Proline synthesis and degradation: a model system for elucidating stress-related signal transduction

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Abstract

A causal relationship between increased proline synthesis and plant tolerance of hyperosmotic stresses has been demonstrated. Nonetheless, the molecular basis of this effect is not yet established. Proline accumulation appears to be mediated by both ABA-dependent and ABA-independent signalling pathways, although the events that occur between the perception of stress and the induction of proline biosynthetic genes are poorly characterized. Recent evidence supports an important role for post-transcriptional events in dehydration- and ABA-induced proline synthesis. Further research concerning the factors that regulate the expression of enzymes involved in proline synthesis and degradation will not only be of value in attempts to increase plant stress tolerance, but may contribute to an improved understanding of at least certain stress-related aspects of the regulatory network which controls plant responses to the environment. In Arabidopsis thaliana, synthesis of the immediate precursor of proline, Δ1-pyrroline-5-carboxylate (P5C), is apparently regulated by a pathway disrupted by mutation of ABI1, a protein serine/threonine phosphatase of the 2C class. Similarities and differences between the signalling events upstream of the regulation of the gene encoding P5C synthetase and model stress-inducible Arabidopsis genes such as RD29A, KIN2, and RAB18 are reviewed. Further analysis of the factors that induce these genes may assist in elucidating the mechanisms involved in stress-induced proline accumulation. Putative stress-regulated promoter elements in the AtP5CS1, AtP5CS2 and AtP5CR genes are identified. Recent evidence that a signal related to proline synthesis and/or degradation selectively increases the expression of stress-related genes underscores the importance of elucidating the signalling events associated with proline accumulation under adverse conditions.

Key words: ABA, Arabidopsis, cyclic ADP-ribose, proline, pyrroline-5-carboxylate synthetase, signal transduction, stress.

Introduction

Environmental stresses such as drought, excessive salinity and low temperature are important factors which limit plant distribution and productivity. It has long been appreciated that the ability of plants to withstand stressful environments is controlled by a number of genes. The multigenic character of stress tolerance is a primary limitation to its manipulation using both traditional breeding approaches as well as more recent transformation technologies. In the light of increasing evidence implicating the existence of a general stress response...
system in plants, it has been proposed that overlapping responses to different environmental stresses may be mediated by common cellular signal transduction pathways (Hare et al., 1997). Recent studies of signalling cascades in higher plants have identified ion channels, intracellular signalling proteins and second messengers as critical components which mediate early events in signal transduction. In the long term, targeting the genes encoding components of stress-related signal transduction pathways may be more profitable than the manipulation of individual genes at the termini of these cascades. This approach was recently shown to be viable in conferring desiccation tolerance to callus tissue of *Craterostigma plantagineum* (Furini et al., 1997) and in increasing the freezing tolerance of non-acclimated *Arabidopsis thaliana* plants (Jaglo-Ottosen et al., 1998). These advances may open the way for the engineering of crops with an increased ability to adapt to several stresses experienced concurrently in the field. Nonetheless, better characterization of the molecular signals involved in stress perception and the molecular events that specify the expression of stress tolerance will be necessary to provide a sound basis on which such strategies to improve agricultural productivity can be founded. For over 40 years, plant physiologists have studied the accumulation of free proline in a number of species subjected to hyperosmotic stress. Terrestrial plants experience dehydration not only under conditions of water deficit and elevated soil salinity, but also following exposure to low temperatures. It is generally accepted that most of the proline accumulated during hyperosmotic stress arises from increased synthesis from glutamate (Hare and Cress, 1997). In plants, proline is synthesized from glutamate via \( \Delta^1 \)-pyrroline-5-carboxylate (P5C) by two successive reductions, which are catalysed by P5C synthetase (P5CS; EC 2.7.2.11/1.2.1.41) and P5C reductase (P5CR; EC 1.5.1.2). Genes encoding these two enzymes have been identified in several plant species and all have been reported to be upregulated in response to water deprivation and/or salinization (Hare and Cress, 1997; Hare et al., 1998). In *Arabidopsis*, P5CS is encoded by two differentially regulated genes, named *AtP5CS1* and *AtP5CS2* (Strizhov et al., 1997; Zhang et al., 1997). Divergence of the physiological function(s) of P5CS isoforms, which have also been observed in alfalfa (*Medicago sativa*) and tomato (*Lycopersicon esculentum*), is suggested by the observation that whereas *AtP5CS1* is expressed in differentiated tissues, but cannot be detected in dividing cell cultures in the absence of stress stimuli, *AtP5CS2* is solely responsible for the synthesis of transcript encoding P5CS in rapidly dividing *Arabidopsis* cell cultures (Strizhov et al., 1997).

Although it is not considered to contribute substantially to proline accumulation, the capacity for proline degradation is known to decrease under prolonged hyperosmotic stress (Hare and Cress, 1997). Proline degradation is catalysed by the sequential action of the mitochondrial enzymes proline dehydrogenase (PDH; EC 1.5.99.8) and P5C dehydrogenase (P5CDH; EC 1.5.1.12). An *Arabidopsis* PDH has been characterized at the molecular genetic level (Kiyosue et al., 1996; Peng et al., 1996; Verbruggen et al., 1996). Levels of transcript encoding PDH decline after prolonged exposure to NaCl- and polyethylene glycol (PEG)-mediated stress. This indicates that the decline in proline degradation is regulated at the genetic level and does not merely result from stress-mediated inactivation of PDH, which is believed to be bound to the inner mitochondrial membrane (Verbruggen et al., 1996). Upon relief from hyperosmotic stress, *AtPDH* transcript levels increase rapidly (Kiyosue et al., 1996; Peng et al., 1996; Verbruggen et al., 1996), concomitant with a decline in *AtP5CS1* transcript abundance (Kiyosue et al., 1996; Peng et al., 1996). A rapid induction of PDH activity is consistent with the long-proposed importance of the proline accumulated during stress as a rapidly mobilizable reserve of carbon, nitrogen and energy during recovery from stress (Hare and Cress, 1997). The isolation of a cDNA encoding plant P5CDH has yet to be reported.

A causal relationship between proline synthesis and tolerance of drought and salt stress has been demonstrated in tobacco (*Nicotiana tabacum*) plants which express a transgene encoding P5CS (Kavi Kishor et al., 1995). Characterization of the signalling events that regulate proline accumulation is justified not only by the possibility that an understanding of the molecular basis of regulated proline accumulation may further enhance the stress-tolerant phenotype observed by these workers, but also by the known sensitivity of proline metabolism to a wide range of adverse biotic and abiotic conditions, many of which do not contain a significant osmotic component (Hare and Cress, 1997). As reviewed below, only limited information exists on the nature of the signal transduction pathway which links the perception of osmotic stress to proline accumulation. Nonetheless, several recent advances provide a foundation on which further details of the stress-induced signalling process may be built.

**Stress-induced proline accumulation is dependent on cycloheximide-sensitive gene activation**

During stress, genes may be induced either as part of a primary response to minimize damage or as a result of secondary effects (e.g., generalized protein denaturation or inappropriate expression resulting from inactivated regulatory proteins) which arise from stress-induced damage. Clearly, any effective physiological response to adverse environmental conditions must be invoked before the onset of significant damage to the stressed tissues. Furthermore, the more rapidly a gene is induced, the less...
likely it is to have arisen from secondary effects. Therefore, independence of the stress-induced modulation of levels of transcripts encoding proline metabolic enzymes from changes in de novo protein synthesis is an important criterion for establishing both whether these events are primary responses to stress and whether the response is likely to be of adaptive significance. The protein synthesis inhibitor cycloheximide (CHX) is most often used as an indicator of the distance of any down-stream event from the initial signal perception event. If the observed effect on transcript level occurs both in the presence and absence of CHX, it is assumed that the reaction does not depend on de novo synthesis of proteins, but instead depends on signal transduction elements that are constitutively present.

The use of transcriptional and translational inhibitors in studies of 10-d-old Arabidopsis plantlets indicated that both de novo transcription and translation are required during the first 4-h of salt stress before proline begins to accumulate (Verbruggen et al., 1993). Transcriptional and translational inhibitors also inhibit proline accumulation in barley (Hordeum vulgare) leaves that have wilted or been treated with ABA (Stewart et al., 1986). In salt-shocked barley leaves, cordycepin (COR)-mediated inhibition of transcription prevents proline accumulation when added after salinization but before the onset of proline accumulation, but not when added after proline begins to accumulate (Stewart et al., 1986). A conflicting situation was found in Arabidopsis, where COR adversely affected proline accumulation even 12 h after the imposition of salt stress (Verbruggen et al., 1993). Cycloheximide delayed proline accumulation in salt-shocked barley leaves, although with time, proline accumulated in CHX-treated leaves at rates comparable to NaCl-treated controls (Stewart et al., 1986). This delay and subsequent accumulation was observed irrespective of whether CHX was added before, during or after salt treatment. Nonetheless, the earlier in the salt treatment that CHX was applied, the longer was the observed delay (Stewart et al., 1986). Therefore, although it is difficult to make a generalized conclusion concerning all plant species, at least certain aspects of the process(es) which signal proline accumulation under salt stress are dependent on the synthesis of new proteins.

In Arabidopsis, detectable accumulation of AtP5CS1 transcript has been observed within 1 h after the imposition of osmotic stress (Yoshida et al., 1995; Savouré et al., 1997; Strizhov et al., 1997). Pretreatment of seedlings with CHX caused only a slight (25%) reduction in AtP5CS1 transcript accumulation during the first hour after exposure to 200 mM NaCl, but prevented a further increase in transcript levels, which peaked 6 h after NaCl addition in seedlings not treated with CHX (Strizhov et al., 1997). This may be interpreted to indicate that two phases of AtP5CS1 induction occur upon salt shock and that only the mechanism which operates during the first hour of stress is independent of protein synthesis. Induction of AtP5CS2 mRNA accumulation is slower and reaches a lower maximum than that observed for AtP5CS1 under identical salt stress conditions (Strizhov et al., 1997). Induction of AtP5CS2 mRNA accumulation displays absolute dependence on protein synthesis (Strizhov et al., 1997). In transgenic Arabidopsis plants which express a fusion of the AtP5CS2 promoter to the β-glucuronidase (GUS) reporter gene, an approximately 5-fold increase in GUS activity was observed within 3 h of dehydration and increased to almost 7-fold of control levels within 24 h (Zhang et al., 1997). Thus, AtP5CS2 transcript accumulation following osmotic stress apparently arises predominantly from transcriptional activation and the factors that facilitate this process are not constitutively present in unstressed cells. The emerging view that the capacity for proline synthesis is not as sensitive to CHX as is the accumulation of free proline is consistent with the proposal that proline synthesis, and not proline levels per se, may be of primary importance in adaptation to stresses which cause cellular dehydration (Hare and Cress, 1997; Hare et al., 1998).

