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Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture

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Abstract

Drought is one of the greatest limitations to crop expansion outside the present-day agricultural areas. It will become increasingly important in regions of the globe where, in the past, the problem was negligible, due to the recognized changes in global climate. Today the concern is with improving cultural practices and crop genotypes for drought-prone areas; therefore, understanding the mechanisms behind drought resistance and the efficient use of water by the plants is fundamental for the achievement of those goals. In this paper, the major constraints to carbon assimilation and the metabolic regulations that play a role in plant responses to water deficits, acting in isolation or in conjunction with other stresses, is reviewed. The effects on carbon assimilation include increased resistance to diffusion by stomata and the mesophyll, as well as biochemical and photochemical adjustments. Oxidative stress is critical for crops that experience drought episodes. The role of detoxifying systems in preventing irreversible damage to photosynthetic machinery and of redox molecules as local or systemic signals is revised. Plant capacity to avoid or repair membrane damage during dehydration and rehydration processes is pivotal for the maintenance of membrane integrity, especially for those that embed functional proteins. Among such proteins are water transporters, whose role in the regulation of plant water status and transport of other metabolites is the subject of intense investigation. Long-distance chemical signalling, as an early response to drought, started to be unravelled more than a decade ago. The effects of those signals on carbon assimilation and partitioning of assimilates between reproductive and non-reproductive structures are revised and discussed in the context of novel management techniques. These applications are designed to combine increased crop water-use efficiency with sustained yield and improved quality of the products. Through an understanding of the mechanisms leading to successful adaptation to dehydration and rehydration, it has already been possible to identify key genes able to alter metabolism and increase plant tolerance to drought. An overview of the most important data on this topic, including engineering for osmotic adjustment or protection, water transporters, and C_4 traits is presented in this paper. Emphasis is given to the most successful or promising cases of genetic engineering in crops, using functional or regulatory genes. as well as to promising technologies, such as the transfer of transcription factors.

Key words: Diffusional and metabolic limitations, genetic engineering, photosynthesis, water deficits, water-saving irrigation.

Introduction

Water scarcity imposes huge reductions in crop yield and is one of the greatest limitations to crop expansion outside present-day agriculture areas. Because the scenarios for global environmental change suggest a future increase in aridity and in the frequency of extreme events in many areas of the earth (IPCC, 2001), irrigation and the use of appropriate crops is an important issue worldwide. Nowadays, approximately 70% of the global available water is employed in agriculture and 40% of the world food is

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produced in irrigated soils. Some irrigation (around 10%) uses water from aquifers, leading to many underground water tables being exploited unsustainably (Somerville and Briscoe, 2001).

It is now recognized that fine-tuning irrigation can improve crop water-use efficiency, allowing a more precise use of water and, at the same time, having a positive impact on the quality of the products. Similarly, modern biotechnology offers new tools for agricultural improvement and sustainability. Whereas the main advances in agriculture during the 1960s were designed for favourable environments, today, crop performance for sub-optimal environments and marginal lands which were bypassed by the 'green revolution' are also being addressed. In recent decades, physiological and molecular bases for plant responses to drought, and concurrent stresses, such as high temperature and irradiance, have been the subject of intense research (see reviews by Chaves *et al.*, 2003; Flexas *et al.*, 2004*a*).

Plant water deficits may occur as a consequence of a seasonal decline in soil water availability, developing in the long term, or may result from drought spells. An increased evaporative demand of the atmosphere, occurring mostly on a daily basis, affects total carbon gain by the crops, even irrigated ones. The timing, intensity and duration of stress episodes are pivotal to determine the effects produced by drought. Plant strategies to control water status and resist drought are numerous (Schulze, 1986). In general, genotypes native from climates with marked seasonality are able to acclimate to the fluctuating environmental conditions, enhancing their efficiency for those conditions (Pereira and Chaves, 1993, 1995). In the case of slowly developing water deficits, plants may also escape dehydration by shortening their life cycle. In the case of rapid dehydration, oxidative stress developing as a secondary effect is potentially very damaging to the photosynthetic machinery (Ort, 2001). The capacity for energy dissipation (Flexas *et al.*, 2002) and metabolic protection (induced or constitutive) against the damaging effects of reactive oxygen species (Foyer and Noctor, 2003) is a key element for the success of plants under drought. Tissue tolerance to severe dehydration is not common in most higher plants, including crops, but do arise in species native from extremely dry environments (Ingram and Bartels, 1996). Understanding the mechanisms underlying those different responses can support the design of new management tools and genotypes for modern precision agriculture.

It is well known that a major effect of decreased water availability is diminished leaf carbon fixation (A) due to stomatal closure, which may start at moderate plant water deficits. At the whole plant level, total carbon uptake is further reduced due to the concomitant or even earlier inhibition of growth. It has been shown that cell division and expansion are directly inhibited by water stress (Zhu, 2001*a*). Slower growth has been suggested as an adaptive feature for plant survival under stress, because it allows plants to divert assimilates and energy, otherwise used for shoot growth, into protective molecules to fight stress (Zhu, 2002) and/or to maintain root growth, improving water acquisition (Chaves *et al.*, 2003). This feature may be relevant for crops intended for drought-prone areas, but inconvenient for regions where only mild and sporadic stress is likely to occur. On the other hand, the ability to accumulate (and later on remobilize) stem reserves is likely to be an important characteristic to maintain reproductive growth under water deficits in various species, like cereals and some legumes (Blum *et al.*, 1994).

Revising the constraints to photosynthesis and the regulatory systems operating under water deficits

Diffusive and metabolic limitations: the role of intercellular CO_2 as mediator of metabolic alterations

Although the nature and timing of the limitations that water deficits impose on leaf carbon assimilation have again been under debate (Tezara *et al.*, 1999; Cornic, 2000; Lawlor and Cornic, 2002; Flexas *et al.*, 2004*b*), namely in what concerns stomatal constraints versus non-stomatal limitations, it is generally accepted that, under field conditions, the decrease in photosynthesis observed in response to moderate soil and/or atmospheric water deficits (leaf relative water contents down to 70–75%) is primarily due to stomatal closure (see Chaves *et al.*, 2002, 2003, for reviews). Although early biochemical effects of water deficits that involve alterations in photophosphorylation were described by Tezara *et al.* (1999), it is not widely accepted that this is the most sensitive water-stress component of photosynthesis (Flexas *et al.*, 2004*b*). Recent work by Bota *et al.* (2004) showed that limitation of photosynthesis by decreased Rubisco activity and RuBP content does not occur until drought is very severe.

Primary events of photosynthesis such as the electron transport capacity are very resilient to drought (Cornic *et al.*, 1989; Epron and Dreyer, 1992) and variations in PSII photochemistry can be explained by changes in substrate availability. In fact, ϕPSII often declines concomitantly with A under water stress, suggesting that the activity of the photosynthetic electron chain is finely tuned to that of CO_2 uptake (Genty *et al.*, 1989; Loreto *et al.*, 1995). Meyer and Genty (1998) found out that the decrease observed in photochemical efficiency in dehydrated or ABA-treated leaves could be almost completely reversed after a fast transition of the leaves to an atmosphere enriched in CO_2. This is an indication that photosynthetic capacity remained high during dehydration and the limitation by CO_2 was the main factor responsible for the decrease in the net photosynthetic carbon uptake rate. A de-activation of the carboxylating enzyme Rubisco by low intercellular CO_2 (C_i)

could account for the metabolic component of photosynthetic inhibition that was not reversed after the fast transition to an elevated CO_2 atmosphere (Meyer and Genty, 1998). Other types of evidence suggest that decreased intercellular CO_2 can play a pivotal role as mediator of biochemical alterations in photosynthesis (Ort *et al.*, 1994) (Fig. 1). According to Vassey and Sharkey (1989), sucrose-phosphate synthase (SPS), a highly regulated enzyme that plays a key role in plant source–sink relationships, seems to be a main target for the biochemical effects of water stress. Following stomatal closure and the fall in CO_2 concentration in the intercellular airspaces of the leaves, a decrease in SPS activity was observed. This effect may lead to a limitation of carbon assimilation by Pi under water deficits, as was observed by Maroco *et al.* (2002) in grapevines, by using the A/C_i analysis for estimating the limitation of A by triose phosphate utilization. However, increasing CO_2 in the surrounding atmosphere can reverse this effect (Sharkey, 1990). Speer *et al.* (1988) also found out that when stomata closed under mild dehydration ($RWC \sim 90$–95%) nitrate reduction in spinach leaves was also inhibited. When those leaves were illuminated in an atmosphere of 15% CO_2, this inhibition was reversed, nitrate reduction occurring then at a normal rate.

A recent survey in different species under drought suggests that metabolic impairment of photosynthesis does not occur until maximum light-saturated stomatal conductance is very low (generally lower than 50 mmol m^{-2} s^{-1}) (Medrano *et al.*, 2002). This agrees with the hypothesis of a CO_2-scarcity mediated effect on metabolism under drought. On the other hand, the limitation to photosynthesis by an increased resistance to CO_2 diffusion in the mesophyll under drought has not deserved enough attention (Centritto *et al.*, 2003). In fact, these authors argue that stomatal resistance is not the only diffusive limitation encountered by CO_2 in its route from the atmosphere to the chloroplasts. The mesophyll resistance to CO_2 transfer can be sufficiently large to decrease the CO_2 concentration from the intercellular spaces (C_i) to the site of carboxylation (C_c) and when not taken into account, can lead to an over-eestimation of the metabolic limitations to carbon assimilation as discussed by Centritto *et al.* (2003) and by Ethier and Livingston (2004).

