]
	Genes involved in hormone catabolism Genes involved in hormone signalling miRNAs	Tissue specific promoter expression Overexpression		CRK45	[44]
Post-transcriptional control		Overexpression of miRNAs	Drought tolerance	miR319 miR169 GmNFYA3	[49**,53,54,55] [50,52]
Epigenetic regulation	DNA methylation	Methyltransferase mutants Use of methylation inhibitor	Hypersensitive to salt stress Increased salinity tolerance	met1-3 5-azaC	[58] [60]
	Histone modification	Histone deacetylase mutant	Salt sensitivity	HDA6	[59]

Abbreviations: RCc3, root specific promoter; pRAB17, drought inducible promoter; DHAR, dehydroascorbate reductase; GST, glutathione-S-transferase; GR, glutathione reductase; VTE, encodes the enzyme homogentisatephytyltransferase (HPT); Fld, Flavodoxin; GalS, Galactinol synthase; RS2, raffinose synthase; HXK, Hexokinase; CRT, calreticulin; BiP, binding protein, a HSP70 molecular chaperone; GRP94, Glucose REGULATED protein, a HSP90 chaperone; pSAG12, senescence-activated promoter; pSARK, senescence-associated receptor kinase; IPT, isopentenyltransferase; pWRKY, root-specific promoter; CKX1, cytokinin oxidase/dehydrogenase; CRK45, cysteine-rich receptor-like protein kinase; NFYA, Nuclear factor A, target of miR169; HDA6, Histone deacetylase; 5-azaC, 5-azacytidine; met, methyltransferase.

More comprehensive lists of genes engineered for abiotic stress tolerance can be found in the articles highlighted in bold.

families, among other signal transducers, has been widely explored [5**]. However, the high degree of cross-talk between regulatory systems often leads to detrimental pleiotropic effects for plant growth under normal conditions, limiting the success of the strategy.

Over 50 families of transcription factors (TFs), including MYC/MYB, NAC, DREB, bZIP, WRKY and AREB, function as downstream integrators of the regulatory networks, controlling in a combinatorial and amplificatory fashion the expression of stress-responsive genes [4,25]. Ectopic constitutive expression of TFs leads to improved abiotic stress tolerance, but usually results in dwarf phenotypes and yield loss [5**,26,27,28*,29**]. Tolerance in the absence of pleiotropic effects could be often achieved, as reported for the constitutive expression of HaHB1 or WRKY30 in Arabidopsis [27,30]. The use of conditional or tissue-specific promoters is a general alternative to bypass negative effects (Table 1) [31].

New intervention points: fine tuning of the stress response

Post-translational modifications: ubiquitination, sumoylation and phosphorylation

Fine-tuning of protein levels and activity by post-translational modifications has recently emerged as essential regulatory mechanisms, thereby constituting interesting intervention targets for a tighter control of the plant defence and acclimation responses [32] (Figure 1 and Table 1).

Ubiquitination induces target protein degradation by the 26S-proteasome, playing a crucial role in the regulation of plant stress responses by modulating the accumulation of signalling components [32]. For instance, MATH-BTB, a component of an E3 ubiquitin ligase complex, promotes degradation under drought of the TF ATHB6, a negative regulator of abscisic acid (ABA) responses. Although ectopic expression of ATHB6 in Arabidopsis caused phenotypic alterations, overexpression of MATH-BTB led in turn to increased drought tolerance with minimal side effects [33*,34]. An alternative strategy to improve stress tolerance is based on ICE1, a TF involved in the expression of a cold-inducible regulon, CBF/DREB1. The RING E3 ligase HOS1 induces the degradation of ICE1 under cold, thereby attenuating the response after prolonged activation. Arabidopsis plants expressing a point-mutant version of ICE1 resistant to ubiquitination led to higher levels of the protein and thus increased freezing tolerance [35].

Sumoylation regulates the activity, function or localization of proteins [36]. Upon sumoylation by the SUMO E3 ligase SIZ1, ICE1 is activated leading to enhanced expression of CBF/DREB1 genes [32]. SIZ1 is also involved in the fine regulation of ABA signalling as the coordinated sumoylation of the TFs MYB30 and ABI5

contributes to a balanced gene expression of ABA-responsive genes [37**]. *Phosphorylation* provides still an additional way for regulating TF activity [38], as it is shown for the case of ABI5 and ICE1, in which phosphorylation is essential for their activity [32]. Mutation of residues substrate for these modifications is a promising strategy as a targeted approach for the fine-regulation of signalling responses [38].