Signal transduction events upstream of P5CS gene induction

Overwhelming evidence supports the conclusion that P5C synthesis is the rate-limiting step in proline synthesis (Kavi Kishor et al., 1995). Although levels of transcript encoding P5CR have been reported to increase under stress in at least two legumes (Hare and Cress, 1997), and P5CR enzyme activity has been shown to increase under stress in several plant species (Mattioni et al., 1997), controversy has arisen concerning whether or not Arabidopsis P5CR transcript abundance is sensitive to salinization. Whereas one group has reported induction of AtP5CR mRNA levels in response to osmotic stress (Verbruggen et al., 1993; Savouré et al., 1997), others have contested a significant accumulation of transcripts encoding P5CR in osmotically-stressed Arabidopsis (Yoshida et al., 1995). Using an Arabidopsis P5CR cDNA as a probe, Mattioni et al. (1997) concluded that an increase in P5CR activity in Triticum durum seedlings during dehydration and salt stresses is unrelated to levels of the corresponding transcript.

In contrast, there is a general consensus that levels of transcripts encoding P5CS are rapidly induced to high levels upon dehydration and exposure to high NaCl concentrations (Yoshida et al., 1995; Peng et al., 1996; Igarashi et al., 1997; Savouré et al., 1997; Strizhov et al., 1997). Limited accumulation of AtP5CS1 transcript is observed after 24 h exposure of Arabidopsis plants to 4°C, although no detectable increase was observed 10 h after commencement of this stress (Yoshida et al., 1995).
In contrast to dehydration stress, 48 h incubation at 4 °C did not induce transcriptional activity of the AtP5CS2 promoter in transgenic Arabidopsis plants which expressed an AtP5CS2::GUS fusion (Zhang et al., 1997). Collectively, these data suggest that different pathways regulate Arabidopsis P5CS transcript accumulation under chilling and osmotic stresses. However, Xin and Browse (1998) have subsequently reported a 3-fold increase in AtP5CSI level and an approximately 10-fold increase in proline content after a 48 h exposure of WT Arabidopsis plants to 4 °C. In rice (Oryza sativa), OsP5CS transcript levels increased within less than 2 h of transfer to 4 °C, which was more rapid than induction by salt or dehydration (Igarashi et al., 1997). Heat treatment (40 °C for at least 24 h) does not induce P5CS gene expression in either Arabidopsis (Yoshida et al., 1995) or rice (Igarashi et al., 1997). Although on the basis of these studies, induction of P5CS transcript accumulation does not appear to be a general stress response, further investigation of the response in a range of species with different levels of tolerance of temperature extremes may be warranted.

Arabidopsis is a chilling- and freezing-tolerant plant (Mäntylä et al., 1995). The molecular basis of the response of this species to low temperature, drought and high salinity has been well characterized. Like AtP5CSI, many commonly studied Arabidopsis genes are responsive to low temperatures, desiccation and salinity. These have been variously designated as RD (responsive to dehydration), KIN (kyn-an-indusoitu; Finnish for cold-induced), RAB (responsive to ABA), COR (cold-regulated), LTI (low-temperature induced) and AtDi (Arabidopsis thaliana drought-induced). In an attempt to synthesize the available data and suggest how recurrent themes of stress-regulated signal transduction which have emerged from the study of these genes may relate to elucidating the pathways that control proline accumulation, our discussion will focus primarily on RD29A (also referred to as COR78, LTI78, LTI140, COR67, and COR160 by various workers), KIN2 (COR6.6, pHH29) and RAB18 (AtDi8).

The involvement of ABA in P5CS gene expression

Abscisic acid action has been strongly implicated in many of the other stresses which have been shown to be capable of inducing proline accumulation (Hare and Cress, 1997). Observations that exogenous application of ABA to unstressed plants can induce biochemical changes similar to those observed under stress, and that treating plants with ABA frequently hardens them against stress, have long suggested that ABA may normally be used to control at least certain aspects of acclimation to stress. Nonetheless, the dominant role played by ABA in mediating responses to water deprivation has often been called into question by investigations which failed to correlate stress-related responses (e.g. inhibition of shoot growth, leaf turgor pressure or stomatal closing) with endogenous ABA concentrations (Hare et al., 1997). As will be discussed below, ABA levels frequently peak shortly after the imposition of stress, but thereafter decline to unstressed levels even under sustained stress. In keeping with these reservations, considerable evidence indicates the existence of ABA-independent dehydration- (Gosti et al., 1995) and cold-induced (Gilmour and Thomashow, 1991; Nordin et al., 1991) signal transduction pathways. Many of the genes which are induced by these stresses in an ABA-independent manner are nonetheless responsive to applied ABA. Dissecting the interactions between ABA-dependent and ABA-independent signalling cascades is currently the focus of extensive investigation (Ishitani et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997).

A causal link between ABA and proline accumulation has been suggested for some, but not all plant species investigated (Dallmier and Stewart, 1992; Xin and Li, 1993; Savouré et al., 1997). In Arabidopsis, exogenously applied ABA increases the levels of AtP5CSI (Yoshida et al., 1995; Savouré et al., 1997; Strizhov et al., 1997) and AtP5CS2 (Strizhov et al., 1997) transcripts. In rice, rapid accumulation of OsP5CS transcript is observed within 2 h of treatment with ABA (Igarashi et al., 1997). Analysis of stress-responses in ABA-related mutants of Arabidopsis provides a powerful means of assessing the functional significance of ABA-mediated processes in acclimation to stress. These mutants can be broadly categorized as those with abnormally low levels of ABA in all tissues (aba) and those which are impaired or deficient in various responses that are regulated by ABA (ABA-insensitive; abi). Investigation of abal (formerly aba), an Arabidopsis mutant which is defective in ABA synthesis, indicated that induction of the accumulation of both free proline and AtP5CSI transcripts by low temperature and sorbitol-mediated dehydration is independent of the endogenous ABA level (Savouré et al., 1997). Somewhat contradictory evidence exists concerning the necessity for ABA synthesis in mediating the increases in AtP5CSI expression following salt stress (Table 1). Whereas the results of Strizhov et al. (1997) indicate an absolute requirement for ABA synthesis in mediating the accumulation of both AtP5CSI and AtP5CS2 transcripts, Savouré et al. (1997) did not observe a decreased abundance of AtP5CSI transcript in NaCl-stressed abal plants relative to WT controls. Salt-stressed abal plants accumulated proline, albeit to lower levels than comparable WT plants (Table 1). The apparent contradiction in the results of these two groups may arise from differences in the duration of the period over which the salinity stress was sustained. Savouré et al. (1997) incubated 10-d-old seedlings in 250 mM NaCl for 24 h, whereas Strizhov et al. (1997) incubated 14-d-old seedlings in 200 mM NaCl for 6 h. Whereas Savouré et al.
Table 1. Overview of the effects of various mutations characterized in Arabidopsis on selected responses to abiotic stresses; where tested, an abi3 mutation did not affect any of the responses listed

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<td>red.</td>
<td>red.</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Hajela et al. (1990)</td>
</tr>
<tr>
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<td>Cold (4°C)</td>
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<td>Lång and Palva (1992)</td>
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<tr>
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<tr>
<td>Cold (4°C)</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>±</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>±</td>
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<tr>
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<td>ud</td>
<td>+</td>
<td>–</td>
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<tr>
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<tr>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>de Bruxelles et al. (1996)</td>
</tr>
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<td>–</td>
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<td>+ +</td>
<td>+</td>
<td>ND</td>
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–, Constitutive level; ±, slight induction; ++, maximal response; ND, not determined; red., reduced level relative to unstressed wild-type (WT) control; ud, not present at a detectable level.
(1997) used the ecotype Landsberg erecta as the WT control, Strizhov et al. (1997) examined *AtP5CS1* transcript levels in seedlings of the WT Columbia.

Unlike *abi1*, *Arabidopsis abi* mutants do not have reduced endogenous ABA levels and their phenotypes cannot be reversed by exogenous application of ABA. The *Arabidopsis ABI1* and *ABI2* genes encode homologous serine/threonine phosphatases of the 2C class (PP2C) which are intermediates in ABA signal transmission primarily in vegetative tissues (Leung et al., 1997). Recent studies indicate that together with at least a third PP2C, ABI1 and ABI2 function redundantly as repressors of ABA signal transduction (Sheen, 1998). Both the *abi1* and *abi2-1* mutations involve conversion of a conserved glycine residue to an aspartate (Leung et al., 1997). This alteration in ABI1 constitutes a gain-of-function, rather than a dominant loss-of-function mutation, despite the elimination of PP2C activity (Sheen, 1998). However, although the ABI1 (wild-type) and ab1 (mutant) proteins inhibit ABA action by different mechanisms, it is likely that ab1 and ABI1 act at the same level in the ABA signal transduction pathway (Sheen, 1998). Many well-characterized stress responses, including drought rhizogenesis, a morphological response associated with proline accumulation (Vartanian et al., 1992), as well as ABA-mediated induction of KIN2 mRNA accumulation and ABA- or stress-induced increases in levels of transcript encoding a homeodomain-containing leucine zipper (HD-ZIP) protein ATHB-7, are compromised in *abi1* but not in *abi2* (Table 1). In contrast, induction of transcript encoding alcohol dehydrogenase (ADH) by exogenous ABA and dehydration is affected in *abi2* but not by mutation of ABI1 (de Bruxelles et al., 1996). Mutations in *ABI1* and *ABI2* have identical effects on the induction of *RAB18* transcript by ABA (Leung et al., 1997; Table 1), although the ABA-dependent accumulation of the same transcript by desiccation is severely inhibited in *abi1*, but not *abi2* (Table 1). Thus, analysis of ABA response mutants permits assignment of different stress responses to particular branches of the signalling network downstream of ABA perception. However, it is worth noting that although ABA-regulated expression of many genes (e.g. *RD29A*, *KIN2*, *ADH*) may be impaired in an *abi1* mutant, their induction by cold or desiccation may not be affected by mutation at this locus (Table 1).

Based on their analysis of the effects of isosmotic concentrations of NaCl and sorbitol on *AtP5CS1* abundance and proline levels in *abi1* and *abi2* (Table 1), Savouré et al. (1997) concluded that an increase in proline upon salinity stress is not controlled at the level of *AtP5CS1* transcript abundance, but possibly at a post-transcriptional level regulated by a pathway disrupted by mutation of *ABI1*. Little is known about post-transcriptional effects governed by ABA, although their importance in fine-tuning of the salt-stress response has recently been emphasized (Moons et al., 1997). Translational effects mediated by pathway(s) affected by mutations in *ABI1* and *ABI2* have been implicated in the expression of both *RD29A* and *RAB18* (Table 1). Nuclear run-on transcription assays indicated that cold-induced increases in *KIN2* transcript levels are primarily due to post-transcriptional control mechanisms (Hajela et al., 1990). Zhang et al. (1997) analysed the promoter activity of the *Arabidopsis AtP5CS2* gene by its fusion to the GUS reporter gene in transgenic *Arabidopsis* and tobacco plants. Although expression of the chimeric gene was induced by dehydration and NaCl stresses, ABA failed to induce GUS activity. Although these workers concluded that induction of *AtP5CS2* gene expression is a specific response to salt and water stresses, Strizhov et al. (1997) observed induction of *AtP5CS2* transcript by ABA in *Arabidopsis* leaves and suspension-cultured cells, although not in roots of 28-d-old seedlings grown in liquid culture. The most obvious way to reconcile these findings is to conclude that ABA is involved in post-transcriptional regulation of *AtP5CS2* gene expression at the level of stabilization of *AtP5CS2* transcript. Interestingly, in the leaves and roots of *Arabidopsis* seedlings, but not in suspension-cultured cells, induction of *AtP5CS1* mRNA accumulation by ABA is far more rapid and extensive than that of *AtP5CS2* (Strizhov et al., 1997).