Under field conditions plants are commonly subjected to multiple stresses in addition to drought, such as high light and heat. The combination of high irradiance (and/or heat) with CO_2 deprivation at the chloroplast (driven by stomatal closure) predisposes the plants for a down-regulation of photosynthesis or for photoinhibition. In fact, under conditions that limit CO_2 fixation, the rate of reducing power production can overcome the rate of its use by the Calvin cycle. Protection mechanisms that prevent the production of excess reducing power are thus an important strategy under water stress. Such protection may be achieved by the regulated thermal dissipation occurring in the light-harvesting complexes, involving the xanthophyll cycle (Demmig-Adams and Adams, 1996; Horton *et al.*, 1996; Ort, 2001) and presumably the lutein cycle (Bungard *et al.*, 1999; Matsubara *et al.*, 2001). These photoprotective mechanisms compete with photochemistry for the absorbed energy, leading to a down-regulation of photosynthesis which is shown by the decrease in quantum yield of PSII (Genty *et al.*, 1989). If the limitation of the rate of CO_2 assimilation is accompanied by an increase in the activity of another sink

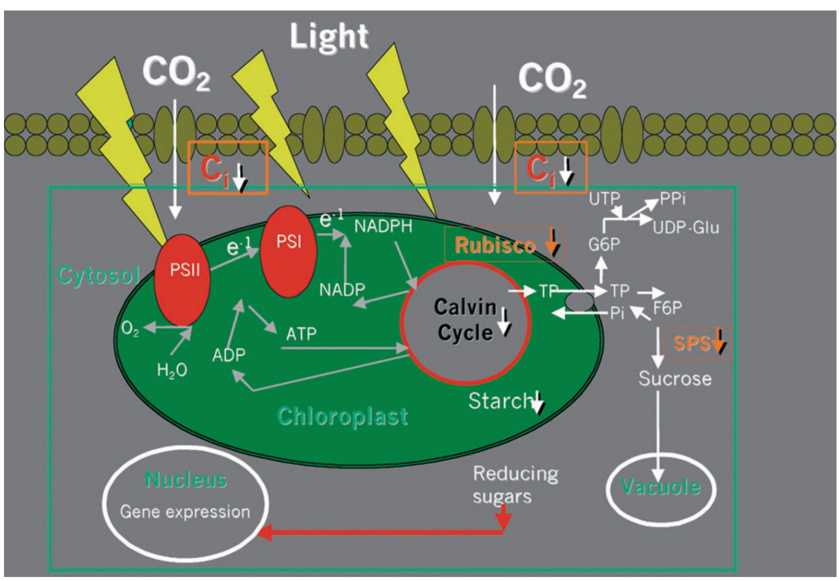

Fig. 1. Under moderate water deficits intercellular CO_2 (C_i) decreases due to stomatal closure, while photosynthetic capacity is maintained. This decrease in C_i may induce reversible inhibition of some enzymes (e.g. SPS). At the same time, starch content decreases and reducing sugars are maintained or even increase. This change in the carbohydrate status can lead to alterations of gene expression.

for the absorbed energy, for example, photorespiration (Genty *et al.*, 1990; Harbinson *et al.*, 1990; Wingler *et al.*, 1999) or Mehler-peroxidase reaction (Biehler and Fock, 1996), the decline in non-cyclic electron transport will be proportionally less than the decrease observed in the rate of CO_2 assimilation. This type of response has mainly been documented in plants native to semi-arid regions. Much less is known about how crop plants cope with excessive light, conditions that may arise even in irrigated field-grown plants during the summer period.

Oxidative stress or redox signalling under drought?

In agriculture, crop survival of a stress episode, such as drought plus high temperature is vital. Protective responses at the leaf level must be triggered quickly to prevent the photosynthetic machinery from being irreversibly damaged. Therefore, signals are key players in plant resistance to stress.

As already mentioned, the over-reduction of components within the electron transport chain, following a drastic decrease in intercellular CO_2 under drought results in electrons being transferred to oxygen at PSI or via the Mehler reaction. This generates reactive oxygen species (ROS), such as superoxide, hydrogen peroxide (H_2O_2) and the hydroxyl radical, that may lead to photo-oxidation, if the plant is not efficient in scavenging these molecules. It is now acknowledged that the redox-state of the photosynthetic electron components and the redox-active molecules synthesized also act as regulatory agents of metabolism (Neill *et al.*, 2002; Foyer and Noctor, 2003).

Redox signals are early warnings, exerting control over the energy balance of a leaf. Alterations in the redox state of redox-active compounds regulate the expression of several genes linked to photosynthesis (both in the chloroplast and in the nucleus), thus providing the basis for the feedback response of photosynthesis to the environment, or in other words, the adjustment of energy production to consumption. It must be pointed out that the data on the redox regulation of photosynthesis genes is still contradictory, suggesting a highly complex signalling network (see the review by Pfannschmidt, 2003). Redox signalling molecules include some key electron carriers, such as the plastoquinone pool (PQ), or electron acceptors (e.g. ferredoxin/thioredoxin system) as well as ROS (e.g. H_2O_2). The PQ redox state was shown to control gene transcription of photosystem reaction centres of cyanobacteria and chloroplasts (Allen, 1993). In particular, a reduced PQ pool activates the transcription of the PSI reaction centre, whereas an oxidized pool activates the transcription of the PSII reaction centre (Li and Sherman, 2000).

The intracellular concentrations of ROS are controlled by the plant detoxifying system, which includes ascorbate and glutathione pools. Accumulating evidence suggests that these compounds are implicated in redox signal transduction, acting as secondary messengers in hormonal-mediated events (Foyer and Noctor, 2003), namely stomatal movements (Pei *et al.*, 2000).

H_2O_2 acts as a local or systemic signal for leaf stomata closure, leaf acclimation to high irradiance, and the induction of heat shock proteins (Karpinska *et al.*, 2000); see also the review by Pastori and Foyer, 2002). The effects of H_2O_2 on guard cells were first reported in *Vicia faba* by McAinsh *et al.* (1996), who found that exogenous applications of H_2O_2 induced an increase in cytosolic calcium as well as stomatal closure. On the other hand, ABA applied to guard cells of *Arabidopsis* was shown to induce a burst of H_2O_2 that resulted in stomatal closure (Pei *et al.*, 2000; Desikan *et al.*, 2004). However, when the production of H_2O_2 exceeds a threshold, programmed cell death might follow.

H_2O_2 and other redox compounds play an important role in the stress perception of the apoplast, which acts as a bridge between the environment and the symplast. Recently it was observed that H_2O_2 is transported from the apoplast to the cytosol through the aquaporins, suggesting that the regulation of signal transduction can also occur via the modulation of transport systems (Pastori and Foyer, 2002). The interplay between the signalling oxidants and their antioxidants counterparts, in particular ascorbic acid (AA), the most important buffer of the redox state in the apoplast, are key factors in the regulation of plant growth and defence in relation to biotic and abiotic stresses, as recently pointed out by Pignocchi and Foyer (2003). These authors propose that the modulation of the apoplast redox state modifies the receptor activity and the signal transduction, leading to the stress response. It was also suggested recently that AA in the apoplast and the enzyme responsible for its redox state, the ascorbate oxidase (AO), are involved in cell division and expansion, processes that are generally affected by diverse stresses, namely drought. For example, the inhibition of cell division was observed when DHA (an oxidized form of AA) accumulates in the apoplast (Potters *et al.*, 2000; Foyer and Noctor, 2003).

Nitric oxide (NO), a reactive nitrogen species, acts as a signalling molecule, in particular by mediating the effects of hormones and other primary signalling molecules in response to environmental stimuli. It may act by increasing cell sensitivity to these molecules (Neill *et al.*, 2003). Recently, NO was shown to play a role as an intermediate of ABA effects on guard cells (Hetherington, 2001; Neill *et al.*, 2003). Likewise H_2O_2, NO may be also involved in stress perception by the apoplast, since this compartment can be a major site of its synthesis. It is also likely that both NO and H_2O_2 are synthesized in parallel and act in a concerted way in a number of physiological responses, including stomatal responses to the environmental stresses. Although the links between dehydration and NO are not yet fully resolved, it seems that some of signalling components down-stream of NO (and H_2O_2) in the ABA-induced stomatal closure are calcium, protein kinases, and cyclic GMP (Desikan *et al.*, 2004). NO also serves as an antioxidant by interacting with ROS produced under different stresses, such as superoxide, and by inhibiting lipid peroxidation. However, if NO is produced in excess it may result in nitrosative stress (see Neill *et al.*, 2003, for a review). The balance between NO and H_2O_2 also seems to play a role in some critical cellular responses, including programmed cell death.

