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Transgenic crops coping with water scarcity

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Water scarcity is a serious problem that will be exacerbated by global climate change. Massive quantities of water are used in agriculture, and abiotic stresses, especially drought and increased salinity, are primary causes of crop loss worldwide. Various approaches may be adopted to consume less water in agriculture, one of them being the development of plants that use less water yet maintain high yields in conditions of water scarcity. In recent years several molecular networks concerned with stress perception, signal transduction and stress responses in plants have been elucidated. Consequently, engineering some of the genes involved in these mechanisms promises to enhance plant tolerance to stresses and in particular increase their water use efficiency. Here we review the various approaches used so far to produce transgenic plants having improved tolerance to abiotic stresses, and discuss criteria for choosing which genes to work on (functional and regulatory genes) and which gene expression promoters (constitutive, inducible, and cell-specific) have been used to obtain successful results.

Contents

Introduction

According to the Food and Agriculture Organization the planet has approximately 1400 million km^3 of water [1]. However, after subtracting the salt water of the oceans and the freshwater locked up in ice caps, only around 9000–14,000 km^3 of freshwater is potentially available for human use [1].

The success of agricultural production depends crucially on water availability. Over 80% of global cropland is rain-fed; however irrigated cropland, constituting about 16% of the total, produces about 40% of the world's food [2]. Agriculture currently uses over 70% (86% in developing countries) of available freshwater [3,4].

The global population is projected to increase from the current 6.7 billion to over 9 billion by 2050. Rising living standards have increased meat consumption, with consequent increased demand for grain for animal feed, which will have a significant impact on agricultural land use [5]. Increased food production implies increased demand for and consumption of water.

Water scarcity, typically accompanied by increasing salinity, is the one of the major causes of poor plant performance and limited

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crop yields worldwide [6] and is the single most common cause of severe food shortage in developing countries. Even in most of the agriculturally productive regions, short periods of water deficiency are responsible for considerable reductions in seed and biomass yields each year [5]. Global climate change, which is increasing temperatures worldwide and changing rainfall patterns, is expected to exacerbate the negative effects of water deficiency in agriculture [7].

To meet the growing demand for food and to contrast the detrimental effects of climate change on crop yields, it is imperative to develop new crops that have improved water use efficiency and have improved resistance to drought stress. These goals might be attained by conventional plant breeding approaches. In fact using the traditional methods of crossing and selecting progeny, breeders have produced new varieties with improved ability to resist stresses [8]. However modern plant biotechnology has a much greater potential by far to produce substantial improvements [9,10].

Mechanisms of drought resistance

Plants are sessile and have had to evolve various mechanisms to enable them to adapt to ever-changing environmental conditions. They have developed two strategies to resist drought: drought avoidance and dehydration tolerance [11]. Drought avoidance refers to a plant's ability to maintain high water status even when water is scarce, for example by growing long roots to reach deep soil moisture, or reducing water loss by restricting the aperture of the stomata on leaf surfaces. In fact, stomata play a major role in plant adaptation to stress. Dehydration tolerance is well illustrated by the so-called 'resurrection plants' which are able to withstand loss of around 90% of their water content yet remain viable and re-grow when moist conditions return; most other plants can withstand only moderate dehydration (about 30% water loss) and still remain viable. Breeding programs have generally sought to improve dehydration avoidance rather than dehydration tolerance as strategies for producing new varieties able to cope with drought stress [11].

At least six signal transduction pathways in plants are involved in responses to drought and closely related responses to high salinity and cold stress [12]. The hormone abscisic acid, synthesized in response to abiotic stress, plays a key role in three of these pathways [12].

The elucidation of mechanisms involved in stress responses has provided valuable insights into how plant responses to abiotic stresses might be improved by genetic engineering. Following the application of microarray technology, several hundred stress-induced genes, mainly in the model plant *Arabidopsis thaliana*, have been identified as candidates for manipulation [12] and have been classified into three groups [13]: (a) genes encoding proteins with a known enzymatic or structural function. Examples include enzymes for synthesis of osmoprotective compounds, late embryogenesis abundant (LEA) proteins, osmotins, chaperons, channels involved in water movements through cell membranes, ubiquitins, proteases involved in protein turnover, and detoxifying enzymes; (b) genes with as yet unknown functions; and (c) regulatory genes, such as those coding for kinases, phosphatases and transcription factors.

Numerous studies have investigated drought resistance mechanisms at the vegetative stage, and such studies are important for improving resistance in plants subject to continuous or sub-continuous water deficit. However for major cereals, resistance to drought is more important at the reproductive stage, to ensure pollen fertility and grain development, and more recent studies have therefore focused on identifying genes that can be modified to improve drought tolerance at this crucial stage [14–18].