Returning to the study of Savouré et al. (1997), it is emphasized that there is no definitive evidence that the role of the signalling pathway affected by mutation in *ABI1* is confined exclusively to action downstream of ABA perception. Indeed, after treatment with ABA, induced *AtP5CS1* transcript levels were not lower in *abi1* than in the WT or *abi1*, although interestingly, ABA-mediated induction of *AtP5CS1* was less effective in *abi2* (Savouré et al., 1997). This mirrors the induction characteristics of *RAB18* for which *abi2* inhibits transcript accumulation by exogenous ABA but not by progressive drought (Gosti et al., 1995; Table 1). The findings of both Savouré et al. (1997) and Strizhov et al. (1997) do not implicate a role for *abi2* in salinity- or dehydration-induced *AtP5CS1* transcript accumulation. In contrast to Savouré et al. (1997), Strizhov and co-workers found reduced levels of both *AtP5CS1* and *AtP5CS2* transcripts in unstressed *abi1* relative to the WT. This suggests that the pathway disrupted by mutation in *ABI1* may normally affect the basic level of expression of these genes. However, control of P5CS gene expression by this pathway during stress is not absolute, since a significant accumulation of both transcripts was noted in NaCl-stressed *abi1* plants (Strizhov et al., 1997; Table 1). Surprisingly, Savouré et al. (1997) observed a slight induction of constitutive *AtP5CS1* transcript levels in unstressed *abi1* seedlings! The basis of this phenomenon is uncertain, although it has been observed for other
ABA-responsive genes, e.g. *KIN2*, *ATHB-7* and *ADH* (Table 1). It may be related to the participation of ABI1 and ABI2 in an autoregulatory circuit that co-ordinates ABA content and flux through ABA signalling pathways with levels of the intermediates that transmit the primary stimulus (Leung et al., 1997). Although this effect was not observed in *abi1*, another explanation is that these *abi* mutants constantly suffer from increased water loss from their aerial parts owing to an inability to regulate stomatal aperture.

In this regard, characterization of proline accumulation and *AtP5CS1* expression in the *Arabidopsis sos1* (salt-overly sensitive) mutant has suggested an important role for turgor reduction in signalling both of these responses. The *sos1* mutant, which is deficient in both Na$^+$ and K$^+$ uptake, accumulates more proline and 2-3-fold higher levels of *AtP5CS1* transcript than the WT under saline conditions (Liu and Zhu, 1997a). These workers proposed that Na$^+$ and K$^+$ are the predominant osmolytes in salt-stressed *Arabidopsis*; and that the higher levels of proline synthesis in *sos1* may arise from a reduced capacity for the mutant to reduce its internal osmotic potential by ion uptake. Both of the studies conducted thus far which have concerned P5CS expression in *abi1* have been conducted *in vitro*. This decreases the likelihood that reduced control over stomatal aperture may account exclusively for the constitutively higher *AtP5CS1* transcript levels observed by Savoure´ et al. (1997). Nonetheless, investigation of the response in undifferentiated tissues may help to establish whether or not the effects of the *abi1* mutation on stress-induced *AtP5CS1* transcript levels and proline accumulation are exercised exclusively at the cellular level or via a more indirect effect on leaf water status. Furthermore, both studies involved examination of responses in whole seedlings. Thus, the effects of *abi1* on these responses in root tissue cannot yet be assessed.

The proposal by Liu and Zhu (1997a) that reduced turgor may signal proline accumulation is not consistent with the demonstration by Savoure´ et al. (1997) that NaCl is a more effective inducer of both free proline and *AtP5CS1* mRNA accumulation than an iso-osmotic concentration of sorbitol (Table 1). However, subsequent workers reported that mannitol induces higher steady-state levels of *AtP5CS1* transcript than does an isosmotic concentration of NaCl (Knight et al., 1997). This was observed at both high (0.666 M) and low (0.333 M) concentration of mannitol and iso-osmotic concentrations of NaCl. Mannitol was also a better inducer of *RD29A* and *RA818* transcript accumulation (Knight et al., 1997). In assessing the involvement of inorganic ions in controlling proline accumulation, it is worth mentioning that differences have been noted in the time-courses of *AtP5CS1* induction by desiccation and salt treatments. Whereas *AtP5CS1* mRNA levels start to accumulate within 1 h after treatment with 250 mM NaCl and continue to accumulate for a further hour, levels subsequently decrease constantly over a 24 h period (Yoshiba et al., 1995). In contrast, desiccation caused a slower accumulation of *AtP5CS1* mRNA, but the maximum level reached 5 h after commencement of the stress was sustained for at least a further 20 h (Yoshiba et al., 1995). Comparison of the effects of these treatments on cell turgor is difficult, although they suggest that osmotic adjustment resulting from ion uptake under salinity stress may account for the more transient accumulation of *AtP5CS1* transcripts after exposure to NaCl. Accordingly, sorbitol is more effective than an iso-osmotic concentration of NaCl in reducing *Arabidopsis* root growth (Cramer and Jones, 1996). However, a contrasting situation was found for rice seedlings: an increase in *OsP5CS* levels was also evident 5 h after the start of dehydration, but reached a maximum after 10 h and then decreased to pre-stress levels by 24 h (Igarashi et al., 1997). Moreover, induction of *OsP5CS* was evident only 10 h after treatment with 250 mM NaCl, but a maximum level attained within 24 h was sustained until at least 72 h after imposition of salt stress (Igarashi et al., 1997). Certainly, characterization of *sos1* has eliminated the likelihood that Na$^+$ accumulation alone signals proline synthesis in *Arabidopsis*. Furthermore, whereas proline and betaine accumulation in gram-negative bacteria is affected by the intracellular concentration of K$^+$, elevated levels of proline synthesis in *sos1* indicate that depletion of intracellular K$^+$ is not a sufficient signal for *AtP5CS1* induction in *Arabidopsis* (Liu and Zhu, 1997a). By examining the effects of the K$^+$ channel blocker tetraethylammonium chloride on mannitol-induced *AtP5CS1* expression, Knight et al. (1997) independently reached the same conclusion that K$^+$ uptake is not essential for *AtP5CS1* expression.

Any attempt to assess the importance of ABA in mediating stress-induced changes in P5CS activity should consider whether the kinetics of transcript accumulation match changes in the level of endogenous ABA during stress. Unfortunately, changes in ABA have not been monitored in any of the studies which have investigated the induction of genes encoding P5CS. In keeping with the transient induction of *AtP5CS1* transcript levels in response to salinization (Yoshiba et al., 1995) and *OsP5CS* levels in response to dehydration (Igarashi et al., 1997), dehydration-, salt- and cold-induced transients in ABA concentrations have frequently been reported (Cowan et al., 1997). In the roots of rice seedlings, salt shock (150 mM NaCl) caused an approximately 10-fold increase in ABA content within 8 h of the imposition of the stress, but these decreased to near control levels by 12 h and remained stable for the next 60 h (Moons et al., 1997). Dehydration stress (0.6 M mannitol) induced an almost 30-fold increase in the level of ABA in roots and an approximately 5-fold increase in shoots of *Arabidopsis* plants within 12 h, although by 24 h after the imposition...
of the stress, the ABA levels had dropped to about half of the respective maxima (de Bruxelles et al., 1996). Low-temperature treatments (0 °C or 4 °C) apparently had no effect on ABA levels in 28-d-old Arabidopsis plants (de Bruxelles et al., 1996), although Láng et al. (1994) reported a 2–4-fold transient increase in shoot ABA content from plants grown at 4 °C. In axenically-grown Arabidopsis plants, levels peaked between 6 h and 24 h of exposure to cold, since at these times, ABA content was comparable to that of controls (Láng et al., 1994). Although it is difficult to draw conclusions, comparison of these data with the time-courses of P5CS gene induction outlined above, which were obtained in separate studies, suggests that ABA content may not be the primary regulator of P5C synthesis under dehydration, salt or cold stresses, at least in rice and Arabidopsis. It may be of relevance to note that detectable induction of both AtP5CS1 and OsP5CS within at least 2 h after treatment with ABA is sustained for at least 24 h in the case of Arabidopsis (Yoshiba et al., 1995), and 48 h in the case of rice seedlings (Igarashi et al., 1997). However, within 72 h after treatment with 1 mM ABA, OsP5CS levels in rice seedlings had declined to pre-treatment levels (Igarashi et al., 1997). In cultured Arabidopsis cells, both AtP5CS1 and AtP5CS2 transcripts accumulate within less than 1 h of exposure to 1 μM ABA, but levels of both transcripts had declined to pre-treatment levels within 48 h (Strizhov et al., 1997).

Transcriptional activation of stress-regulated genes

Recent studies have begun to unravel some of the mechanisms whereby water stress elicits its effects on gene expression by transcriptional activation (Shinozaki and Yamaguchi-Shinozaki, 1997). Analyses of the promoters of several dehydration-inducible genes which do not require protein synthesis for induction have revealed that they contain a cis-acting ABA responsive element (ABRE; PyACGTGGC). A large family of bZIP proteins (contain a conserved basic domain followed by a minimum of three leucine residues located at intervals of seven amino acids), exhibit a relaxed DNA binding specificity for sequences containing the ACGT core, although none has yet been shown unambiguously to mediate induction by ABA in vivo. Coupling DNA elements, located upstream or downstream from the ABRE, are required in conjunction with an ABRE to generate an ABA-responsive complex (Shen and Ho, 1997). Analysis of the 5'-untranslated regions (5'-UTRs) of the AtP5CS1 (Genbank Accession No. AC003000), AtP5CS2 (Zhang et al., 1997) and AtP5CR (Verbruggen et al., 1993) genes indicated that a perfect match with the consensus ABRE sequence is found only in the 5'-UTR of AtP5CS2 at a position approximately 805 nucleotides upstream of the second (more downstream) transcription initiation site (Zhang et al., 1997). Two sequences which contain the CCACC core of the ABA coupling element CE1 (TGCCACCGG; Shen and Ho, 1997) are found within 70 nucleotides upstream of this putative ABRE (PD Hare, unpublished results). The apparent inability of ABA to activate transcription of AtP5CS2 (Zhang et al., 1997) is not consistent with a functional role for this putative ABA-responsive complex.

Besides genes containing ABREs, a second class of ABA-dependent genes, best exemplified by RD22, requires the synthesis of protein factors before osmotically-induced gene expression (Shinozaki and Yamaguchi-Shinozaki, 1997). Dehydration- and ABA-inducible MYC and MYB homologues have been shown to bind and activate the RD22 promoter, which lacks ABREs (Abe et al., 1997). Cold-, dehydration- and ABA-responsive bZIP transcription factors (Kusano et al., 1995; Nakagawa et al., 1996), have also been proposed to act in this ABA-dependent pathway which requires protein synthesis upstream of ABA-mediated gene induction (Shinozaki and Yamaguchi-Shinozaki, 1997). A role for ABA in stress-induced P5CS gene expression, as well as the requirement for protein synthesis in full induction of this response, implicates the involvement of Pathway I defined by Shinozaki and Yamaguchi-Shinozaki (1997) in regulating P5CS expression.