Because nitrite can act as a precursor of NO, nitrate reductase (NR)-dependent NO production is now receiving much attention. Since the activity of NR is highly regulated by the environment (including nitrate supply, light, temperature, CO_2, cytosolic pH) this may be reflected in NO production and regulatory functions, such as those exerted on stomatal aperture (Garcia-Mata and Lamattina, 2003). It was also suggested that NO might operate over long distances, acting for example as root signal via nitrite coming from the roots to the shoot via the xylem stream. It would then produce NO in the guard cells. This evidence suggests that besides the role of NR in the co-ordination of C to N metabolism, this enzyme might also participate in the regulation of stomatal response to ABA and other stress factors.

Finally, NO also seems to play a role in the root response to drought and other stresses, namely by inducing adventitious root development (Pagnussat *et al.*, 2002).

Sugar signalling

The carbohydrate status of the leaf, which is altered in quantity and quality by water deficits, may act as a metabolic signal in the response to stress (Koch, 1996; Jang and Sheen, 1997; Chaves *et al.*, 2003). The signalling role of sugars under this context is not totally clear. In general, drought can lead either to increased (under moderate stress) or to constant (under intense stress) concentration of soluble sugars in leaves, in spite of lowered carbon assimilation, because growth and export are also inhibited. Under very severe dehydration soluble sugars may decrease (Pinheiro *et al.*, 2001). However, starch synthesis is, in general, strongly depressed, even under moderate water deficits (Chaves, 1991).

An increase in acid invertase activity was observed in leaves of droughted plants, coinciding with the rapid accumulation of glucose and fructose in maize leaves (Trouverie *et al.*, 2003) and with the accumulation of glucose, fructose, and sucrose, in both leaf blades and petiole of lupins (Pinheiro *et al.*, 2001). The trend of changes observed in sucrose of the leaf petioles is anti-parallel to the changes in leaf blades, suggesting that, under severe stress, leaves are increasing export (Pinheiro *et al.*, 2001). Interestingly, the activity of acid vacuolar invertase was highly correlated with xylem sap ABA concentration (Trouverie *et al.*, 2003). Recent molecular analysis indicated that ABA is a powerful enhancer of the IVR2 vacuolar invertase activity and expression (Trouverie *et al.*, 2003).There is also the indication of a direct glucose control of ABA biosynthesis. An increase in the transcription of several genes of ABA synthesis by glucose was observed in *Arabidopsis* seedlings (Cheng *et al.*, 2002). Modulation of the expression of ABA signalling genes by glucose and ABA was also reported. Other evidence indicates that CO_2, light, water, and other environmental signals can be integrated and perceived as sugar signals (Pego *et al.*, 2000), suggesting that different signal types may be perceived by the same receptor or that the signal pathways converge downstream (Ho *et al.*, 2001). On the other hand, sugars travelling in the xylem of droughted plants or sugars that might increase dramatically in the apoplast of guard cells under high light are likely to exert an important influence on stomatal sensitivity to ABA (Wilkinson and Davies, 2002).

Crosstalk between the sugar and plant hormone pathways, namely those of ABA and ethylene (Pego *et al.*, 2000; see also the review by Leon and Sheen, 2003) was also revealed. It was shown, for example, that glucose and ABA at high concentrations act in synergy to inhibit growth, whereas at low concentrations they can promote growth. On the other hand, it was demonstrated that the glucose inhibition of growth could be overcome by ethylene, although, in general, this hormone acts as a growth inhibitor (Leon and Sheen, 2003). Responses and interactions appear to be both dependent on concentrations and on the particular tissue; an example of the latter is the opposite effect of ABA on growth of shoot and root (Sharp, 2002).

Sugars are also involved in the control of the expression of different genes related to biotic stress, and lipid and nitrogen metabolism (Koch, 1996; Jang and Sheen, 1997). They also affect the expression of genes encoding photosynthesis via a complex and branched pathway. Depletion of sugars triggers an increase in photosynthetic activity, presumably due to a de-repression of sugar controls on transcription, and an accumulation of sugars, due to a lower consumption of photoassimilates, have the opposite effect (Pego *et al.*, 2000).

Chloroplast resistance to dehydration and rehydration: the importance of membrane stability

Contrary to poikilohydrous plants that change their tissue water potential in parallel with that of the soil and/or air, quickly recovering from dehydration, higher plants can buffer to a certain extent the variations in plant water status. As already discussed, this can be achieved by preventing water loss through stomatal closure or by improving water acquisition from drying soil, either via a process of root osmotic adjustment or via an additional investment in the root system.

When water deficits become too intense (generally agreed to be in the range of leaf *RWC* lower than 70% (Kaiser, 1987; Chaves, 1991) or too prolonged, leaves can wilt, cells shrink, and mechanical stress on membranes may follow. Because membranes play a central role in various cellular functions, in particular those membranes with embedded enzymes and water/ion transporters, the strain on membranes is one of the most important effects of severe drought and survival. Recovery under these conditions is closely linked to plant capacity to avoid or to repair membrane damage, maintaining membrane stability during dehydration and rehydration processes. Speer *et al.* (1988) found out that photosynthetic membranes from spinach leaves wilted slowly under natural conditions and were damaged earlier (i.e. become transiently permeable) than the plasma membrane. Chloroplastic membranes, and their membrane bound-structures, are especially susceptible to oxidative stress because large amounts of ROS can be produced in these membranes. ROS can cause an extensive peroxidation and de-esterification of membrane lipids, as well as protein denaturation and DNA mutation (Bowler *et al.*, 1992). On the other hand, intense shrinkage leads to an increased concentration of internal solutes that may reach toxic concentrations for certain proteins/enzymes (Speer *et al.*, 1988), thereby intensifying detrimental effects on photosynthetic machinery, the cytosol, and other organelles. Upon the decrease in cellular volume, cell contents become viscous, increasing the probability of molecular interactions that can lead to protein denaturation and membrane fusion (Hoekstra *et al.*, 2001).

Interestingly, studies of oxidative stress have shown that some antioxidants or their transcripts (e.g. glutathione reductase, GR or ascorbate peroxidase, APX) may be higher during recovery than during the drought period, as observed, for example, in cotton (Ratnayaka *et al.*, 2003) or in pea plants (Mittler and Zilinskas, 1994). This might suggest that either the stress had induced an antioxidant response that 'hardens' the plants for future stressful conditions (Ratnayaka *et al.*, 2003) or/and that antioxidant protection is pivotal under the recovery phase. A broad range of compounds has been identified as playing a protective role on membranes and macromolecules. They comprise proline, glutamate, glycine-betaine, carnitine, mannitol, sorbitol, fructans, polyols, trehalose, sucrose, and oligosaccharides. All these compounds enable the proteins to maintain their hydration state (Hoekstra *et al.*, 2001). Upon further drying, sugars may replace the water associated with the membrane macromolecules, therefore maintaining their structural integrity. In particular, the hydroxyl groups substitute water in the maintenance of hydrophilic interactions with membrane lipids and proteins. Dehydrins are supposed to protect proteins against denaturating agents, therefore stabilizing membranes, through ion sequestration and replacement of hydrogen bonding (Close, 1996). Small heatshock proteins (HSPs) might act as molecular chaperones, both during dehydration and rehydration processes. Generally, HSPs are able to maintain partner proteins in a folded-competent state, minimizing the aggregation of non-native proteins and degrading and removing them from the cell (Feder and Hofmann, 1999). Among compatible solutes, sugars, especially the non-reducing disaccharides but also tri- and tetrasaccharides and fructans, are the most effective for preserving proteins and membranes under low water content (below 0.3 g H_2O g^{-1} DW). At this water content, water dissipates from the water shell of macromolecules and therefore, the hydrophobic effect responsible for structure and function is lost (Hoekstra *et al.*, 2001).

In the work done by Speer *et al.* (1988) it is also inferred that membrane damage (namely the chloroplast envelope) was more pronounced during rapid rehydration than during the preceding dehydration process. During rehydration, water replaces the sugar (or other compatible compound) at the membrane surface and, during this process, a transient membrane leakage takes place (Hoekstra *et al.*, 2001). When dehydration is too intense, giving rise to some rigidification of membranes, an irreversible leakage happens, followed by lethal injury. It seems that membrane fluidity is an important factor in resistance to injury. The effects of rehydration on membranes might explain the retardation of recovery after rewatering, often observed after prolonged and/or intense drought. It was also suggested that the degree of reversibility of the effects of dehydration is more species specific than the effects of dehydration itself, which might reflect differences in leaf structure rather than biochemical differences among species (Speer *et al.*, 1988).

Long-distance signalling: the root chemical signals

The importance of the chemical signals synthesized in the roots for the plant feedforward response to water stress has been under debate for some time (Wilkinson and Davies, 2002). Root-to-shoot signalling requires that chemical compounds travel through the plant in response to stress sensed in the roots. These signals may either be positive, in the sense that something is added to the xylem flow, or negative, if something is taken away (or not produced) from the xylem stream.