In the following we review the various genetic engineering approaches used in recent years to obtain drought-tolerant plants.

Improving response to water stress by manipulating single action genes

Early attempts to develop transgenic plants resistant to water stress focused on single action genes responsible for the modification of a single metabolite or protein that would confer increased tolerance to drought stress. Recent reviews [13,19] document progress in this area.

Osmoregulation is one of the most effective ways evolved by stress-tolerant plants to combat abiotic stress, but most crop plants lack the ability to synthesize the osmoprotectants naturally produced by stress-tolerant plants. Therefore genes concerned with the synthesis of osmoprotectants have been incorporated into transgenic plants to confer stress-tolerance (reviewed in [13,19]). Overproduction of compatible solute osmoprotectants such as amino acids (e.g. proline), quaternary and other amines (e.g. glycinebetaine and polyamines), and sugars and sugar alcohols (e.g. mannitol, trehalose and galactinol) has been achieved in various target plants. Glycinebetaine in particular has been extensively studied as a compatible solute, both by genetically engineering its biosynthesis in agriculturally important species and by its exogenous application [20]. When maize plants were transformed with the *betA* gene from *Escherichia coli* that encodes choline dehydrogenase, they accumulated glycinebetaine in tissues and were more tolerant to drought stress than wild-type plants at different developmental stages. Most importantly their grain yield was 10–23% higher than that of wild-type plants after three weeks of drought stress [21].

In some cases the accumulation of compatible solutes also protects plants against damage by reactive oxygen species (ROS) [22]; in other cases the solutes have chaperone-like activities that protect other proteins maintaining their structure and function [23,24].

Genes coding for heat-shock proteins, molecular chaperones and LEA proteins (reviewed in [13,19]) have been extensively used to improve drought responses in plants. An interesting recent example is the use of RNA chaperones of bacterial origin by Castiglioni *et al.*, to confer abiotic stress tolerance in several species, and improved grain yield in maize under water-limiting conditions [25]. These authors demonstrated that constitutive expression of two cold shock proteins – CspA from *E. coli* and CspB from *Bacillus subtilus* (both RNA chaperones) – conferred abiotic stress tolerance to transgenic *Arabidopsis*, rice, and maize. They obtained a greater than 20% increase in maize grain yield under water-limiting conditions in field trials, without observing pleiotropic effects on plant development. The improvement in drought response was observed in the late vegetative/flowering period as well as the grain-fill period: during these periods, three consecutive days of wilting can reduce grain yield by 30–50% [26]. Stress tolerance conferred by manipulation of cold shock proteins

is not only novel, but also appears as a highly promising approach to improving plant productivity in suboptimal growth conditions.

Improving response to water stress by manipulating regulatory genes

The transference of a single gene encoding a specific stress protein does not always result in sufficient expression to produce useful tolerance, because multiple and complex pathways are involved in controlling plant drought responses [27] and because modification of a single enzyme in a biochemical pathway is usually contrasted by a tendency of plant cells to restore homeostasis [28]. Targeting multiple steps in a pathway may often modify metabolite fluxes in a more predictable manner. Another promising approach is therefore to engineer the overexpression of genes encoding stress-inducible transcription factors. Transcription factors typically regulate several genes and are likely to be used extensively in the next generation of genetically modified crops [29,30]. Numerous transcriptional regulators are known to be involved in plant responses to drought stress [31]; most fall into one of the large transcription factor families (AP2/ERF, bZIP, NAC, MYB, MYC, Cys_2His_2 zinc-finger, NFY and WRKY); and some *cis*-elements, bound by these transcription factors, have been identified [31]. For example abscisic acid-responsive elements (ABRE) [32] are 5′ upstream regions of abscisic acid-responsive genes that are bound by AREB/ABF transcription factors belonging to the basic leucine zipper family. These mediate at least one of the abscisic acid-dependent pathways involved in responses to drought stress. Another *cis*-element is the dehydration responsive element/C-repeat (DRE/CRT) which is involved in one of the abscisic acid-independent pathways [29]. Various DRE/CRT-binding proteins, coding for ERF/AP2 transcription factors, are induced by desiccation, salt treatment, and cold in some plant species [31].