Most strikingly, there are several perfect matches between consensus MYB recognition sequences (Iturriaga et al., 1996; Abe et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Uimari and Strommer, 1997) and sequences in the 5'-UTRs of AtP5CS1, AtP5CS2 and AtP5CR (PD Hare, unpublished results). It seems likely that the stress-inducible AtMYB2 protein from Arabidopsis (Abe et al., 1997) may regulate expression of AtP5CS1, since NaCl-mediated induction of both genes is more extensive in sos1 (Liu and Zhu, 1997a) and sos2 (Zhu et al., 1998) than in WT Arabidopsis seedlings. It is tempting to speculate that there may be special significance in the use of MYB-type transcription factors in the regulation of the expression of genes responsible for proline synthesis. As has been found for mammalian MYB-class proteins, plant MYB-type transcription factors appear to be subject to redox regulation and will only bind to specific DNA sequences when in the reduced state (Martin and Paz-Ares, 1997; Williams and Grotewold, 1997). Given the apparent importance of proline synthesis in the modulation of cellular redox potential during stress (Hare and Cress, 1997; Hare et al., 1998), placing the synthesis of proline directly under the control of redox potential would seem a likely mechanism by which proline biosynthetic capacity might be auto-regulated. In Arabidopsis, AtMYB2 co-operates with RD22BP1, a dehydration- and ABA-inducible basic helix-loop-helix leucine zipper (bHLH-ZIP) MYC-related protein, in transcriptional activation of RD22 (Abe et al., 1997).
Investigation of whether AtP5CS2, KIN2 RD29A V AtP5CS1 eskimo1 Arabidopsis AtP5CS2 ACCGACA A is found 320 nucle-

-UTRs of genes which contain DREs in their 5’-UTRs of Arabidopsis (Shinozaki and Yamaguchi-Shinozaki, 1997). The CACATG motif recognized by RD22BP1 is DRE-responsive gene products which bind to the ABRE,

cold-induced proline accumulation and Stress- and ABA-related signal transduction COR15a

near the termini of ABA-dependent and -independent (Table 1) to indicate that proline biosynthesis does not

the existence of which cannot be reconciled with the view (Jaglo-Ottosen et al., 1997). However, many of the mutations enhance or reduce the response to more than one of the three stimuli. These must define shared components in the cold-, NaCl- and ABA-regulated signalling pathways, the existence of which cannot be reconciled with the view that osmotic and cold signalling invoke parallel ABA-dependent and ABA-independent pathways. Thus, mutational analysis suggests that rather than acting in parallel by operating separately on discrete DNA sequence motifs, the DRE consensus DRE motif occur in any of the 5’-UTRs or AtP5CS1, AtP5CS2 or AtP5CR (PD Hare, unpublished results). The sequence AACCAGACAA is found 320 nucleo-

tides upstream of the second, downstream transcription initiation site of AtP5CS2. The recent characterization of a constitutively freezing-tolerant Arabidopsis mutant named eskimo1 (esk1; Xin and Browse, 1998) seems consistent with a lack of involvement of a pathway which targets the DRE in mediating the induction of AtP5CS1 by dehydrative stresses. Mutation of the ESK1 locus increases constitutive AtP5CS1 transcript levels approximately 8-fold, but has no effect on the expression of RD29A, KIN2 or two other cold-regulated genes (COR47 and COR15a), which are upregulated in CBF1-over-

expressing lines (Jaglo-Ottosen et al., 1998).

Stress- and ABA-related signal transduction

The identification of stress-responsive promoter elements has opened the way for characterization of the events near the termini of ABA-dependent and -independent stress-induced signalling pathways. Several stress-responsive gene products which bind to the ABRE, DRE/C-repeat and MYB or MYC target sequences have been identified (Abe et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Jaglo-Ottosen et al., 1998). As will be discussed below, recent studies have indicated the power of single cell analyses in deciphering the events closer to the site(s) of stress perception. Since the mechanisms whereby plants translate physical parameters such as temperature extremes, water availability or ionic strength into physiological responses are largely unknown, genetic approaches are likely to be particularly valuable in defining the primary biochemical events in stress-related signalling.

Ishitani et al. (1997) recently reported the use of misexpression of a luciferase (LUC) transgene under the control of the RD29A gene promoter to identify 103 Arabidopsis mutants with aberrant transcriptional responses to low temperature, ABA and NaCl stress. The RD29A promoter contains the DRE/C-repeat and ABRE motifs. Examination of the separate effects of low temper-

ature, osmotic stress and ABA on RD29A expression in these cos, los or hos (constitutive, low or high expression of osmotically responsive genes, respectively) mutants indicated that certain of the mutations only affect RDA29:::LUC expression in response to low temperature or NaCl. This conforms with the view that ABA-independent signal transduction pathways act in parallel to ABA-dependent cascades (Shinozaki and Yamaguchi-Shinozaki, 1997). However, many of the mutations enhance or reduce the response to more than one of the three stimuli. These must define shared components in the cold-, NaCl- and ABA-regulated signalling pathways, the existence of which cannot be reconciled with the view that osmotic and cold signalling invoke parallel ABA-dependent and ABA-independent pathways. Thus, mutational analysis suggests that rather than acting in parallel by operating separately on discrete DNA sequence motifs, ABA-dependent and ABA-independent pathways interact extensively before converging on the promoters of stress-related genes.

Characterization of proline synthesis in the range of cos, hos and los mutants is warranted, since like RD29A, AtP5CS1 is subject to control by both ABA-dependent and ABA-independent mechanisms. The demonstration that non-acclimated esk1 plants have an approximately 35-fold higher level of free proline and an 8-fold higher level of AtP5CSI transcript than non-acclimated WT plants (Xin and Browse, 1998) implicates a role for proline biosynthesis in mediating cold tolerance. Previously, Savouré had interpreted their finding that cold-induced proline accumulation and AtP5CSI mRNA levels in an ABA-deficient mutant incapable of cold acclimation are comparable to the levels in WT plants (Table 1) to indicate that proline biosynthesis does not play an important role in the tolerance of low temperatures. ESK1 is believed to be a repressor of AtP5CSI.
expression. Like \( \textit{AtP5CS1}, \textit{RAB18} \) is constitutively expressed at a higher level in \( \textit{esk1} \) than in the WT, although mutation of \( \textit{ESK1} \) did not cause constitutive expression of either \( \textit{RD29A} \) or \( \textit{KIN2} \) (Xin and Browse, 1998). These workers have extended the proposal by Ishitani et al. (1997) that is not appropriate to consider the events that result in cold acclimation as a simple linear pathway such as suggested by Shinozaki and Yamaguchi-Shinozaki (1997). Despite the value of this model in an heuristic sense, it does not incorporate ABA-dependent gene expression in response to low temperature. Based on their observation that \( \textit{esk1} \) does not affect genes such as \( \textit{RD29A} \) and \( \textit{KIN2} \) (which have been implicated in all previous models of both ABA-dependent and ABA-independent signalling), and their observations that other freezing-tolerant mutants are affected in neither proline biosynthetic capacity nor \( \textit{RD29A} \) or \( \textit{KIN2} \) expression, Xin and Browse (1998) have proposed that at least four separate and possibly overlapping pathways participate in cold acclimation.

In view of the evident complexity in the network of signalling chains that control genetic responses to stress, further investigation of the events that regulate proline accumulation may shed some light on how these chains are intertwined at the molecular level to co-ordinate a single response to stimuli such as a change in endogenous ABA concentration or cell turgor. As will be elaborated below, an important role for Ca\(^{2+}\) is now well established for ABA-dependent gene induction. Stretch-activated Ca\(^{2+}\) channels have also been identified (Cowan et al., 1997). Tightly regulated changes in cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_c\)) have been identified as important components of signalling pathways induced in response to the imposition of several environmental stresses associated with proline accumulation (Hare et al., 1997). For instance, characterization of the \( \textit{sos3} \) mutant of \textit{Arabidopsis} has confirmed the long-postulated importance of Ca\(^{2+}\) in tolerance of NaCl, through its effects on K\(^+\)/Na\(^+\) selectivity (Liu and Zhu, 1997b). The \( \textit{SOS3} \) gene product shares significant sequence similarity with a subunit from yeast calcineurin, which is Ca\(^{2+}\)/calmodulin (CaM)-dependent phosphatase (Liu and Zhu, 1998). Pardo et al. (1998) recently confirmed the importance of intracellular Ca\(^{2+}\) in affecting plant salinity tolerance through their demonstration that overexpression of constitutively activated yeast calcineurin in tobacco plants substantially increased growth under saline conditions. The ability of Ca\(^{2+}\) to completely suppress the growth defect of \( \textit{sos3} \) plants on media that contains low K\(^+\) to ameliorate the hypersensitive phenotype of \( \textit{sos3} \) plants grown in the presence of 50 mM NaCl (Liu and Zhu, 1997b) is not observed for \( \textit{sos1} \) or \( \textit{sos2} \) (Zhu et al., 1998). Double mutant analysis indicates that \( \textit{sos1} \) is probably epistatic to \( \textit{sos3} \) (Liu and Zhu, 1997b) and that \( \textit{SOS1} \) and \( \textit{SOS2} \) participate in the same pathway (Xhu et al., 1998). Like \( \textit{sos1} \) (Liu and Zhu, 1997a), \( \textit{sos2} \) is characterized by a higher level of \( \textit{AtP5CS1} \) expression than the WT, although constitutive levels of \( \textit{AtP5CS1} \) transcript are lower in \( \textit{sos2} \) than in \( \textit{sos1} \) (Zhu et al., 1998). It will be interesting to discover whether \( \textit{sos3} \) has an elevated capacity for proline biosynthesis.

Increased exogenous Ca\(^{2+}\) not only increased the K\(^+\)/Na\(^+\) ratio, but also enhanced proline content in alfalfa callus cultures (Shah et al., 1990). However, since extracellular Ca\(^{2+}\) exists at millimolar concentrations, and cells must thus constantly remove Ca\(^{2+}\) to keep the [Ca\(^{2+}\)]\(_c\) at levels where it can function effectively in signal transduction, such studies are difficult to interpret in the context of a role for Ca\(^{2+}\) in mediating the molecular events associated with proline accumulation and other stress responses. With the advent of more sophisticated approaches, substantial progress has recently been made in identifying some of the biochemical changes that are involved in the induction of \( \textit{AtP5CS1}, \textit{RD29A}, \textit{RAB18} \), and \( \textit{KIN2} \) genes. These data, which are summarized in Table 2, provide a sketchy framework on which to base further investigations.