Hormones may become important controllers of plant metabolism under poor growth conditions, such as imbalances in light, nutrients, and water availability (Weyers and Paterson, 2001), where developmental plasticity could provide benefits through altered growth, optimizing the response to the environment (Trewavas, 1986). Hormones, with particular relevance to ABA, but also cytokinins and ethylene, have been implicated in the root–shoot signalling, either acting in isolation or concomitantly. This long-distance signalling by hormones may be mediated by reactive oxygen species (Lake *et al.*, 2002). One example of the combined action of hormones in root–shoot communication is that increased cytokinins concentration in the xylem sap was shown to promote stomatal opening directly as well as to decrease stomatal sensitivity to ABA (see the review by (Wilkinson and Davies, 2002). The central role of ABA in this process has been extensively reviewed recently, covering aspects as different as biosynthesis, compartmentation within the cell/tissue, modulation by different factors and co-ordination of the responses at the whole plant level (see the reviews by Hartung *et al.*, 2002; Wilkinson and Davies, 2002). Since the mid-1980s chemical compounds synthesized in drying roots, namely ABA or its conjugates (glucose esters), were shown to act as long-distance signals inducing leaf stomatal closure (Blackman and Davies, 1985) or restricting leaf growth, by arresting meristematic development (Gowing *et al.*, 1990, see also Davies and Zhang, 1991, for a review). Such knowledge has enabled it to be understood how some plant responses to soil drying can occur without significant changes in the shoot water status. This is the case of 'isohydric' plants that are able to buffer their leaf water potential by controlling stomatal aperture via feed-forward mechanisms.

Further work has shown that ABA transport into the root xylem can be modulated by the environment, namely through xylem pH, and also that the sensitivity of guard cells to ABA and changes in pH seem to be dependent on the time of the day (Wilkinson and Davies, 2002). Under water deficits an increase in xylem pH can occur, enhancing ABA loading to the root xylem (Hartung and Radin, 1989; Hartung *et al.*, 2002). Water stress may also reduce ABA catabolism and prevent rhizosphere- and phloem ABA from entering the symplast, thus enhancing the ABA root signal (Wilkinson and Davies, 2002). Environmental conditions that stimulate transpiration (e.g. VPD) also increase leaf sap pH, such increases in sap pH being correlated with reductions in stomatal conductance. Davies *et al.* (2002) and Wilkinson and Davies (2002) speculated that differences in species in relation to stomatal sensitivity to ABA may be related with different degrees of alkalinization in response to soil drying. On the other hand, an increase in xylem sap pH may act alone as a drought signal to reduce leaf expansion via an ABA-mediated mechanism, as found in barley ABA-deficient mutants and in tomato (Bacon *et al.*, 1998).

In a recent review Sharp (2002) proposed that the role of ABA in the control of shoot and root growth under water stress is an indirect one, resulting from the inhibitory effect of ABA on the synthesis of ethylene. Because ethylene inhibits growth, an insufficient ABA accumulation would result in an ethylene inhibition of shoot growth, whereas, in roots, the higher accumulation of ABA would prevent the ethylene-mediated inhibition of growth. Translocation of ABA from roots to shoots, in addition to producing stomatal closure and therefore turgor maintenance would, to some extent, counter-balance the inhibition of shoot growth by ethylene (Sharp, 2002). Considering that ABA ultimately co-ordinates whole plant performance, by regulating the partition of assimilates between the shoot and root, this ABA long-distance signalling could be described as a typical 'resource allocation' hormonal action.

Applications to water-saving agriculture

Improving plant trade-off between assimilated carbon and water by using controlled irrigation

The understanding of the factors that regulate the trade-off between carbon assimilation and water loss, and those that drive partitioning of assimilates between reproductive and non-reproductive structures in relation to water availability are essential to identify the technologies for matching water input with plant requirements. Irrigation strategies that exploit the knowledge of a plant's long-distance signalling system are increasingly being used to get improved crop water use efficiency under sustained or improved quality of the product (Davies *et al.*, 2002; Loveys and Ping, 2002). Indeed, it was demonstrated that large unregulated fluxes of water are not essential to plant functioning and that water can be saved by manipulating stomatal functioning (Loveys and Davies, 2004). A measure of successful regulation of carbon assimilation under variable water availability is the plant ability to maintain an equilibrium among the intervening processes, namely CO_2 diffusion, light harvesting, photochemistry, and biochemistry (Geiger and Servaites, 1994), so that the flux through each component of the process is in balance with the others, except for brief periods of transition. When water deficits start to build up, leaf stomatal conductance usually decreases faster than carbon assimilation, leading to increased water use efficiency, WUE (Chaves *et al.*, 2004). It is also well known that when irrigation is above the optimum, an excessive shoot growth can occur at the expense of roots and fruits (Zhang, 2004). Manipulation of pre- and post-flowering water use in crops can be used to increase harvest index (HI) and by using methods of controlled irrigation the optimized water use by stomata can lead to an increase in WUE, without a significant decrease in production and eventually with beneficial effects in quality.

Closure of stomata under dehydrating conditions is the result either from a feedback response to the generation of water deficits in the leaf itself that is transmitted to the guard cells, or from a feed-forward control before any alteration in leaf tissue water status takes place (Schulze, 1986). These feed-forward responses of guard cells comprise the responses to high vapour pressure deficit, whose mechanisms area still under debate (Franks and Farquhar, 1999) and dehydration taking place elsewhere in the plant, namely in the roots (Davies and Zhang, 1991). In addition to stomatal closure, shoot growth is slowed down at a very early stage of water stress (Hsiao, 1973; Kramer, 1983). As discussed in the previous section, strong evidence has accumulated suggesting that this kind of response to decreasing soil water may be mediated by long-distance signals produced in drying roots, namely of chemical origin (such as the hormone ABA or cytokinins) and transported to the shoot in the transpiration stream (Wilkinson and Davies, 2002). They will provide to the shoot a measure of the water available in the soil. However, ABA signalling is a complex process which involves not only the up-regulation of ABA biosynthesis and transport via the xylem to the leaf, but ultimately depends on homeostasis of xylem sap along the length of the transport system and on the variable role of anion trapping (Wilkinson and Davies, 2002). In fact, a large proportion of ABA transported from the roots is catabolized in the cells of the leaf in a process termed ABA filtration (Wilkinson, 2004). The pH of the xylem sap and of the leaf apoplast was shown to prevent ABA from entering the apoplast via the xylem. This is based on the 'anion trap' concept (Wilkinson and Davies, 2002), which establishes that ABA accumulates in the most alkaline compartments of the cells. The arrival of these signals at the guard cells (Alvim *et al.*, 2001) or the growing tissues (Wilkinson, 2004) is therefore ultimately governed by the apoplastic pH,. Environmental factors (such as PPFD, temperature or VPD) that influence shoot physiological processes will interact with factors that affect the rhizosphere, determining the final apoplastic pH. As a consequence, plant WUE will reflect the multiple environmental stimuli perceived and the ability of the particular genotype to sense the onset of changes in moisture availability and therefore fine-tune its water status in response to the environment (Wilkinson, 2004).

This knowledge has inspired a special kind of deficit irrigation, the so-called partial root-zone drying (PRD), where each side of the root system is irrigated during alternate periods. In PRD the maintenance of the plant water status is insured by the wet part of the root system, whereas the decrease in water use derives from the closure of stomata promoted by dehydrating roots (Davies *et al.*, 2000). Large-scale implementation of PRD irrigation in vineyards has already taken place in Australia (Loveys and Ping, 2002). This irrigation type has been further studied in grapevines (Souza *et al*., 2003; Santos *et al*., 2003) and in other crops, such as tomato (Davies *et al.*, 2000; Mingo *et al.*, 2003), raspberries (Grant *et al.*, 2004), orange trees (Loveys and Davies, 2004) or olive trees (Mentritto *et al.*, unpublished data). Although the nature of the signals is not totally clear, it is recognized that stomatal closure and growth inhibition are likely to be responding simultaneously to different stimuli, some of which may operate through common signal transduction systems (Webb and Hetherington, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000). Physiological data that are being accumulated (e.g. in grapevines under PRD) point to subtle differences between PRD and the deficit irrigation (DI), where the same amount of water is distributed by the two sides of the root system (Souza *et al.*, 2003; Santos *et al.*, 2003). These differences include some reduction of stomatal aperture in PRD (more apparent when measurements of stomatal conductance are done under constant light and temperature, rather than under the fluctuating conditions prevailing in the field), a depression of vegetative growth, and an increase in cluster exposure to solar radiation, with some potential to improve fruit quality (Table 1). An interesting finding is the link found between the intensity of the PRD stomatal response and *VPD*, high *VPD* intensifying PRD stomatal closure compared with the controls (Loveys and Davies, 2004). These authors suggest that the enhanced response of stomata to VPD in PRD irrigation could be related to an increased ability of the xylem to supply ABA.