Regulation of hormone metabolism and signalling

The phytohormones are key regulators of plant physiology, development and growth, as well as mediators of the response to environmental stress by integrating environmental stimuli with regulatory networks [39]. Manipulation of hormone metabolism and signalling processes thus represents a promising alternative towards obtaining enhanced stress tolerance. However, a tight spatio-temporal regulation is necessary in order to minimize possible negative effects on growth and development [39,40**,41,42].

ABA is a key hormone in plant stress adaptation to drought conditions. Strategies relying on the over-expression of genes involved in ABA biosynthetic and catabolic pathways resulted in increased drought tolerance, but led to impaired growth under control conditions due to pleiotropic effects even when using inducible promoters [40**,43]. A more tight control of ABA levels and signalling was achieved by overexpressing *CRK45* (a stress-inducible kinase involved in ABA signalling) which resulted in enhanced drought tolerance indicating that CRK45 could fine-tune endogenous ABA levels [44]. Similarly, in order to minimize negative effects, *IPT* (isopentenyltransferase mediating the rate-limiting step in CK biosynthesis) was expressed under the control of stress-inducible promoters in crops leading to increases in CK content, antioxidant scavenging and changes in root growth, which contributed to improved grain yield under drought conditions [39,45,46]. Similar approaches were applied for other stress-related hormones like salicylic acid, jasmonic acid, brassinosteroids and ethylene [39,47] (Table 1).

Regulation by small RNAs

Small non-coding RNAs, such as microRNAs (miRNAs) and endogenous small interfering RNAs (siRNAs), are 20–24 nucleotides single-stranded RNAs that regulate the expression of target (complementary) genes by affecting mRNA levels, chromatin remodelling, DNA methylation, histone modification or by mediating translational repression [48,49**].

miRNAs are involved in almost every biological and metabolic process, including regulation of plant development [50]. Recent studies identified several stress-regulated miRNAs mostly targeting TFs, depicting their role as master regulators at the core of stress gene-regulatory networks [50,51]. Transgenic approaches have thus

Box 1. Epigenetic control of gene expression

Epigenetic processes involving chromatin remodelling by DNA methylation or histone modifications play essential roles in modulating gene activity in response to environmental stimuli, not only affecting abiotic stress tolerance but also influencing flowering time and thus grain yield [57,58].

Modulation of DNA methylation via methyltransferase loss-of-function mutants, methylation-resistant DNA targets or chemicals, results in altered expression of stress-responsive genes [59]. For instance, the use of the methylation inhibitor 5-azacytidine resulted in increased tolerance to salinity in rice [60]. Typically, DNA methylation drives reversible changes in gene activity by silencing loci, but it is also associated to re-location of mobile elements such as transposons leading to alteration of the chromatin organization and gene expression [61].

The reversible acetylation/deacetylation or methylation/demethylation of specific lysine, and lysine or arginine residues on core histones, respectively, lead to transient changes in gene expression of TFs under drought stress [2*,59]. For instance, Arabidopsis histone deacetylase mutants exhibit altered seed germination and salt stress response, as well as ABA-induced and salt stress-induced gene expression [59].

Therefore, modulation of the epigenetic status to alter rapidly and reversibly gene expression by the targeted control of methylation and acetylation of genes, represents a promising approach for improvement of stress endurance (Figure 1) [59].

been recently applied to overexpress or knock down specific miRNAs or their targets (Table 1) [49**,51,52]. For instance, over-expression of miR169c and miR319 yielded tomato plants tolerant to drought stress, and rice with enhanced cold, salt and drought tolerance, respectively [53–55]. However, a more thorough understanding of the networks of miRNA gene regulation is essential to avoid off-targets and minimize pleiotropic effects leading to growth or developmental handicaps [50,54,56].

Several siRNAs accumulating under stress conditions have been recently identified in plants, and their role in stress signalling networks are starting to be unveiled as summarized in recent reviews [49**,50], opening up new perspectives for developing synthetic siRNA-based targeted approaches (Figure 1).

Perspectives: synthetic biology approaches

Synthetic biology applies basic engineering principles to the rational design and development of new biological modules from natural existing components, thus facilitating the *de novo* engineering of genetic switches and circuits, and the manipulation of signalling processes [68,69,70*]. This emerging field has already given rise to a wide variety of biotechnological applications in bacterial, yeast and mammalian cell systems, ranging from material sciences and production of chemicals, to metabolic engineering, high-end molecular biology tools and biomedical applications [71].