Knight et al. (1997) used intact transgenic \textit{Arabidopsis} seedlings that express the soluble Ca\(^{2+}\)-sensing luminescent protein aequorin in the cytosol to demonstrate that a transient increase in [Ca\(^{2+}\)]\(_c\) upon mannitol or NaCl treatments can be substantially inhibited by pre-treatment with the Ca\(^{2+}\)-channel blocker lanthanum (La\(^{3+}\)) and to a lesser extent, the Ca\(^{2+}\)-chelator EGTA (Knight et al., 1997). This increase in [Ca\(^{2+}\)]\(_c\) participates in NaCl- and dehydration-mediated upregulation of proline biosynthesis, since the induction of \( \textit{AtP5CS1} \) transcript by either stressor can be inhibited by La\(^{3+}\) (Knight et al., 1997). Mannitol-, but not NaCl-induced \( \textit{RD29A} \) and \( \textit{RAB18} \) transcript accumulation was also inhibited by La\(^{3+}\) (Table 2). Induction of expression of \( \textit{AtP5CS1} \) by mannitol was also inhibited by EGTA and the Ca\(^{2+}\)-channel blockers gadolinium (Gd\(^{3+}\)) and verapamil (Knight et al., 1997).

The observation that Ca\(^{2+}\) transients were of a similar magnitude and duration in response to iso-osmolar concentrations of mannitol and NaCl, yet mannitol induced a greatly increased abundance of \( \textit{AtP5CS1} \) transcript than an iso-osmolar concentration of NaCl, led Knight and co-workers to conclude that a factor(s) other than [Ca\(^{2+}\)]\(_c\) participates in the discrimination between dehydration and salinity signals in \textit{Arabidopsis}. Furthermore, a substantial transient in [Ca\(^{2+}\)]\(_c\) obtained by adding external Ca\(^{2+}\) was not adequate for full induction of \( \textit{AtP5CS1} \) transcript accumulation, thus indicating that additional signalling factor(s) are required for the response. Although the demonstration that both La\(^{3+}\) and EGTA inhibit the NaCl- and mannitol-induced elevations in [Ca\(^{2+}\)]\(_c\) in \textit{Arabidopsis} seedlings indicates the involvement of extracellular Ca\(^{2+}\) in this response, the observation
that neither of these inhibitors abolishes the response totally led these workers to investigate the additional involvement of intracellular Ca\(^{2+}\) stores.

In animal systems, many extracellular stimuli are known to activate phosphoinositide-specific phospholipase C (PLC), which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate to generate two second messengers, inositol 1,4,5-trisphosphate (IP\(_3\)) and 1,2-diacylglycerol (DAG). While DAG activates protein kinase C (PKC), IP\(_3\) activates receptors coupled to Ca\(^{2+}\) channels to induce the release of Ca\(^{2+}\) from intracellular stores. The Ca\(^{2+}\), in turn, activates the enzymes responsible for the ensuing cellular response. Since previous studies had indicated that osmotic stress enhances the competence of IP\(_3\)-sensitive vacuolar Ca\(^{2+}\) channels to respond to IP\(_3\), Knight et al. (1997) assessed the change in [Ca\(^{2+}\)]\(_c\) in the microdomain adjacent to the tonoplast in Arabidopsis plantlets exposed to mannitol. In contrast to what had previously been found for low temperature treatment, where the peak height of the Ca\(^{2+}\) response at the cytosolic face of the vacuolar membrane was c. 70% of that observed in the cytosol, the [Ca\(^{2+}\)]\(_c\) in the vacuolar microdomain was equal to or higher than that observed in the cytosol. It also exhibited a faster rate of increase and was of longer duration (Knight et al., 1997). Despite these differences in cold- and dehydration-induced Ca\(^{2+}\) transients, it is interesting to note that previous researchers, using the same approach as Knight and co-workers, had demonstrated that increases in [Ca\(^{2+}\)]\(_c\) in cold-shocked Arabidopsis seedlings can be blocked by La\(^{3+}\) and Gd\(^{3+}\) as well as by an extracellular Ca\(^{2+}\) chelator (Polisensky and Braam, 1996).

Use of inhibitors of PLC and myo-inositol-1-phosphatase suggested that a significant release of vacuolar Ca\(^{2+}\) following dehydration of Arabidopsis seedlings occurred through IP\(_3\)-dependent Ca\(^{2+}\) channels (Knight et al., 1997). Nonetheless, a direct role for the activation of a phosphoinositide-sensitive vacuolar Ca\(^{2+}\) channel in contributing to the induction of AtIP5CS1 gene expression by mannitol and salt treatments was not investigated (Knight et al., 1997). It would be interesting to know whether or not the effects of inhibition of phosphoinositide signalling can be reversed by Ca\(^{2+}\) or intermediates of the phosphatidylinositol cycle as well as whether any effects on stress-inducible gene expression might be mimicked or suppressed by protein kinase and/or phosphatase inhibitors. Whereas NaCl-stressed sos1 seedlings displayed AtIP5CS1 expression relative to comparable WT plantlets, accumulation of transcript encoding an AtPLC1S, a PLC which is responsive to ABA, dehydration, salt and cold stresses (Hirayama et al., 1995), was not affected in sos1 (Liu and Zhu, 1997a). However, a sos2 mutant which has elevated AtIP5CS1 mRNA levels relative to the WT, although not as high as those found in sos1, is characterized by higher levels of AtPLC1S expression than comparable salt-stressed WT plants (Zhu et al., 1998). The expression of RD29A is unaffected in sos1 and sos2 (Table 2; Liu and Zhu, 1997a, Zhu et al., 1998).
involvement of phosphoinositide signalling in the regulation of AtP5CS1 expression thus requires further investigation, although transcriptional activation of the gene encoding a stress-inducible PLC does not appear to be required for AtP5CS1 expression. A role for PKC in plant stress tolerance is suggested by the demonstration that H7, a preferential PKC inhibitor, slightly reduces freezing tolerance in Arabidopsis and inhibits KIN1 and KIN2 transcript accumulation in cold-treated plants (Tähtiharju et al., 1997).

A further significant advance in characterizing the biochemical events which trigger the induction of stress-responsive genes has arisen through single-cell microinjection experiments using tomato hypocotyls (Wu et al., 1997). Separate co-injection of either ABA or Ca²⁺ together with fusions of the GUS gene to the promoters of RD29A and KIN2, activated expression of both reporter genes. The ABA-mediated induction of both genes was blocked by EGTA. This is consistent with the demonstration that inclusion of Ca²⁺-containing medium can induce the expression of an ABA-responsive gene in maize protoplasts (Sheen, 1996). Thus, in contrast to the apparent situation for AtP5CS1, at least part of the cellular apparatus responsible for RD29A or KIN2 expression appears to be constitutively primed to respond to increases in a universal signalling intermediate with minimal intrinsic informational specificity (Table 2).

Based on a previous demonstration that Ca²⁺ release from plant vacuoles may be triggered by cyclic ADP-ribose (cADPR), Wu and co-workers further demonstrated that cADPR can substitute for ABA or Ca²⁺ in stimulating RD29A and KIN2 expression. Prevention of cADPR-mediated induction of both genes not only by EGTA, but also by an analogue of cADPR and a specific inhibitor of cADPR activity, strongly implicate cADPR as an intermediate in ABA signal transduction which elicits its effects via intracellular Ca²⁺ release. Furthermore, a bioassay indicated that amounts of cADPR in Arabidopsis plants increased in response to ABA treatment and before ABA-induced gene expression (Wu et al., 1997). Microinjection of ADP-ribose cyclase activated expression of both reporter genes in the absence of ABA. This enzyme not only synthesizes cADPR from NAD⁺, but also catalyses the synthesis of nicotinic acid adenine dinucleotide (NAAD⁺) from NAD⁺ (Graef et al., 1998). The effectiveness of only nanomolar concentrations of NAAD⁺ in activating Ca²⁺-release in animal cells is well documented. Since cADPR and NAAD⁺ mobilize intracellular Ca²⁺ stores by totally independent mechanisms, and these are pharmacologically distinct from those activated by IP3 (Graef et al., 1998), differential regulation of ADP-ribose cyclase potentially holds an important clue concerning how cells may differentiate between different Ca²⁺-mobilising signals.

Importantly, the experiments of Wu and co-workers have demonstrated that ABA-induced RD29A and KIN2 expression is cell autonomous and the primary stimulus does not necessarily need to act downstream of Ca²⁺ release in order to induce expression of RD29A and KIN2. Nonetheless, in view of the findings of Ishitani et al. (1997) concerning the regulation of RD29A by ABA, as well as by cold and salt stresses, there may be considerable modification of the signal upstream of cADPR-mediated Ca²⁺ release. Regarding the findings of Knight et al. (1997), it is noteworthy that IP3 also induced expression of RD29A::GUS and KIN2::GUS, and that this could be blocked by heparin, a competitive antagonist of the IP3 receptor (Wu et al., 1997). However, since heparin had no effect on cADPR- or ABA-activated gene expression, and AtPLC1S transcript accumulates in response to treatment with ABA (Hirayama et al., 1995), it was concluded that IP3 may be involved in a secondary, rather than a primary response involved in transcriptional activation of both genes (Wu et al., 1997). The effects of agonists and antagonists of ryanodine receptors, the putative targets of cADPR action, were not reported by Wu et al. (1997). However, others have independently shown that such an inhibitor, ruthenium red, partially inhibits KIN2 transcript accumulation and freezing tolerance in cold-stressed Arabidopsis, albeit not as effectively as La³⁺ or Gd³⁺ (Tähtiharju et al., 1997).

Besides changes in [Ca²⁺], protein kinase and phosphatase actions have been implicated in ABA signal transduction, although the relationship of kinase/phosphatase balance to [Ca²⁺] is not yet well characterized. Plants possess at least four distinct types of serine/threonine-specific protein phosphatases, which can be distinguished on the basis of their substrate specificity, metal requirements and inhibitor sensitivity. Further micro-injection experiments using inhibitors of protein kinases (staurosporine and K252a) and phosphatases (okadaic acid; OKA) suggested that an OKA-sensitive phosphatase(s) may act upstream of cADPR, Ca²⁺ release and protein kinase action in the induction of RD29A and KIN2 expression (Table 2). Induction of ABA-responsive gene expression appears to be positively regulated by protein kinases and negatively regulated by protein phosphatases. Accordingly, Sheen (1996) has demonstrated that, in maize leaf protoplasts, the ABA-responsive barley HVA1 promoter can be activated in the absence of ABA by overexpressing the catalytic domain of certain Ca²⁺-dependent protein kinases (CDPKs). Five CDPKs are differentially upregulated by cold stress in Arabidopsis and W7, an inhibitor of CDPKs and CaM, prevented cold acclimation as well as the induction of KIN1 and KIN2 expression (Tähtiharju et al., 1997). The WT ABI1, and two mutant abi1 proteins all significantly blocked CDPK-mediated HVA1 expression (Sheen, 1998). The action of ABI1 and ABI1 proteins downstream of the
positively-acting CDPK action in ABA signal transmission is also consistent with the insensitivity of ABI1 and ABI2 to OKA (Leung et al., 1997). Despite several different approaches, no evidence has been obtained for a direct role for Ca\(^{2+}\) in regulating ABI1 activity (Sheen, 1998). Recent findings implicate the CHX-independent activation of phospholipase D (PLD) in ABA signalling in barley aleurone cells (Ritchie and Gilroy, 1998). Application of phosphatidic acid (PPA), the product of PLD-mediated hydrolysis of phospholipids, simulates ABA-activated processes and these can be overcome by inhibition of PLD activity (Ritchie and Gilroy, 1998). The involvement of PLD activity in stress-regulated gene expression remains to be investigated. Regarding the findings of Knight et al. (1997), it is noteworthy that DAG and PPA are readily interconvertible (Ritchie and Gilroy, 1998). These workers presented evidence that it is unlikely that a classical DAG/IP\(_3\) signalling pathway operates in the ABA response of barley aleurone protoplasts.