There is also evidence that PRD can increase fruit quality in tomato, presumably as a result of differential effects on vegetative and reproductive production (Davies *et al.*, 2000). The root system also seems to be significantly altered in response to partial dehydration, not only in respect to total extension and biomass but also in architecture (Dry *et al.*, 2000; TPd Santos *et al*., unpublished results; MA Bacon and WJ Davies, personal communication). It is likely that this alteration in the root characteristics

Table 1. *Effect of controlled irrigation on physiological responses of field-grown grapevines*

Maximum and minimum values of leaf net photosynthetic rates (A_{field}) and stomatal conductance ($g_{\text{s field}}$) measured at midday, from mid-June to mid-September 2000, in the grapevine cultivar Moscatel, under different irrigation treatments, FI, DI, PRD, and NI. $A_{\text{controlled}}$ and $g_{\text{s controlled}}$ measured under controlled conditions of light (1200 μmol m^{-2} s^{-1}) and temperature (25 °C) at the end of August (mean values ± SE). Maximum and minimum values of leaf predawn water potential (Ψ_{pd}) for the same period as above and for sap flow measurements done during August. Discrimination of ^{13}C in the berries, measured at harvest, in September (mean values ± SE). Leaf area per vine measured at harvest and percentage of sun-exposed cluster at maturation (mean values ± SE). (Data from Souza *et al*., 2003; Santos *et al*., 2003).

	Full irrigation (FI)	Deficit irrigation (DI)	Partial root-zone drying (PRD)	Non-irrigated (NI)
A_{field} (μmol m^{-2} s^{-1})	16.3−10.0	13.3−11.3	14.6−8.3	12.5−3.3
$g_{\text{s field}}$ (mol m^{-2} s^{-1})	0.30−0.28	0.23−0.19	0.19−0.15	0.13−0.07
$A_{\text{controlled}}$ (μmol m^{-2} s^{-1})	13.3±0.5	12.0±1.1	11.5±0.9	9.5±0.9
$g_{\text{s controlled}}$ (mol m^{-2} s^{-1})	0.35±0.04	0.33±0.05	0.25±0.05	0.14±0.01
Ψ_{pd} (MPa)	−0.10 to −0.18	−0.14 to −0.44	−0.14 to −0.30	−0.22 to −0.64
Sap flow (g h^{-1} m^{-2})	402−356	275−196	145−130	109−101
Berries $\delta^{13}C$ (‰)	−26.3±0.17	−25.9±0.28	−23.7±0.07	−22.4±0.69
Leaf area (m^2 per vine)	6.3±0.26	4.9±0.15	4.3±0.21	3.6±0.18
Exposed clusters (%)	9.8±2.8	12.9±3.2	16.8±3.5	22.8±3.8

and in the source/sink balance plays an important role in plant performance under PRD.

In some crops, such as cereals (Blum *et al.*, 1994; Gent, 1994) and some legumes (Chaves *et al.*, 2002), reserves accumulated in the stem before anthesis can be utilized for grain filling in addition to current assimilates, therefore contributing to important gains in HI. Under stress conditions (Blum *et al.*, 1994) or high respiration rates (for example, high temperatures) stem reserves are essential to complete grain filling (Gent, 1994). The potential for storing reserves in the stem is dependent on stem length and weight density, although these characteristics per se are not sufficient to ensure that those reserves would be translocated to the fruit. Mobilization of reserves is dependent on sink strength, which varies with the genotype and is affected by the environment (e.g. water availability). On the other hand, the stem (in particular, the stem stele, which is associated with the vascular tissue) is especially well protected against environmental stress. In fact, studies in lupin subjected to drought indicated that the stem stele never dropped its relative water content (RWC) below 83%, whereas the other organs in the plant exhibited values below 60%, namely the leaves 57%, the roots 58%, and the stem cortex 58% (Pinheiro *et al.*, 2004). It can be speculated that this response is associated with the protection given by the accumulation of assimilates, mainly glucose, fructose, and sucrose whose concentration in the stem stele doubles under water deficits (Pinheiro *et al.*, 2001). These sugars could also act as signals for the observed induction of protective proteins such as late embryogenesis abundant (LEA) proteins, much more pronounced in the stele than in the cortex (C Pinheiro *et al*., unpublished data).

Controlled soil drying was shown to promote the remobilization of carbon reserves during late grain filling in wheat and improve HI, especially when the crop is grown under high nitrogen (Yang *et al.*, 2000, 2001). In fact, under such conditions, a mild soil drying counteracts the delay in senescence of vegetative tissues that usually accompanies the heavy use of N, and improves remobilization of stem reserves to the grains. Stay-green for too long results in the non-remobilization of pre-anthesis reserves in leaves, glumes, and stems, which may account for 30–47% of the carbon in protein and 8–27% of the carbon in carbohydrates deposited in the grain (Gebbing and Schnyder, 1999). In China, if crop maturation is delayed, dry winds at the end of the growing season can dehydrate wheat very rapidly and reduce grain yield. Yang *et al.* (2001) showed that, by applying a moderate soil drying and thus inducing an earlier senescence, they could accelerate grain filling and therefore improve yield.

However, in regions without the constraints described above extending the grain filling period, and therefore delaying leaf senescence, could benefit yield by allowing more time for the translocation of assimilates to the grain (Richards *et al.*, 2001). This can be achieved either by controlling irrigation and/or by selecting genotypes for stay-green capability.

Genetic engineering for improved plant response to water deficit: recent advances

In the past decade most of the genetic engineering work that has been successful in agricultural terms was directed towards crop resistance to biotic stresses or to technological properties (see the review by Sonnewald, 2003). The studies addressing plant resistance to abiotic stress, namely in relation to drought, have been confined so far to experimental laboratory work and to single gene approaches, which has led to marginal stress improvement (Ramanjulu and Bartels, 2002). However, recent advances suggest that rapid progress will be possible in the near future, with large economical impact in many areas of the globe (Dunwell, 2000; Garg *et al.*, 2002; Wang *et al.*, 2003) (Table 2). In fact, even modest improvements in crop resistance to water deficits and in water use efficiency will increase yield and save water. One of the major challenges of this technology is to develop plants not only able to survive stress, but also able to grow under adverse conditions with reasonable biomass production, overcoming the negative correlation between drought resistant traits and productivity, which was often present in past breeding programmes (Mitra, 2001). Such a compromise requires improved efficiency in maintaining homeostasis, detoxifying cells from harmful elements (like ROS), and recovering growth that is arrested upon acute osmotic stress (Xiong and Zhu, 2002). This also means that there is the need to introduce sets of genes that govern quantitative traits, a technological approach that has already proved to be successful, for example, in the case of transgenic rice with introduced provitamin A (Ye *et al.*, 2000). The progressive cloning of many stress-related genes and responsive elements, and the proof of their association to stress-tolerant QTLs (Quantitative Trait Loci), suggests that these genes may represent the molecular basis of stress tolerance (Cattivell *et al*., 2002). On the other hand, the identification of QTLs associated with drought tolerance is also an important tool for marker-assisted selection (MAS) of tolerant plants. These studies have been conducted on a broad variety of species (see for instance Casasoli *et al*., 2004; Lanceras *et al*., 2004; Tuberosa *et al*., 2002). A lot of work has been done on this topic and will not be covered here; however, it is clear that the combination of traditional and molecular breeding (MAS and genetic engineering) will allow a more rapid way to improve abiotic stress tolerance in agricultural crops.

The increasing knowledge of stress adaptation processes and the identification of key pathways and interactions involved in the plant response to the stress conditions is being exploited to engineer plants with higher tissue tolerance to dehydration or with drought avoidance characteristics (Laporte *et al.*, 2002). The latter is, of course, more

Table 2. *Recent achievements in improving drought tolerance in crops through genetic engineering*

The genes used were originated from plants or bacteria and accounted for various cellular responses ending up in increased drought tolerance.

Gene/enzyme	Organism of origin	Target plant	Effect	Author
Functional proteins				
Superoxide dismutase (MnSOD)	*Nicotiana plumbaginifolia*	Alfalfa	Better performance in the field under drought	McKersie *et al*. (1996)
HVA1 (group 3 *Lea* gene)	Barley	Rice	Constitutive expression leads to protein accumulation in leaves and roots and improved recovery after drought and salt stress	Xu *et al*. (1996)
Myo-inositol *O*-methyltransferase (*IMT1*)	*Mesembryanthemum crystallinum*	Tobacco	Enhanced photosynthesis protection and increased recovery under drought, through the accumulation of D-ononitol.	Sheveleva *et al.* (1997)
Trehalose-6-P synthase, Trehalose-6-P phosphatase	Bacteria	Tobacco	Better photosynthetic efficiency and higher dry weight under drought stress	Pilon-Smiths *et al*. (1998)
HVA1 (group 3 *Lea* gene)	Barley	Wheat	Constitutive expression (*ubi*P) improved biomass productivity and water use efficiency under water-stress	Sivamani *et al*. (2000)
Aldose/aldehyde reductase (*MsALR*)	Alfalfa	Tobacco	Detoxification effect (reduced amounts of reactive aldehydes derived from lipid peroxidation) leading to tolerance to multiple stresses, including drought	Oberschall *et al*. (2000)
NADP-malic enzyme	Maize	Tobacco	Drought avoidance phenotype through decreased stomatal conductance and increased fresh weight per unit water consumed. Growth and rate of development similar to wild type	Laporte *et al*. (2002)
Fusion gene with Trehalose-6-P synthase and Trehalose-6-P phosphatase (*TPSP*) regulated by ABA inducible promoter or small subunit *rbcS* promoter	*E. coli*	Rice	Sustained plant growth and reduced photo-oxidative damage under drought and other abiotic stresses. Improved photosynthetic activity also under non-stress conditions.	Garg *et al.* (2002)
Mannitol-1-phosphate dehydrogenase (*mtlD)*	*E. coli*	Wheat	Improved drought tolerance with mannitol accumulation at a concentration insufficient for osmotic adjustment	Abebe *et al*. (2003)
Aquaporin NtAQP1	Tobacco	Tobacco	Over-expression of NtAQP increased membrane permeability for CO_2 and water, and increased leaf growth	Uehlein *et al*. (2003)
Regulatory proteins				
Calcium dependent protein kinase (*OsCDPK7*)	Rice	Rice	Over-expression of *OsCDPK7* led to induced expression of a glycine rich protein (*salT*) and LEA proteins (*rab16A*, *wsi18*) under stress. Increased salt and drought-tolerance.	Saijo *et al*. (2000)
CBF1 (DREB1B) (driven by P35SCaMV)	*Arabidopsis*	Tomato	Increased resistance to water-stress, but dwarf phenotype. Higher levels of proline than controls, and faster closure of stomata under water stress. Higher catalase activity and lower (McAinsh *et al*., 1996), with or without stress	Hsieh *et al*. (2002)

difficult to achieve, because it is linked to whole-plant morphological and physiological characteristics (Altman, 2003).