The first examples of transcription factor engineering to improve abiotic stress tolerance were overexpression of the ERF/AP2 factors CBF1, DREB1A and CBF4. Overexpression of these factors resulted in cold, drought and salt tolerance in *Arabidopsis* [33–35] and it was later shown the similar tolerance could be induced in many crop plants by overexpression of these factors [36,37]. Numerous transgenic *Arabidopsis* varieties with improved drought tolerance due to overexpression of various stress-regulated transcription factors have been reported, but similar results have also been obtained in crop plants [38].

Typically a gene coding for a transcription factor in *Arabidopsis* is isolated, characterized and shown to improve drought response when overexpressed. The gene is then transferred to a crop plant where it often confers the same drought-tolerant phenotype. The *HRD* gene, coding for an AP2/ERF-like transcription factor [39] exemplifies this approach. *Arabidopsis* plants with a gain-of-function mutation in the *HRD* gene (*hrd-D* mutants) are drought-resistant, salt-tolerant, and overexpress abiotic stress marker genes. Overexpression of the same gene in rice significantly improves water use efficiency both under well-watered conditions (50–100% increase) and under drought (50% increase). These plants also show enhanced photosynthetic assimilation and reduced transpiration [39]. *HRD* gene overexpression conserves drought tolerance in both dicots and monocots.

In other cases a gene coding for a transcription factor is isolated and characterized in *Arabidopsis,* but its orthologue gene in the crop plant of interest is identified and made to overexpress. For example Nelson *et al.* [40] showed that overexpression of the *Arabidopsis* CAAT box-binding transcription factor AtNF-YB1 confers improved performance in *Arabidopsis* under drought conditions. They next overexpressed the orthologue of AtNF-YB1 (called ZmNF-YB2) in maize and found that, under simulated drought conditions, the altered maize plants produced up to 50% more than unmodified plants [40].

In other cases, studies of responses to abiotic stress directly on the crop plant of interest have contributed to the identification of candidate genes to overexpress. A recent example is the study of Oh *et al.* [17], which showed that independent constitutive expression of the stress-inducible genes *AP37* and *AP59* in rice – which code for transcription factors belonging to the AP2 family – resulted in increased tolerance to drought and salinity at the vegetative stage. This study also provides a good example of how gene modification can result in opposing effects in response to drought in the reproductive and vegetative organs of a plant. Thus in plants transgenic for AP37, grain yield increased by 16–57% over controls under severe drought conditions, whereas in plants transgenic for AP59 grain yield was reduced by 23–43% compared with controls, under both normal and drought conditions [17], while at the vegetative stage overexpression of the two genes resulted in increased tolerance.

In recent years much attention has focused on the transcription factors involved in regulating stomatal movements [41–44]. Stomata are pores in the plant epidermis that regulate CO_2 uptake for photosynthesis and water loss by transpiration; pore size is controlled by turgor-driven volume changes in the two guard cells that surround each stoma [45]. The highly specialized guard cells integrate signals from the plant and from the environment to regulate aperture size and help ensure plant survival under various conditions [46].

The transcription factor SNAC1 (stress-responsive NAC1) influences stoma aperture size in the rice plant. Transgenic plants that overexpressed SNAC1 had improved drought resistance and a 22–34% increase in seed-setting (both compared to control) during severe in-the-field drought conditions at the reproductive stage [42]. Such plants show increased sensitivity to abscisic acid and have more stomata closed under both normal and drought conditions than wild-type plants [42]. The rice variety in which this work was done is not widely grown commercially, so the team is now generating transgenic plants from commercial rice varieties [47].

Constitutive, inducible and cell-specific promoters

All the approaches to improving water stress tolerance reviewed above involved constitutive overexpression of genes. This implies that the gene construct introduced into the target plant also contains a gene promoter that ensures constitutive transcription of the gene. Commonly used promoters are Cauliflower mosaic virus 35S (CaMV35S) [48] or promoters for constitutively expressed plant genes like ubiquitin [49], actin [50], and cytochrome c [51]; these are usually effective in producing transgenic plants with the required stress-tolerant properties. In some cases however, constitutive expression of a gene normally only induced by stress has negative effects – so-called pleiotropic effects [34,52–54] – on growth and development when stress is not present. One

solution is to use inducible (rather than constitutive) promoters that allow expression of a transgene only when it is required, while it is silenced otherwise. For example constitutive expression in *Arabidopsis* of *DREB1/CBF3*, a gene coding for a transcription factor induced by osmotic stress, confers tolerance to stress, but causes severe growth retardation under normal growth conditions [34]. However, if this gene is expressed under the control of an osmotic stress-inducible promoter – RD29A was the promoter used – no growth retardation occurs, while the plant is highly resistant to several stressing conditions when these occur [34].