The findings of Knight et al. (1997) and Wu et al. (1997) clearly provide a valuable basis for further detailed characterization of second messengers involved in stress-related signalling in plants. Nonetheless, it is worth mentioning that the response of [Ca\(^{2+}\)]\(_c\) to osmotic stress does not appear to be uniform and may vary with species, cell type, tissue or developmental stage. Using ratiometric fluorescent imaging, Cramer and Jones (1996) observed a rapid reduction of [Ca\(^{2+}\)]\(_c\) in cells from the meristematic region of Arabidopsis roots exposed to NaCl. Iso-osmotic concentrations of NaNO\(_3\), KCl and sorbitol reduced [Ca\(^{2+}\)]\(_c\) to the same extent, thus indicating that this was primarily an osmotic effect. A concentration effect was noted for ABA treatments, although [Ca\(^{2+}\)]\(_c\) always ultimately declined in response to ABA. Use of an abil mutant indicated that the reductions in [Ca\(^{2+}\)]\(_c\), elicited by osmotic stress in root cells were unlikely to be mediated by changes in endogenous ABA (Cramer and Jones, 1996). However, ABA-mediated PPA release apparently reduces [Ca\(^{2+}\)]\(_c\) in gibberellin-treated barley aleurone protoplasts (Ritchie and Gilroy, 1998). It appears that of the many different Ca\(^{2+}\)-regulated pathways that occur in a single cell, some may override others depending on the cell type or developmental stage.

Cytokinins and auxins

Although cytokinins (CKs) are generally considered to be antagonists of ABA, in contrast to ABA, there is a poor understanding of the precise role of CKs in plant stress responses (Hare et al., 1997). Even less well characterized is the role that auxins may play in adaptation to environmental stresses. Conflicting evidence regarding a role for exogenously applied CK in inducing proline accumulation has been reviewed (Hare et al., 1997). A recent report (Peters et al., 1997) may contribute to resolution of the seemingly paradoxical effects of enhanced CK levels on stress response pathways. In the leaves of Mesembryanthemum crystallinum, CK-induced increases in free proline as well as the abundance of transcript encoding PEPCase and the activity of this enzyme are differentially affected depending on whether BA is applied to root or shoot tissues (Peters et al., 1997). It is pertinent to note that at the concentrations used, BA applied to the roots decreased leaf water content, while application to the shoots had no effect on this parameter. Thus, as has already been suggested above for the effects of the abil mutation, a component of the higher induction of proline accumulation in leaves from plants treated with CK at the roots may arise as a secondary effect related to a loss of cell turgor.

In Arabidopsis, CK does not affect the accumulation of AtP5CS1 mRNA in roots, but caused some reduction of AtP5CS1 transcript levels in leaves at least after 6 h treatment (Strizhov et al., 1997). Levels had apparently recovered to those found in untreated leaves within 24 h. In contrast, BA caused a significant induction of AtP5CS2 mRNA transcript in leaves, but not in roots (Strizhov et al., 1997). Transfer of cultured cells to CK-supplemented medium after they had been washed in hormone-free medium, had no effect on levels of either transcript encoding P5CS when monitored throughout a 48 h period (Strizhov et al., 1997).

Auxin (2,4-D) caused a slight (approximately 3-fold) induction of AtP5CS1 mRNA accumulation in leaves and roots of 28-d-old Arabidopsis seedlings. Application of 2,4-D strongly stimulated AtP5CS2 mRNA accumulation in leaf tissue, but had no effect on, or caused even slight suppression of, the abundance of this transcript in roots (Strizhov et al., 1997). The Arabidopsis axr2 mutant accumulates lower levels of both AtP5CS1 and AtP5CS2 transcripts after a 6 h exposure to NaCl (Strizhov et al., 1997). Interpretation of this effect is complicated by the likelihood that, like abil, this is a neomorphic (gain-of-function) mutation. Thus, AXR2 may not normally function in hormone action (Estelle and Klee, 1994). Moreover, axr2 is resistant not only to auxin, but also to ethylene and ABA (Estelle and Klee, 1994). In contrast to abil (Strizhov et al., 1997; but see Savoure et al., 1997) for a different conclusion regarding AtP5CS1), the axr2 mutation does not affect the basic level of both AtP5CS1 and AtP5CS2 transcripts in the absence of stress (Strizhov et al., 1997). It was suggested that axr2 and abil may disrupt a common signalling pathway downstream of ABA perception, which is not affected by abil2 or abil3 mutations. The aux1 mutant, which in all likelihood is deficient in an auxin transporter, is resistant to auxin, ethylene and CK but displays normal sensitivity to ABA in a root elongation assay (Estelle and Klee, 1994). After salt stress, aux1 accumulated AtP5CS1 and
AtP5CS2 transcripts to levels similar to those observed in WT Arabidopsis (Strizhov et al., 1997).

**Signal transduction events upstream of PDH gene induction**

Following prolonged dehydration or salt stress, expression of the proline biosynthetic genes is activated, expression of the gene encoding PDH is suppressed (Kiyosue et al., 1996; Peng et al., 1996; Verbruggen et al., 1996). Upon rehydration after desiccation (Kiyosue et al., 1996) or PEG-mediated dehydration (Verbruggen et al., 1996), as well as after relief from salinization (Peng et al., 1996), AtP5CS1 transcript levels decline and expression of the AtPDH gene is rapidly induced to high levels. Although P5CDH has yet to be characterized at the gene level, and the relative importance of P5CR gene induction is questionable (Yoshiba et al., 1995; Hare and Cress, 1997), P5CS and PDH appear to catalyse the rate-limiting steps in proline synthesis and degradation, respectively. The reciprocal regulation of the genes encoding these enzymes introduces the possibility that their relative levels may be co-ordinated by the same signalling cascade. Recent studies of phytochrome A signal transduction suggest that the same pathway, involving both Ca²⁺ and cGMP, both activates a gene encoding ferredoxin NADP⁺ oxidoreductase and mediates repression of an asparagine synthetase gene in the light (Mustilli and Bowler, 1997). Furthermore, specific PP2C activity apparently negatively regulates a single ABA signalling pathway that controls both gene activation and repression (Sheen, 1998). Although abiotic stresses are known to cause both an increased and reduced abundance of discrete gene products, repression of gene expression by adverse conditions is seldom investigated. Investigation of the regulation of PDH expression may not only provide insight into the mechanisms of stress-mediated gene repression, but may also shed light on the interesting question of whether plants possess rehydration-specific signalling pathways, or whether physiological responses that follow relief from stress simply involve derepression of genes which are downregulated or silenced under stress. The possible involvement of rehydration-related signalling pathways is not indicated in the graphical representation of the current knowledge of the factors which control proline synthesis and degradation provided in Fig. 1.

In view of the downregulation of PDH activity following prolonged stress, it was surprising that Kiyosue et al. (1996) isolated a PDH cDNA clone (ERD5; early responsive to dehydration) by differential screening of a library prepared from Arabidopsis plants which had been dehydrated for 1 h. Nonetheless, Northern analysis confirmed that the abundance of ERD5 transcript (henceforth referred to as AtPDH) increased transiently 1 h after desiccation, before decreasing to non-detectable levels within 10 h after the commencement of the stress (Fig. 1). Although AtPDH levels are rapidly induced by exogenously applied proline (Kiyosue et al., 1996; Peng et al., 1996; Verbruggen et al., 1996), the transient induction of AtPDH mRNA accumulation precedes detectable proline accumulation by at least 4 h and occurs simultaneously with the accumulation of AtP5CS1 mRNA (Kiyosue et al., 1996). A transient increase in AtPDH transcript levels was also observed within 2 h after exposure to 4°C, and within 1 h of incubation at 40°C, with levels continuing to increase for at least a further hour in the case of heat treatment (Kiyosue et al., 1996). Neither of these temperature extremes were as effective as dehydration in inducing AtPDH expression. Interestingly, the same anomalous induction of AtPDH expression was also observed in both WT and esk1 Arabidopsis plants after incubation at 4°C for 2 d (Xin and Browse, 1998). These findings, together with the rapid induction of AtP5CS transcripts discussed above, might be interpreted to indicate an important role for cycling between proline and its precursors in ensuring metabolic homeostasis under moderate environmental fluctuations or as an early response to severe stress (Hare et al., 1998). This may be accomplished through associated effects on adenylate charge as well as the level of reduction of pyridine nucleotides, particularly NADP⁺.

Strong induction of AtPDH expression upon rehydration after desiccation occurs within 2 h and reaches a maximum level between 24 h and 48 h after rehydration. Within 10 h after rehydration, free proline levels declined to approximately one-fifth of the level accumulated after dehydration, and continued to decline until 48 h after rehydration, when AtPDH transcript abundance is still more than 10-fold higher than at the end of the dehydration stress (Kiyosue et al., 1996). It is tempting to speculate that induction of AtPDH transcript accumulation upon relief from dehydration is mediated by a rehydration-related signal transduction pathway. When incubated in liquid medium supplemented with 10 mM proline, axenically-grown Arabidopsis plants accumulate substantial amounts of the imino acid within 12 h (Verbruggen et al., 1996). However, induction of AtPDH mRNA levels apparently saturates at levels in excess of 25 mM exogenous proline (Kiyosue et al., 1996), which are unlikely to occur in vivo under stress conditions. Furthermore, induction of AtPDH abundance by t-proline may be transient: levels decreased after 10 h exposure to 260 mM proline (Kiyosue et al., 1996). Nonetheless, this decline was not observed when AtPDH accumulation was induced by n-proline, possibly because this isomer is not metabolized. Irrespective of whether or not AtPDH expression upon rehydration is primarily induced by free proline after suppression by dehydration has been relieved, the demonstration that repression of AtPDH expression by salt stress overrides induction by exogenous...
proline (Peng et al., 1996) indicates some interaction between the signalling events downstream of stress perception and assessment of free proline levels. The recent characterization of esk1, which has elevated levels of AtP5CS1 expression (Xin and Browse, 1998), substantiates this proposal. The observation that mutation of ESK1 causes a 35-fold higher level of free proline without any effects on AtPDH transcript levels prompted investigation of whether the esk1 mutation acts to prevent the induction of AtPDH by proline. Ten hours after watering WT plants with a 100 mM proline solution, proline levels were about one-third of the constitutive level found in esk1 and AtPDH transcript levels were significantly increased. In contrast, the AtPDH level in esk1 was only slightly increased (Xin and Browse, 1998). Thus, further characterization of esk1 may open the way for dissecting the reciprocal control of proline synthesis and degradation under sustained stress. Exogenous proline does not seem to affect the expression of the AtP5CR (Verbruggen et al., 1993) and AtP5CS2 (Zhang et al., 1997) genes, although the effects of proline on AtP5CS1 expression do not appear to have been investigated.