The recent progress in gene discovery and knowledge of signal transduction pathways is raising the possibility of engineering important traits by manipulation of one single gene, downstream of signalling cascades, with putative impact on more than one stress type. Moreover, in genetic engineering, it is important to mimic nature and activate, at the correct time, only the genes that are necessary to protect the plants against stress effects. This may be achieved by using appropriate stress-inducible promoters and will minimize effects on growth under non-stressing conditions, which is essential for agricultural crops. It is also desirable to target the desired tissue/cellular location, to control the intensity and time of expression, and to ensure that all the metabolic intermediates are available, so that no negative effects will arise (Holmberg and Bulow, 1998). Finally, to be able to prove that a transgenic plant is more resistant to water stress than the wild type, one needs a rigorous evaluation of the physiological performance as well as the water status of transformed plants. This will avoid ambiguous interpretations of the gene effects on plant drought resistance, such as those often appearing in the literature (see, for example, the comment by Blum in www.plantstress.com/admin/Files/Hsieh_PlantPhysiol_130.htm and Hsieh *et al.*, 2002). In other words, the impact of the introduced genes must be separated in their direct versus indirect effects (for example, increased resistance of the photosynthetic apparatus versus effects on plant or leaf size, phenology etc.).

Among the genes that are known to respond to drought stress and which are being manipulated by genetic engineering, some encode enzymes involved in metabolism (for example, linked to detoxification or osmotic response), others are active in signalling, or in the transport of metabolites (for example, the proline transporter) or in regulating plant energy status. Some genes do not have a well-established function, such as those encoding the LEA proteins, but result in protection of the cellular machinery against various stresses (Bray, 1997; Xu *et al.*, 1996).

Engineering for osmotic adjustment and/or protection of macromolecules: Engineering for increasing osmolytes, such as mannitol, fructans, trehalose, ononitol, proline, or glycinebetaine, among others may increase resistance to drought, although the protection mechanisms are still not fully understood (Ramanjulu and Bartels, 2002). If the osmolyte accumulation is sufficient to decrease cell osmotic potential thereby enabling the maintenance of water absorption and cell turgor at lower water potentials (Morgan, 1984), one can talk of osmotic adjustment. When the accumulation is low, it is reasonable to ascribe osmolytes a function in protecting macromolecules (such as, for example, enzymes) either by stabilizing proteins or by scavenging reactive oxygen species produced under drought (Shen *et al.*, 1997*a*; Zhu, 2001*b*). Although the benefits of osmolyte accumulation for crop yield are the subject of some controversy (Serraj and Sinclair, 2002), some results of genetic transformation point to advantages for plant performance under drought, which may open avenues for the future. Still, transgenic plants that have been engineered to overproduce osmolytes often exhibit impaired growth in the absence of stress. This is probably due to the involvement of osmolytes in signalling/regulating plant responses to multiple stresses, including reduced growth that may be part of the plant adaptation strategy against stress, as suggested by Maggio *et al.* (2002).

The **raffinose family** oligosaccharides, such as raffinose and galactinol, are among the sugars involved in desiccation tolerance. Taji *et al.* (2002) engineered *Arabidopsis* plants for over-expression of *AtGolS* 1, 2, or 3, all genes coding for galactinol synthase from *A. thaliana*. The overexpression of *AtGolS2* did increase endogenous galactinol and raffinose in transgenic plants and was found to reduce transpiration from leaves and to improve drought tolerance. These compounds seem to act as osmoprotectants, rather than by providing osmotic adjustment (Taji *et al.*, 2002).

Expression of bacterial **fructan** in tobacco and sugar beet led to an improved growth under water deficits in transgenic plants than in the wild type (Pilon-Smits *et al.*, 1995, 1998).

Mannitol, the most widely distributed sugar alcohol in nature (Stoop *et al.*, 1996), was demonstrated to scavenge hydroxyl radicals and stabilize macromolecular structures, such as phosphoribulokinase (a thiol-regulated enzyme), thioredoxin, ferredoxin, and glutathione (see for example, Shen *et al.*, 1997*a*, *b*). The protective effect seems to result from the formation of hydrogen bonds between macromolecules and osmolytes under limited water availability, thus preventing the formation of intramolecular H-bonds that could irreversibly modify the three-dimensional molecular structures. Recently, Abebe *et al.* (2003) achieved a significant improvement of wheat tolerance to water and salt stress through the ectopic expression of the *mtlD* gene (mannitol-1-phosphate dehydrogenase) from *E. coli*. The authors found that the amount of mannitol accumulated (0.6–2.0 μmol g^{-1} FW) was too low to ensure protection through osmotic adjustment, but was effective in improving stress tolerance. Lines containing over 0.7 μmol g^{-1} FW in the flag leaf, started showing side effects of mannitol accumulation and lines with over 1.6 μmol g^{-1} FW in the flag leaf showed severe abnormalities, including sterility. This was accompanied by an exceptionally low sucrose content. The plants with the lower mannitol contents (up to 0.7 μmol g^{-1} FW), however, did not suffer from the adverse effects of excess mannitol, which would deplete the sucrose pool and negatively impact the growth of wheat plants. Because mannitol is a naturally occurring sugar-alcohol and is used as an additive in many processed foods, its overexpression may prove to be a useful tool to enhance crop resistance to drought and salt. The overexpression of *IMTI1* (inositol methyl transferase) gene, from the ice plant *Mesembryanthemum crystallinum* into tobacco, led to the accumulation of another sugar-alcohol, the methylated form of inositol, **D-ononitol,** leading to an increased tolerance to drought and salt stress (Sheveleva *et al.*, 1997).

Trehalose, a non-reducing disaccharide of glucose, has been shown to stabilize biological structures and macromolecules (proteins, membrane lipids) in different organisms during dehydration (Crowe *et al.*, 1992). Through the regulated over-expression of a fusion gene containing the coding regions of both *otsA* and *otsB* (trehalose-6-P synthase and trehalose 6-P-phosphatase) of *E. coli*, Garg *et al.* (2002) showed that trehalose has a primary positive effect in transformed plants under abiotic stress conditions. This effect was linked to the maintenance of an elevated capacity for photosynthesis under stress. The positive effect of trehalose accumulation (an increase in 3–9-fold compared with the wild type) was observed under salt, drought, and low-temperature conditions. Under drought, trehalose accumulation accounted for an increased protection of Photosystem II against photo-oxidative damage, as assessed by *in vivo* chlorophyll fluorescence (ϕ_{PSII} and F_v/F_m). These effects were observed both when the fusion gene was directed to the chloroplast (with a transit peptide and under the control of the promoter of the small subunit of *rbcS*) or to the cytosol (under the control of an ABA-inducible promoter). The reason why photosynthetic capacity was preserved in drought-stressed transgenic rice is, however, not clear; is it because shoot water status was improved, or is it simply because, under a dehydration intensity similar to that affecting the wild-type plants, the photosynthetic apparatus is protected against oxidative stress? It may be speculated that because the transgenic lines with gene expression in the chloroplast showed protection against drought at lower trehalose concentrations than those with cytosolic expression, the second hypothesis is the most likely.

Garg *et al.* (2002) also found an increase in other soluble carbohydrates after exposure to abiotic stress (20% higher concentrations in transformed than in wild-type plants). These results are consistent with the hypothesis raised by Paul *et al.* (2001), working with tobacco plants expressing *E. coli* trehalose biosynthetic genes, that trehalose may play a role in the modulation of carbon metabolism in response to external factors, through sugar-sensing mechanisms. The work by Garg *et al.* (2002) confirmed some beneficial effects observed in earlier transformation work done by Pilon-Smits *et al.* (1998) in tobacco. However, very significant progress was achieved by comparison with previous studies, where undesirable pleiotropic effects, including stunted growth and the formation of abnormal leaves, occurred in plants where the two enzymes involved in the trehalose biosynthesis were overexpressed (Goddijn *et al.*, 1997; Holmstrom *et al.*, 1996). If these studies are confirmed by field trials, they increase the possibility for cultivating rice, a major staple crop worldwide, in rainfed conditions or in saline soils (Penna, 2003).