The maturity level reached by plant systems biology platforms and bioinformatics tools contributes to a more comprehensive knowledge of cellular regulatory

Box 2. Unmet challenges: translation from lab to field

An ever-increasing world population with enhanced requirements for a rich diet and diversion of resources to bioenergy production in the context of a limited agricultural area, and water and soil resources, demands increasing crop productivity [62–64]. Moreover, abiotic stress leads to extensive yield losses, accounting for up to 80% of potential productivity figures for major crops [3]. Consequently, there is plenty of room for improvement by application of genetic engineering approaches for enhanced stress tolerance and better plant use of natural resources (sustainability) [62,65]. However, successful transfer of traits shown to be efficient in laboratory conditions to crops in the field has proven elusive. Despite numerous promising lines currently being assayed in field trials [62,66], there have been yet no reports of the release to the market of stress tolerant genetically engineered varieties.

In this respect, a series of factors contribute to the limited success of the applied strategies. Although laboratory assessment of plant tolerance is normally biased towards survival rates upon harsh stress experimental setups usually involving individual stress sources, crop yield is the most important factor of agronomical interest, and plants in the field are exposed to a combination of stresses gradually increasing in intensity and with synergistic or even antagonistic effects on plant defence systems (crosstalk with developmental pathways) [7,29**,62]. The developmental stage is also a determining factor, as a stress situation in the flowering period results in high yield penalties. However, laboratory experiments are usually conducted in vegetative growth phases and are not targeted to seed production but rather to leaf or root physiology [7].

A more comprehensive strategy needs to be deployed to tackle the complexity of stress responses and yield, in the frame of multifactorial interacting response pathways. Thorough phenotyping approaches in greenhouse conditions [67], combined with data mining from systems biology projects are to provide with more comprehensive models of regulatory networks and cultivar specific traits necessary for applying an integrated biotechnology approach combining conventional and molecular breeding with genetic engineering [66]. These issues considered, the years to come could witness a second green revolution aimed at reducing growth penalties under normal conditions while increasing crop productivity under stress.

components and processes, thus providing an endless source of biological parts for the construction of synthetic tools and the principles for the design of engineering strategies [68]. First reports of synthetic biology approaches in plants include the development of synthetic regulatory elements (promoters and other cis-elements, synthetic small RNAs, among others) and switches for the spatiotemporal manipulation of gene-expression and engineering of signalling networks [72*]. These tools allow for multi-gene approaches in metabolic engineering and introduction of clusters of direct response genes or regulatory components, which require tight and coordinated regulation of the individual genes [72*]. Runguphan *et al.* (2010) reported the successful introduction of a synthetic biosynthesis pathway for the production of halogenated alkaloids in the medicinal plant *Catharanthus roseus* [73]. Moreover, Nielsen *et al.* have successfully relocated an entire cytochrome P450 monooxygenase pathway from the endoplasmic reticulum to the chloroplasts, thereby rendering the production of defense secondary metabolites light-driven (Nielsen *et al.* DOI: 10.1021/sb3001128r, 2013 ACS Synthetic Biology). The development of biosensors for the

analysis of the perception and signalling relays of molecular and physical cues will help understanding stress regulatory networks and thus facilitate the identification of useful intervention points for genetic engineering strategies. By interfacing mammalian and plant synthetic biology, Wend *et al.* (2013) developed a synthetic quantitative, time-resolved auxin biosensor that allows monitoring auxin dynamics in living cells [74]. Antunes *et al.* (2011) designed a fully synthetic signal transduction pathway by rewiring the endogenous components of the CK signalling network with a bacterial metabolite sensing system, thereby being able to link the detection of a metabolite to the expression of targets genes [75].

In this article we give an overview of emerging strategies for improving plant abiotic stress tolerance based on the intervention of novel regulatory targets responsible for the modulation and fine-tuning of plant stress responses, such as post-transcriptional modifications, hormonal networks and small RNAs. In this context, integration of synthetic biology approaches and tools into genetic engineering programs will provide with novel functionalities and allow for a more precise and controlled intervention at different levels in the networks by combining specificity, crosstalk and amplification features. This opens up unprecedented opportunities for the rational design and development of stress tolerant crops.

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