Hormonal regulation of AtPDH expression is presently not well characterized. A 10 h treatment with ABA, BA and 2,4-D had no apparent effects on AtPDH transcript levels in 28-d-old Arabidopsis plantlets incubated for 24 h or 72 h in 200 mM NaCl, although the effects of nitrogen status on OAT expression were not investigated. The relative positions of ABI1 and AXR2 cannot be defined with certainty. Since the abi1 and axr2 mutations, which abrogate P5CS gene expression, are neomorphic mutations, ABI1 and AXR2 may not necessarily normally participate in the regulation of proline synthesis in Arabidopsis. Glutamate; Orn, ornithine; Pro, proline; PTR, post-transcriptional regulatory event that does not affect transcript abundance; VP, verapamil; →, positive action; ↔, negative action; ?, uncertain.
Signal cross-talk

A primary limitation to facile elucidation of the processes that regulate free proline levels under both optimal and stressful conditions is the realization that the multitude of signals which regulate plant growth and development are not transduced via linear pathways operating in parallel. Instead, plant responses to environmental stimuli are integrated with endogenous developmental programmes by a complex network which is characterized by extensive ramification and redundancy. Since the various inputs into this network modulate each other, both positively and negatively, the overall context of this web of interacting components, and no single factor in isolation, is likely to regulate any of the physiological changes that accompany the imposition of hyperosmotic stress. Determining how the multitude of individual chains downstream of the stimuli that regulate proline synthesis and degradation are intertwined is thus an important consideration in interpreting the effects of hyperosmotic stress on these processes.

Investigation of the factors which regulate the expression of genes involved in proline synthesis and degradation is complicated by the fact that their products have important housekeeping functions in the absence of stress. In contrast, many well characterized stress-inducible genes, including RD29A and RAB18 (Table 1) appear to be induced de novo under adverse conditions. The roles of proline synthetic and degradative enzymes in stress-related responses must be superimposed upon the plant’s continuous requirements for protein synthesis during growth. For instance, it may be of interest to note that the AtP5CS2 promoter contains two transcription start sites. Dehydration stimulates transcription predominantly from the downstream site (Zhang et al., 1997).

Considerable circumstantial evidence supports an important role for proline synthesis in regulating several physiological responses, including developmental transitions, even in the absence of stress (Hare and Cress, 1997). Considerable flexibility in the signalling events which effect the induction of genes involved in proline biosynthesis is anticipated. Indeed, informational and functional redundancy, superimposed upon subtle modulation by developmentally regulated processes, may account for much of the contradictory evidence outlined above regarding the involvement of ABA levels and ABI1 in mediating AtP5CS1 expression (Savoure´ et al., 1997; Strizhov et al., 1997), as outlined above. Such redundancy is also consistent not only with the existence of plant isoforms of P5CS, but also with the increasingly accepted view that the accumulation of organic solutes such as proline may fulfill multiple roles in facilitating acclimation to stress (Hare et al., 1998).

Since light quality and quantity are perhaps the most significant extrinsic cues which affect plant growth and development, establishing the most important sites of cross-talk between phytochrome and stress-related pathways is likely to be of prime importance in dissecting the intricate network of interactions that regulate plant responses to adverse conditions. The importance of light in mediating proline accumulation is well documented and recent studies implicate the involvement of phytochrome (Hare et al., 1997). Exposure of light-adapted Arabidopsis plants to darkness caused an approximately 50% reduction in AtP5CS1 mRNA levels, but did not affect AtP5CS2 mRNA abundance (Strizhov et al., 1997). Phytochrome effects are also inextricably intertwined with the action of hormones. For example, light differentially affects the absolute levels of both ABA and CKs, while CK downregulates PHYA (phytochrome A) gene expression (Thomas et al., 1997).

The involvement of [Ca\(^{2+}\)], and protein phosphorylation in the regulation of almost all cellular signal transduction pathways makes them obvious candidates for mediating interacting pathways between various stimuli. Nonetheless, there is currently a poor understanding of the mechanisms of how the various ubiquitous components identified using pharmacological approaches interact in the context of a signalling pathway comprising macro-molecular intermediates. Microinjection technology offers a powerful approach to dissect the individual components of separate pathways. For example, in contrast to what was found using RD29A and KIN2 promoters, expression of a fusion of chlorophyll a/b binding protein promoter to GUS (CAB::GUS) is induced by Ca\(^{2+}\), but not by cADPR (Wu et al., 1997). Thus, although phytochrome can induce [Ca\(^{2+}\)]\(_e\) transients, stress-induced cADPR apparently elicits a unique cytoplasmic Ca\(^{2+}\) wave that results in activation of at least a subset of ABA-responsive genes, but not certain PHYA-responsive genes. Furthermore, the inability of IP\(_3\) to activate CAB::GUS suggests that it too does not participate in the Ca\(^{2+}\)/CaM-dependent branch of PHYA signal transduction. Contrariwise, although heterotrimeric G-proteins have been implicated in the short-term regulation of stomatal aperture, and are known to be the most upstream component of the PHYA signal transduction machinery (Mustilli and Bowler, 1997), a lack of any effect of microinjection of either an activator or antagonist of G-protein action on RD29A and KIN2 transcriptional activation negates a role for G-proteins in ABA signal transduction events that control the expression of these genes (Wu et al., 1997). Neither RD29A nor KIN2 were activated when PHYA was microinjected into hypocotyl cells of a tomato mutant in which PHYA-regulated processes are severely compromised (Wu et al., 1997).

The level of reduction of the cellular NADP pool, which has been suggested to be a primary cue for proline synthesis (Hare and Cress, 1997), apparently plays an important role in modulating the expression of PHYA-
inducible genes that are responsible for the assimilation of light energy (Mustilli and Bowler, 1997). Carbohydrate status exerts an additional level of control over photoregulated genes. The possible involvement of hoxose sensing in mediating plant responses to adverse conditions has been discussed recently, specifically in the context of metabolic and signalling effects associated with stress-induced osmolyte accumulation (Hare et al., 1998). Consistent with the suggestion that sucrose is a positive effector of proline accumulation, much circumstantial evidence implicates the importance of integrating proline synthesis with the energy assimilating capacity of the plant. A recombinant P5CS from Vigna aconitifolia is feedback inhibited by ADP, thus indicating its sensitivity to cellular energy status (Zhang et al., 1995). In view of the different experimental conditions used by Verbruggen et al. (1993) and Yoshiba et al. (1995), it is tempting to speculate that their contrasting observations regarding NaCl-mediated induction of AtP5CR transcript accumulation may arise from differences in the carbohydrate status of the stressed plants.

It should be emphasized that focus on the regulation of P5CS, P5CR and PDH activities alone is unlikely to account fully for the regulation of proline accumulation. The apparent presence of a proteinaceous inhibitor of P5CS in plant cells (Kavi Kishor et al., 1995; Zhang et al., 1995) introduces the possibility that this may be an important component in regulating proline synthesis at a post-translational level. Furthermore, our discussion thus far has not included the regulation of inter-and intracellular proline transport (Hare et al., 1998) or the contribution of ornithine, an intermediate in arginine biosynthesis and degradation, to P5C synthesis. In Arabidopsis, 14C-isotope tracer experiments established that glutamate, ornithine and arginine all contribute to increased proline biosynthesis following a PEG-mediated reduction in water potential. In 7-d-old seedlings grown in a nitrate-replete nutrient medium, more than four times the amount of proline was made from uniformly labelled glutamate as from uniformly labelled ornithine or arginine (Chiang and Dandekar, 1995). After 24 h of PEG-induced water stress, the radioactivity incorporated into proline from glutamate increased almost 6-fold, whereas the respective increases in 14C incorporation in plants incubated in ornithine and arginine were approximately 10- and 33-times greater than unstressed controls (Chiang and Dandekar, 1995). Thus, although proline synthesis from glutamate during stress may still be quantitatively more important than synthesis from ornithine, these data emphasize the poor appreciation of the relative contributions of both pathways of P5C synthesis under optimal and adverse conditions. The route for P5C synthesis appears to be developmentally regulated. An Arabidopsis cDNA encoding ornithine 6-aminotransferase (OAT; EC 2.6.1.13) has recently been isolated (Roosens et al., 1998). Together with P5CS, OAT plays an important role in proline synthesis during osmotic stress in seedlings, although in mature plants proline accumulation occurs despite the apparent absence of AtOAT transcript and no change in OAT activity (Roosens et al., 1998).

In V. aconitifolia, expression of P5CS appears to be responsive not only to osmotic stress, but also to plant nitrogen status (Delauney et al., 1993). Salt stress and nitrogen starvation induced P5CS mRNA levels and depressed levels of transcript encoding OAT. Conversely, plants fed with NH4NO3 had considerably higher OAT transcript levels, while P5CS mRNA levels were reduced (Delauney et al., 1993). Subsequent studies by this group indicated that a 24 h treatment with 5 mM glutamine did not affect the expression of an AtP5CS2::GUS fusion (Zhang et al., 1997), although the effects of nitrogen metabolites on AtP5CSI promoter activity have not been investigated. Recent studies of transgenic tobacco plants with very low nitrate reductase activity have indicated an important role for nitrate, and not metabolites thereof, in signalling co-ordinated changes in several enzyme activities and levels of transcripts involved in carbon and nitrogen metabolism (Scheible et al., 1997). Nitrate may directly affect P5CS gene expression through its contribution to nutrient assessment pathways that appear to interact extensively with both light and hormonal action (Thomas et al., 1997; Fig. 1). It may also be of relevance to note that transgenic tobacco plants which express an antisense mRNA encoding nitrite reductase accumulate proline to levels almost 5-fold in excess of those found in the WT (Vaucheret et al., 1992). This line had reduced ammonium and glutamine levels and enhanced nitrate reductase activity. Unfortunately, nitrate levels were not assessed.

**Signalling downstream of proline synthesis and/or degradation**

Traditionally, stress physiologists have considered proline to be a compatible solute which stabilizes subcellular structure and attenuates water loss under hypersmotic conditions (Hare and Cress, 1997). Nonetheless, increased stress tolerance of transgenic tobacco plants which express a V. aconitifolia P5CS transgene (Kavi Kishor et al., 1995) is unlikely to arise exclusively from osmotic effects associated with the levels of proline accumulated (Hare et al., 1998). In keeping with the emerging view that the metabolic effects of osmolyte accumulation may be equally, if not more important than their traditionally accepted role in osmotic adjustment (Hare et al., 1998), it has been suggested that stress-induced changes in proline synthesis and degradation may affect plant gene expression (Hare and Cress, 1997).