Betaines, ectoine, and proline are among the compatible solutes that also accumulate in plants as a widespread response against environmental stress (Chen and Murata, 2002; Rontein *et al.*, 2002). Some crop plants have low levels of these compounds, and engineering their biosynthetic pathways is a potential way to improve stress tolerance. For instance, in wheat, the accumulation and mobilization of proline was found to correlate with the level of tolerance towards water stress (Nayyar and Walia, 2003), the tolerant genotype being more responsive to ABA. Overexpressing the gene *P5CS* from *Vigna aconitifolia* in tobacco led to a 2-fold increase in proline and a better growth under water and salt stress (Kavi Kishor *et al.*, 1995). A number of genes involved in the biosynthetic pathways of such compounds, such as choline-oxidase or sorbitol-6-phosphate dehydrogenase, have been tested in transgenic plants with positive results in increasing stress tolerance (Chen and Murata, 2002). In some cases, the accumulation of these solutes is marginal, implying that they were not acting through an effect of osmotic adjustment (Holmstrom *et al.*, 1996).

A group of proteins commonly involved in the enhancement of stress tolerance are the **LEA proteins**. The role of LEA proteins was suggested as chaperones, in binding water, in protein or membrane stabilization, and in ion sequestration (Cushman and Bohnert, 2000). Rice and wheat plants expressing the barley group 3 LEA gene *HVA1* in leaves and roots showed improved osmotic stress tolerance and improved recovery after drought and salinity stress (Xu *et al.*, 1996; Sivamani *et al.*, 2000). Group 2 of the LEA proteins, the dehydrins (also known as the Lea D11 family) has been commonly observed accumulating in response to dehydration or low temperature (Close, 1997). With one or more copies of a putative amphipathic α-helix-forming domain (the K-segment), dehydrins are the best-studied LEA proteins. They have been considered as having a role as surfactants, preventing the coagulation of numerous macromolecules (Close, 1997).

Other proteins may also play a role in protection against drought. This is the case of some **heat shock** (HS) proteins, including small HS (smHS) such as the *At-HSP17.6A* class from *Arabidopsis thaliana*, which, upon over-expression, could increase salt and drought tolerance, presumably due to its chaperone activity demonstrated *in vitro* (Sun *et al.*, 2001). Their action includes preventing protein degradation and assisting the refolding of proteins denaturated during stress. In transgenic tobacco plants, the enhanced accumulation of the chaperone-binding protein BiP, of the endoplasmic reticulum (shown to be induced by a variety of environmental stresses), conferred tolerance to water stress (Alvim *et al.*, 2001). Under progressive drought, leaf BiPs concentration was correlated with shoot water content and photosynthetic rates were maintained in stressed transgenic plants to values similar to those measured in wild-type well-watered plants.

NtC7, a gene encoding a membrane-located receptor-like protein, with transmembrane domains, was also found to induce, in transgenic tobacco plants, a marked increase in tolerance to mannitol-induced osmotic stress, with rapid recovery from severe wilting, whereas wild-type plants showed leaf necrosis (Tamura *et al.*, 2003). The authors suggested that the *NtC7* gene is involved in the signalling pathway that activates genes responsive to osmotic stress (independently of ion homeostasis), presumably as part of the osmosensor system. Osmotic adaptation may occur through mechanic-sensitive signalling, in which alterations in turgor could be the starting point for a signalling cascade, by generating a signal eventually triggering conformational changes in membrane proteins. In potato, mechanical stress has an early cellular response of the significant and rapid synthesis of superoxide radicals (Johnson *et al.*, 2003).

Protection against excessive accumulation of ROS has been achieved by overexpressing a stress-inducible **aldehyde dehydrogenase** gene, already present in *Arabidopsis* *thaliana* (Sunkar *et al.*, 2003). The function of this enzyme is to catalyse the oxidation of various toxic aldehydes, accumulated as a result of side reactions of ROS with lipids and proteins. Transgenic lines showed improved tolerance when exposed to dehydration, as well as to other types of stress (salt, heavy metals, H_2O_2) and this was accompanied by a decreased accumulation of lipid peroxidation-derived toxic aldehydes. Transgenics also survived for longer periods of drought than wild-type plants. The authors claim that these findings may lead to applications in crop plants, such as maize, wheat or soybean. In addition, the ectopic expression, in tobacco, of the alfalfa aldose/aldehyde reductase *MsALR*, provided tolerance to multiple stresses, including drought stress, with reduced amounts of reactive aldehydes generated from lipid peroxidation (Oberschall *et al.*, 2000). Manipulation of ROS scavenging enzymes, yielding the effective reduction of ROS concentration, however, may lead to increased susceptibility to biotic stress, since cell wall fortification, as a barrier to pathogen penetration, is increased by ROS (Xiong *et al.*, 2002). On the other hand, manipulation of ROS scavenging enzymes aiming to reduce oxidative damage is limited by the high number of isoforms and by their location in different sub-compartments and membranes (Bohnert and Sheveleva, 1998).

Engineering for water transporters: Water transport in plants uses both the apoplastic and the symplastic routes. This means that a high number of water molecules have to cross numerous cell membranes. This process is facilitated by aquaporins, membrane-intrinsic proteins found in all living organisms and forming water-permeable complexes (Uehlein *et al.*, 2003). The apoplastic water potential influences the phosphorylation status of aquaporins, so that its ability to transport water increases when phosphorylated. Therefore, aquaporins are likely to play an important role in the control of cellular water status in response to water deficits (Assmann and Haubrick, 1996; Bray, 1997). Differential expression of genes that encode different aquaporin isoforms during plant development were shown to be associated with different physiological processes, including stomatal opening (Chrispeels and Agre, 1994). However, the relationship between the role of aquaporins in the regulation of plant water status and the regulation of aquaporin gene expression is still unclear (Aharon *et al.*, 2003). For example, the over-expression in tobacco of the *Arabidopsis* aquaporin *AthH2*, which encodes PIP1b aquaporin, improved growth performance under non-stress conditions, but it was not effective under drought or salt stress (Aharon *et al.*, 2003).

Aquaporins may also transport other small molecules such as glycerol, solutes and ions (Tyerman *et al.*, 2002) and they show cytosolic pH-dependent gating (changes in the conductance of individual water channels), a feature providing a mechanism of co-ordinated inhibition of plasma membrane aquaporins upon cytosol acidosis (Tournaire-Roux *et al.*, 2003). This behaviour justifies the reduced ability of roots to absorb water under flooding conditions, as a consequence of anoxia.

Recently it was found that the tobacco aquaporin NtAQP1 acts as a CO_2 membrane-transport-facilitating protein, playing a significant role in photosynthesis and in stomatal opening (Uehlein *et al.*, 2003). The overexpression of *NtAQP1* in tobacco raised membrane permeability for CO_2 and water, and increased leaf growth (Uehlein *et al.*, 2003), a feature that may have an impact in plant performance under drought. Photosynthesis increased in these transgenic plants by 36% under ambient CO_2 (380 ppm) and by 81% at elevated CO_2 (810 ppm). This was accompanied by an increase in stomatal conductance in both situations. Therefore, the increase in photosynthesis may result from a combination of more open stomata and a higher mesophyll conductance, resulting from the decreased membrane resistance to CO_2. Both effects led to an increase in CO_2 availability to the cells.

Engineering for C_4 traits: The ability to optimize net carbon gain and therefore increase WUE under reduced water availability is critical for plant survival (Chaves *et al.*, 2004). In species with C_4 photosynthesis high photosynthetic rates can be associated with low stomatal conductance, leading to high WUE (Cowan and Farquhar, 1977; Schulze and Hall, 1982). Manipulating WUE is a highly complex desideratum, because it implies co-ordinated changes relating to stomatal aperture and photosynthesis. Following various attempts to use conventional hybridization to get C_3–C_4 hybrids, several groups have successfully transformed C_3 plants to acquire C_4 characteristics (see the review by Matsuoka *et al.*, 2001). Ku *et al.* (1999), for example, introduced in rice the phosphoenolpyruvate carboxylase (PEPC) from maize, achieving a high-level expression of the PEPC protein (1–3-fold that of maize leaves). Although no significant effects were observed in the rates of photosynthesis, the transformed rice plants exhibited a reduction in the O_2 inhibition of photosynthesis characteristic of C_3 plants that may attain 40% of potential photosynthesis. These transgenic plants may theoretically have some advantage over the wild type, especially under low CO_2 conditions, prevalent for example under water deficits, when carbon loss associated with photorespiration becomes maximal. Some beneficial effects of the introduction of PEPC were observed under supra-optimal temperatures in transgenic tobacco and potato (see Matsuoka *et al.*, 2001). The hypothesis underlying this response is that PEPC participates in the initial CO_2 fixation or it increases CO_2 in the vicinity of Rubisco.