Similar results have been obtained in crop plants. In tomato, overexpression of the *Arabidopsis CBF1* gene, encoding a transcription factor belonging to AP2/ERF family, confers increased drought, cold and oxidative stress tolerance compared to wild-type plants, but plant growth is severely affected [52]. By contrast, when the same gene was placed under the control of a synthetic promoter derived from the barley *HVA22* gene, it was expressed mainly under abiotic stresses, so that the plant had the same tolerance characteristics towards stresses, but plant growth under normal conditions was not affected [53]. This approach was also applied to the rice *OsNAC6* gene, which codes for a transcription factor involved in responses to abiotic stresses. Its overexpression under the control of the CaMV35S promoter resulted in decreased plant growth and productivity, while expression of the same gene under the control of either of the two stress-inducible promoters *LIP9* or *OsNAC6*, improved stress tolerance, without affecting plant growth [54].

Stress-inducible promoters usually maintain low levels of expression of the downstream genes under normal growth conditions, but even low expression levels may have negative effects if the gene of interest has pleiotropic effects under conditions in which its expression is not necessary. A promoter that is completely silenced under normal growth conditions, but is active in response to drought, abscisic acid and increased salinity, is that of the rice *OsLEA3-1* gene [55]. However this promoter also has the drawback that it is activated only after 6 days of drought stress, or after 18 hours of salt treatment. These times are too long for an efficient response. An ideal stress-inducible promoter would be completely silenced under normal conditions, but induced by stress in a fairly short time (a few hours) after stress onset. The promoter of the *Arabidopsis AtMYB41* gene, which is not expressed in any tissues under standard growth conditions but is highly induced in response to drought, salt and abscisic acid [56], may therefore be a very useful promoter.

Cell-specificity is another aspect that needs to be considered in choosing a promoter. Because of the fundamental role played by guard cells in integrating internal and environmental signals, they appear to be attractive targets for engineering drought response. Genetic engineering of target genes in guard cells requires effective expression systems with suitable promoters, because constitutive promoters (e.g. CaMV35S) are not always functional or can have negative effects on plant growth and productivity. However, very few guard cell-specific plant promoters have been identified. One that has been investigated is the *Arabidopsis AtMYB60* promoter which shows high guard cell-specificity [41]. As *AtMYB60* expression is rapidly down-regulated by abscisic acid and dehydration stress, it is promising as a research tool for the targeted guard cell gene silencing of negative regulators of stress response (see below). Constitutive guard cell-specific promoters are also promising for engineering positive stomata responses to drought. Examples include pGC1 [57] and pCYP [58,59].

Gain of function versus loss of function

Most approaches to abiotic stress resistance have introduced genes with a constitutive or inducible promoter, resulting in gene overexpression. In some cases, however, stress resistance has been conferred by gene down-regulation. This may be achieved by RNA interference [60], co-suppression [61] or loss-of-function mutants [41–43]. The major role of short single-stranded RNA molecules (miRNAs) in stress responses has only been recently elucidated and is reviewed in [62].

Use of loss of function to achieve stress resistance is exemplified by an *Arabidopsis* mutant harboring a knock-out mutation in the *AtMYB60* gene, coding for an R2R3MYB transcription factor [41]. As noted above, the *AtMYB60* gene is guard cell-specific. Stoma size in the mutant is 30% smaller than in wild-type plants, so transpiration is reduced resulting in greater tolerance to dehydrating conditions than wild-type plants. The exact role of the AtMYB60 transcription factor has not been fully elucidated. Its expression is up-regulated by signals that induce stoma opening, like white and blue light, and negatively down-regulated by desiccation and abscisic acid treatment – signals that promote stoma closure. It is possible that this transcription factor is an up-regulator of stoma opening that is silenced in stress conditions. Technological transfer of the AtMYB60 strategy to crop plants is in progress.

A similar phenotype has recently been described in a rice *dst* mutant [43]. The corresponding gene encodes a zinc finger drought and salt tolerance (DST) transcription factor that down-regulates stoma closure through modulation of H_2O_2 homeostasis. In the mutant, stoma closure is increased and stoma density reduced, resulting in enhanced drought and salt tolerance.

Conclusion

The green revolution of the 20th century markedly increased crop production through genetic improvements to major food crops, increased mechanization, improved pest control and improved soil fertility. In April 2000, Kofi Annan, then Secretary General of the United Nations, called for a blue revolution in agriculture to generate 'more crop per drop' [1]. Although this revolution is not yet with us, 'the seeds have at least been planted' as recently noted by Pennisi [47].

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