Evidence consistent with this proposal was recently provided by Iyer and Caplan (1998). Intermediates in...
proline synthesis and catabolism such as glutamate and PSC, or an analogue of PSC, 3,4-dehydropoline (DHP), selectively increase the expression of certain stress-regulated genes in rice (Iyer and Caplan, 1998). Treatment with 1 mM PSC or DHP simulated induction of salT, DHN4 and RAB16A (originally RAB21) accumulation by 75 mM NaCl, but decreased levels of a transcript encoding S-adenosylmethionine synthetase (Iyer and Caplan, 1998) and had no effect on levels of Em, another osmotically regulated mRNA. Furthermore, DHP decreased levels of transcript encoding hsp70, a heat shock protein which can be induced by severe osmotic stress. Unlike induction of salT by NaCl, accumulation of salT transcript by PSC is CHX-insensitive (Iyer and Caplan, 1998). However, as was observed for DHN4 mRNA abundance, CHX reduced the maximal level of salT transcript induced by PSC. It has recently been suggested that changes in cellular redox potential, mediated by altered flux through the proline biosynthetic and catabolic pathways may signal these changes in gene expression (Hare et al., 1998). Although redox control of plant gene expression is presently not as well characterized as in animal cells, evidence implicating the involvement of the redox status of chloroplastic thioredoxin and plastoquinone in the regulation of photosynthetic genes has been presented (Hare and Cress, 1997). Given the dependence of animal ADP-ribose cyclase on NAD⁺ and NADP⁺ availability (Graeff et al., 1998), and the levels of these oxidized pyridine nucleotides in plant tissues (Gibon and Larher, 1997), it is tempting to speculate that proline synthesis may provide the substrate for NAADP⁺ or cADPR synthesis and that these agents of Ca²⁺ release (Wu et al., 1997) subsequently induce stress-regulated genes. A role for the availability of substrate in regulating ADP-ribose cyclase activity is consistent with the demonstration that NADase, an enzyme that degrades NAD⁺⁺, the precursor of cADPR, blocks induction of R2D24A and KIN2 by ABA (Wu et al., 1997). Unfortunately, the effects of reducing NAADP⁺ levels were not reported.

Exogenous proline (100 mM) decreases protein synthesis in maize suspension-cultured cells (Xin and Li, 1993). In rabbit reticulocyte lysates, inhibitory effects of PSC on translation can be prevented by an NADPH-generating system (Mick et al., 1988). On the basis that the inhibition of globin synthesis in the cell-free system by 1 mM PSC was greater than that observed with equimolar oxidized glutathione or NADP⁺, Mick et al. (1988) concluded that PSC was not acting solely by its ability to generate NADP⁺ during its catabolism to produce proline. Nonetheless, consideration of the notion that proline and its precursors may participate in a substrate cycle (Hare and Cress, 1997; Hare et al., 1998) may account for their observations without eliminating this possibility. Whereas the conversion of PSC to proline generates a single NADP⁺, further metabolism of the proline to glutamate and its conversion back to proline generates an additional two NADP⁺. A 43% decrease in the NADPH levels of sheath segments from rice plants grown in the presence of PSC (Iyer and Caplan, 1998) is consistent with this proposal. In tissue with a high rate of cycling between proline and its precursors, the apparent discrepancy between the effects of equimolar concentrations of PSC and NADP⁺ observed by Mick et al. (1988) may be ascribed to non-stoichiometrical amounts of NADP⁺ generated per mole of PSC reduced to proline. Studies involving inhibitors of mitochondrial electron transport, which are known to block proline degradation, are consistent with a fairly substantial turnover of the free proline pool in unstressed rice seedlings (Alia and Pardha Saradhi, 1993). Nonetheless, this interpretation is confounded somewhat by the demonstration that DHP is more effective than PSC in induction of salT transcript accumulation, although PSC was more effective in increasing DHN4 and RAB16A levels. As pointed out by Iyer and Caplan (1998), if DHP is converted to proline and subsequently into PSC, all three inducers of salT expression should be equally effective. In contrast, PSC was more effective than proline (Iyer and Caplan, 1998).

Treatment of rice plants with proline, PSC and DHP apparently did not affect ABA levels, although this was only monitored at a single time (24 h) after commencement of the treatment (Iyer and Caplan, 1998). Xin and Li (1993) found no induction of ABA accumulation in maize cultured cells 6 h, 12 h or 24 h after treatment with proline. Levels of rice salT transcript increase not only in response to NaCl, proline, PSC, and DHP, but also after treatment with jasmonic acid (JA) and ABA (Moons et al., 1997). Although treatments with 1 mM PSC or DHP increased levels of salicylic acid in blades of rice plants by almost 6- and approximately 12-fold, respectively (Iyer and Caplan, 1998), neither salicylic acid nor ethylene were inducers of salT expression (Moons et al., 1997). Interestingly, whereas ABA induces salT transcript accumulation in root and most extensively in the sheath of rice seedlings through activation of the salT promoter, salT protein does not accumulate in root or shoot tissues of ABA-treated seedlings (Moons et al., 1997). In contrast to this ABA-related post-transcriptional negative effect on salT expression, JA-induced salT transcript accumulation in shoots is associated with a dose-dependent increase in salT protein abundance (Moons et al., 1997). An excess of ABA over JA reduces salT transcript abundance in roots possibly through an effect on mRNA stability. In the presence of inhibitors of JA synthesis, ABA application can elicit the complete disappearance of the salT message (Moons et al., 1997). Exactly how a PSC-related signal may interfere with this antagonism between ABA and JA action is uncertain. Nonetheless, it is worth noting that induction of the salT transcript by DHP or PSC in sheaths of rice plants requires at least 24 h exposure (Iyer et al., 1998).
and Caplan, 1998), whereas in roots of rice seedlings, ABA-and JA-induced salT transcript accumulation can be detected within 4 h and reaches high levels after 8 h (Moons et al., 1997). Clearly, the effects of P5C on salT protein levels warrants investigation. Using two-dimensional protein profiles, Xin and Li (1993) found that none of the ABA-regulated proteins in maize suspension-cultured cells displayed detectable changes in cells treated with 100 mM proline for 12 h.

The responsiveness of rice RAB16A to ABA application is well-documented (Ono et al., 1996). In barley aleurone protoplasts, the tyrosine phosphatase inhibitor phenylarsine oxide (PAO) can completely block ABA-induced mitogen-activated protein kinase (MAPK) activation and expression of an ABA-inducible gene detected using rice RAB16A as probe (Knetsch et al., 1996). This led to the conclusion that ABA-mediated induction of RAB16 gene expression requires activation of a MAPK via a tyrosine phosphatase(s). Considerable evidence implicates the involvement of MAPK cascades in plant stress responses (Hare et al., 1997). Hallmark features of MAPK cascades are their capacity for signal amplification and their ability to integrate multiple signals transmitted by various second messengers. Thus different signalling pathways appear to converge at MAPKs, which distribute the varying signals to different downstream targets. Since any single MAPK may have multiple substrates, it may set in motion a wide range of events. It seems reasonable to propose that tyrosine phosphatase and MAPK activities may act downstream of the signal proposed to arise from proline synthesis and degradation (Fig. 2A). Nonetheless, the possibility cannot be eliminated that the P5C-mediated induction of RAB16A transcript accumulation in rice plants (Iyer and Caplan, 1998) may arise through a mechanism distinct from that downstream of ABA perception. Furthermore, although the time-course and concentration dependence of MAPK activation by ABA are consistent with a role for MAPK activation in the induction of barley RAB16 accumulation, an absolute requirement for MAPK action in this process has not been established (Fig. 2B). Sensitivity of P5C-responsive rice RAB16A transcript accumulation to PAO has not been investigated. Despite evidence that ABA may regulate proline synthesis, the rapidity of MAPK activation (within less than 1 min after ABA treatment) argues strongly against a model in which ABA-elicited accumulation of RAB16A transcript is mediated through an effect related to proline synthesis. Strong induction of AtP5CS1 gene expression by spraying Arabidopsis plants with 1 mM ABA was reached only after 2 h, with a maximum level of induction being reached only after 5 h (Yoshiba et al., 1995), while ABA-mediated induction of AtP5CS2 transcript accumulation appears to be even less rapid (Strizhov et al., 1997). The CHX-independence of RAB16A induction by P5C was not investigated by Iyer and Caplan (1998). Controversy surrounds whether ABA-mediated induction of rice RAB16A is sensitive to inhibition of protein synthesis (Nakagawa et al., 1996). However, the identification of an ABA-, NaCl- and dehydration-inducible rice bZIP protein, OSBZ8, which binds to the ABRE in the RAB16A promoter (Nakagawa et al., 1996) may open the way for further characterization of the intriguing involvement of proline metabolism in regulating stress-inducible gene expression.

**Conclusion**

Deciphering the signalling events that regulate stress-induced proline accumulation has considerable potential for both basic and applied research into plant stress tolerance. Since both induction and repression of gene expression appear to be co-ordinated in mediating this response, identification of signalling components that elicit proline synthesis and degradation is likely to provide a useful paradigm for investigating how plants co-ordinately increase and decrease the expression of specific genes both under stress and during recovery from adverse conditions. Signalling processes associated with relief from stress have until now been largely ignored, despite their likely importance in future efforts to increase plant stress tolerance. Further characterization of the events that underlie P5CS gene expression may also provide insight into stress-related changes in gene expression at the post-transcriptional level. Considerable evidence now indicates the importance of this poorly understood aspect of both ABA-dependent and -independent signalling events during stress. The identification of putative cis-acting promoter elements in proline biosynthetic genes provides a rational basis for assignment of particular stress-regulated transcription factors to their regulation. As they become available, this approach will become applicable for other genes involved in proline synthesis and degradation. Identification of the molecular features associated with proline-mediated induction of PDH transcript accumulation may assist in clarifying the mechanism(s) whereby proline, P5C and analogues thereof selectively induce stress-related genes. It is proposed that a signalling cascade activated by stress-regulated changes in proline synthesis and/or degradation may modify the throughput of parallel cascades upstream of at least certain stress-inducible genes. This would provide a regulatory mechanism of continuously ensuring that the genetic response to stress is appropriate to the prevailing environmental conditions.

Characterization of proline biosynthetic and degradative capacity in Arabidopsis mutants other than aba1, abi1, abi2, axr2, esk1, sos1, and sos2 will undoubtedly enable different aspects of the regulation of proline synthesis and degradation to be assigned to various signalling pathways disrupted in mutant lines. There can be little doubt that
Fig. 2. Possible interactions between P5C- and ABA-mediated induction of RAB16A transcript accumulation. (A) Abscisic acid and a signal derived from elevated P5C levels (Iyer and Caplan, 1998) may both act at a point upstream of MAPK induction (1, 2), or both signalling routes may be independent (3). (B) The actions of ABA or a P5C-related signal on RAB16A transcript levels may be independent of MAPK activation, although the P5C-related signal may or may not affect ABA-mediated activation of a phenylarsine oxide (PAO)-sensitive protein tyrosine phosphatase upstream of RAB16A transcript accumulation (Knetsch et al., 1996). It is proposed that P5C-induced increases in gene expression may arise from an increase in NADP⁺ levels generated from increased NADP⁺ concentrations resulting from activation of proline synthesis. Not indicated is the potential involvement of phosphatidic acid in propagating the ABA signal that leads to RAB16 protein accumulation in barley aleurone cells (Ritchie and Gilroy, 1998).

these studies will not only complement, but also be complemented by further characterization of the host of stress-inducible plant genes which have been identified over the past decade. Mutants also provide a powerful means of assessing the functional significance of stress-induced shifts in proline metabolism in maintaining metabolic homeostasis, although interpretation of data obtained using this approach may be complicated by the apparent redundancy in the pathways that signal changes in proline synthesis and degradation. Notwithstanding the enormous challenges that lie ahead for workers in this area, improved understanding of stress-related signal transmission is likely to pay substantial rewards in the form of increased agricultural productivity.

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