A recent paper by von Caemmerer (2003) suggests, based on a modelling exercise, that C_4 photosynthesis in a single C_3 cell, although theoretically inefficient due to the absence of appropriate structural features of C_4 plants (see the review by Leegood, 2002), may ameliorate the

CO_2-diffusion limitations of C_3 leaves. Again, this could be beneficial under water-limited conditions, when stomata close and intercellular CO_2 decreases drastically.

An alternative strategy to improve WUE would be to enhance photosynthetic capacity in C_3 crop plants by expressing improved forms of Rubisco, exhibiting higher relative specificity for CO_2 compared with O_2, such as those encountered in rodophyte algae, or to increase the catalytic rate of Rubisco (Spreitzer and Salvucci, 2002; Parry *et al.*, 2003). There is also scope for over-expressing Rubisco activase, which seems to be more susceptible to extreme environments, namely high temperatures (Feller *et al.*, 1998; Rokka *et al.*, 2001).

Engineering via signal components and transcription factors: In spite of the complex nature of the physiological adaptation of plants to the stress conditions and the difficulty of understanding the regulatory mechanisms behind adaptation, there are already a number of genes that have been found to be involved in the signal transduction pathways. They play important roles downstream of signalling cascades, which could be used to engineer a higher ability for plant protection from abiotic stress (Iba, 2002; Zhu, 2002). The modulation of these genes has been reported to improve abiotic stress tolerance in a number of plant species with positive effects, sometimes regarding more than one stress type (Dubouzet *et al.*, 2003).

Multiple stress stimuli lead to Ca^{2+} influx in the cell and to its increased concentration in the cytoplasm. A number of transport proteins such as the aquaporins, H^+-ATPases and ion channels, responsible for cytosolic osmoregulation and involved in stress adaptation, are regulated by calcium-dependent protein kinases (CDPKs). Saijo *et al.* (2000) investigated the function of the rice cold- and salt-inducible OsCDPK7, and found that its over-expression in transgenic rice plants conferred salt and drought-tolerance, apparently through the induced expression of LEA proteins, namely *rab16A* (group 2 LEA protein), *salT* (a glycine-rich protein) and *wsi18* (group 3 LEA protein). This effect, however, was only observed in the rice cells after stress stimuli, pointing to a strong post-translational control and OsCDPK7 activation after the stress-induced calcium influx. The over-expression of OsCDPK7 did not significantly affect plant development and fertility.

The transfer of individual genes to plants, for acquiring higher stress tolerance, has so far only had a limited impact; however, the simultaneous transcriptional activation of a subset of those genes, by transferring transcription factors, has been revealed as a promising strategy (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998).

There are several classes of transcription factors (TFs) playing major roles in dehydration and desiccation (Ramanjulu and Bartels, 2002). In *Arabidopsis*, the TFs DREBs/CBFs specifically interact with the dehydration responsive element/C repeat (DRE/CRT) *cis*-active element, controlling the expression of many stress-inducible genes. DREB/CBF proteins are encoded by AP2/EREBP multigene families and mediate the transcription of a number of genes, such as *rd29A*, *rd17*, *cor6.6, cor15a*, *erd10*, *kin1*, *kin2*, and others, in response to cold and water stress (Ingram and Bartels, 1996; Liu *et al.*, 1998; Seki *et al.*, 2001; Thomashow *et al.*, 2001). A novel transcriptional regulator of the DRE/CRT class of genes, FIERY2 (*FRY2*), acts by repressing stress induction of the upstream DREBs/CBFs TFs (Xiong *et al.*, 2002). Recessive mutations in *FRY2* result in super-induction of the DRE/CRT class of stress-responsive genes. Because FRY2/CPL1 contains dsRNA-binding domains, Xiong and Zhu (2002) speculated that dsRNA could be a regulator of the phosphatase enzymatic activity of FRY2/CPL1. RNA could then regulate hormone and stress responses in plants, as it does in animals. As cited by Xiong and Zhu (2002), some components in mRNA processing (such as the cap-binding protein ABH1 and Sm-like snRNP protein SADI) are specifically involved in ABA and stress responses.

The over-expression in *Arabidopsis* of DREB1 and DREB2 improved tolerance to dehydration (Liu *et al.*, 1998). Under the control of a constitutive promoter, DREB1A was, however, detrimental when stress was not applied, although it had a positive effect for plants under stress. The use of the stress-inducible promoter *rd29A*, instead of the CaMV 35S promoter, to over-express DREB1A minimized the negative effects on plant growth (Kasuga *et al.*, 1999). DREB genes under the control of *rd29A* are presently being tested on tropical rice (Datta, 2002).

The *Arabidopsis* CBF1 (DREB1B) ectopically expressed in tomato, resulted in enhanced resistance to water-deficit, although growth retardation was observed as well as reduced fruit and seed numbers when under the control of the 35S promoter (Hsieh *et al.*, 2002). An ABA-inducible promoter did not affect plant morphology or growth, but was less effective under stress conditions. In transgenic CBF1 tomato under water-deficit, stomata closed faster than in wild-type plants and proline concentration was higher, while catalase activity increased and H_2O_2 decreased compared with wild plants. Another gene, *CBF4*, found to be up-regulated only by drought (and not cold) when over-expressed in transgenic *Arabidopsis* was able to activate genes involved in both drought adaptation and cold acclimation (Haake *et al.*, 2002). The authors proposed that plant responses to cold and drought evolved from a common CBF-like transcription factor, first through gene duplication and then through promoter evolution.

An homologous gene isolated from rice, *OsDREB1A*, and tested in *Arabidopsis* indicated a functional similarity to the *Arabidopsis* DREB1A, although in microarray and RNA blot analyses some differences were observed regarding the induced target genes (Dubouzet *et al.*, 2003). The authors suggested that *OsDREB1A* is potentially useful for producing transgenic monocots tolerant to drought, high-salt, and/or cold stresses.

Improved osmotic stress tolerance was achieved by 35S:AtMYC2/AtMYB2 in transgenic plants, as assessed by electrolyte-leakage tests (Abe *et al.*, 2003). Constitutive expression of TFs, however, usually leads to growth retardation (Abe *et al.*, 2003; Hsieh *et al.*, 2002; Kasuga *et al.*, 1999). The *Arabidopsis* MYB TF proteins AtMYC2 and AtMYB2 were found to function as transcriptional activators in ABA-inducible gene expression (Abe *et al.*, 2003). This role points to a novel regulatory system for gene expression in response to ABA, other than the ABRE (abscisic acid responsive element)-ZIP regulatory system (Wang *et al.*, 2003).

The over-expression of bZIP (basic region leucine zipper) TFs, binding to ABRE *cis*-elements (e.g. *ABF3* and *AREB2/ABF4*) were found to cause ABA hypersensitivity, reduced transpiration rate, and enhanced drought tolerance in transgenic plants (Kang *et al.*, 2002).

Conclusions

Most of the terrestrial plants have evolved either to escape drought by appropriate phenology or to avoid drought, by developing strategies that conserve water or optimize its acquisition. This requires early warning systems and different types of signalling. In general, plants also have to cope with the interaction of other stresses that often arise concomitantly with drought, and ultimately involve oxidative stress. Protective responses at the leaf level must then be triggered quickly in response to the stress effectors to prevent the photosynthetic machinery being irreversibly damaged. Therefore, signals are key players in plant resistance to stress. It is now apparent that redox signals are early warnings, exerting control over the energy balance of a leaf, and alterations in the redox state of redox-active compounds regulate the expression of several genes linked to photosynthesis and other metabolic pathways. It is also known that plant responses to stresses arise from the interplay between different signalling pathways.

The importance of the long-distance signalling for the plant feed-forward response to water stress is acknowledged, namely the role played by chemical signals synthesized in the roots and transported to the shoot via the xylem sap. Novel management techniques that exploit the knowledge of plant's long-distance signalling are increasingly being applied to get improved plant trade-off between carbon assimilated and water used, while sustaining yield and improving the quality of the crop products.

On the other hand, because drought-tolerance traits, 'drying without dying' as described by Alpert and Oliver (2002), are not common in higher plants, genetic engineering to introduce these traits may be a way forward for marginal environments, complementing the breeding work and marker-assisted selection for tolerance that explores the natural allelic variation at genetically identifiable loci. Moreover, QTL mapping allied with comparative mapping and map-based cloning in plants may be used to screen genes important in the response to stress. The molecular understanding of stress perception, signal transduction, and transcriptional regulation of these genes, may help to engineer tolerance to multiple stresses. Engineering a single gene, such as a Group 3 *LEA* gene or one affecting sugar metabolism, or playing a role as an anti-oxidant, proved to alter metabolism, but in most cases only led to marginal stress improvement. However, recent advances suggest that rapid progress will be possible in the near future. It may be possible to achieve multiple tolerance mechanisms for one or more abiotic stresses, with sufficient success for commercial exploitation through co-transformation or gene pyramiding. Moreover, the upstream targeting of regulatory networks may have a more consistent role in providing tolerance, either through protection or repair mechanisms. Advances in the molecular biology of stress response in tolerant organisms are raising a number of possibilities concerning regulatory genes that may be used in agricultural programmes, not only to ensure survival under water deficit but also to guarantee a reasonable productivity under reduced water availability.

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