Role of abscisic acid (ABA) and Arabidopsis thaliana ABA-insensitive loci in low water potential-induced ABA and proline accumulation

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Abstract

The mechanisms by which plants respond to reduced water availability (low water potential) include both ABA-dependent and ABA-independent processes. Pro accumulation and osmotic adjustment are two important traits for which the mechanisms of regulation by low water potential, and the involvement of ABA, is not well understood. The ABA-deficient mutant, aba2-1, was used to investigate the regulatory role of ABA in low water potential-induced Pro accumulation and osmotic adjustment in seedlings of Arabidopsis thaliana. Low water potential-induced Pro accumulation required wild-type levels of ABA, as well as a change in ABA sensitivity or ABA-independent events. Osmotic adjustment, in contrast, occurred independently of ABA accumulation in aba2-1. Quantification of low water potential-induced Pro accumulation in five ABA-insensitive mutants, abi1-1, abi2-1, abi3, abi4, and abi5, revealed that abi4 had increased Pro accumulation at low water potential, but a reduced response to exogenous ABA. Both of these responses were modified by sucrose treatment, indicating that ABI4 has a role in connecting ABA and sugar in regulating Pro accumulation. Of the other abi mutants, only abi1 had reduced Pro accumulation in response to low water potential and ABA application. It was also observed that abi1-1 and abi2-1 had increased ABA accumulation. The involvement of these loci in feedback regulation of ABA accumulation may occur through an effect on ABA catabolism or conjugation. These data provide new information on the function of ABA in seedlings exposed to low water potential and define new roles for three of the well-studied abi loci.

Key words: ABA (abscisic acid), ABA sensitivity, abi mutants, abiotic stress, Arabidopsis thaliana, low water potential, osmotic adjustment, proline.

Introduction

Low water potential ($\psi_w$) and subsequent changes in turgor and water content elicit many responses that allow plants to adapt to stress and, to a certain extent, continue to grow and develop. The large and rapid accumulation of ABA occurring in response to low $\psi_w$ is a critical component in the activation of downstream responses at both the physiological level, such as stomatal regulation (Assmann, 2003), and the molecular level through numerous changes in gene expression (Bray, 1993, 2003; Shinozaki et al., 2003; Zhu, 2002). ABA-dependent responses interact with ABA-independent events and changes in sensitivity to ABA to generate a co-ordinated low $\psi_w$ response.

In general, two key sets of observations indicate that one must look beyond ABA accumulation itself to understand the low $\psi_w$ response. First, some low $\psi_w$ responses do not require wild-type levels of ABA. For example, analysis of several Arabidopsis thaliana mutants with reduced ABA accumulation at low $\psi_w$ has shown that a number of low $\psi_w$-induced genes are induced to wild-type or near wild-type levels despite the reduced level of ABA (Shinozaki et al., 2003). Also, Assmann et al. (2000) reported that the Arabidopsis ABA-deficient mutant aba1, as well as the ABA-insensitive mutants abi1 and abi2, was unaffected in stomatal closure in response to reduced humidity (although all of these mutants are impaired in low $\psi_w$-induced stomatal closure). Second, ABA application to unstressed plants does not always simulate the effects of low $\psi_w$. This
has been demonstrated for the expression of the stress- and ABA-regulated gene *le25* (Imai *et al*., 1995) and ABA-mediated root growth maintenance and shoot growth repression in response to low ψ<sub>w</sub> (Sharp and LeNoble, 2002; Sharp *et al*., 1994). Whether or not these differences are the result of sensing and signalling events that do not involve ABA or are the result of altered response to ABA under low ψ<sub>w</sub> is unclear. For many low ψ<sub>w</sub> responses, such as Pro accumulation and osmotic adjustment, little or no data are available that would allow the relative importance of ABA accumulation, changes in ABA sensitivity, and ABA-independent regulation to be assessed.

In *Arabidopsis*, a series of ABA-insensitive (*abi*) mutants have been isolated. The *abi* mutants all exhibit ABA-insensitive seed germination, but differ in their effects on other ABA-regulated responses (Finkelstein *et al*., 2002). *ABI1* and *ABI2* are type 2C protein phosphatases that act as negative regulators of ABA signalling (Leung *et al*., 1997). *ABI1* and *ABI2* are expressed at a higher level in vegetative tissues than in seeds and are induced by ABA and osmotic stress (Leung *et al*., 1997). *ABI3* encodes a B3 domain transcription factor (Giraudat *et al*., 1992) and is expressed mainly in seeds and meristematic tissue with a low level of expression in vegetative tissue (Finkelstein *et al*., 2002). *ABI4* encodes an APETALA2 domain transcription factor (Finkelstein *et al*., 1998) and *ABI5* encodes a bZIP domain transcription factor (Finkelstein and Lynch, 2000). *ABI4* and *ABI5* are expressed most abundantly in developing seeds, but both also have low levels of vegetative expression (Finkelstein *et al*., 1998; Finkelstein and Lynch, 2000). Despite the importance of understanding the signalling events regulating plant stress responses, the low ψ<sub>w</sub> responses of *abi1* through *abi5*, have not been fully characterized. *abi1-1*, *abi2-1*, and *abi3* have all been reported to have altered responses to exogenous ABA, in addition to effects on seed germination (Finkelstein, 1994; Finkelstein and Somerville, 1990; Suzuki *et al*., 2001), raising the possibility that the *abi* mutants could affect ABA-regulated low ψ<sub>w</sub> responses as well. However, Cramer (2002) found no difference in dry weight, ABA, and ion content between *abi1-1*, *abi2-1*, *abi3*, and wild type exposed to high salinity.

Pro accumulation is an important low ψ<sub>w</sub> response; although its function and regulation are not well understood. Pro is a compatible solute that can have a major role in osmotic adjustment (Voetberg and Sharp, 1991) and may also have a number of other protective roles. These include protecting protein and membrane structure (Chen and Murata, 2002; Yancey *et al*., 1982), scavenging reactive oxygen (Hong *et al*., 2000; Smirnoff and Cumbes, 1989), and eliminating excess reductant or regulating cellular redox status (Hare *et al*., 1998). In addition, Pro, or its catabolic intermediate Δ<sup>1</sup>-pyrroline-5-carboxylate (P5C) has been proposed to have a role in the metabolic signalling of carbohydrate status (Hellmann *et al*., 2000).

There is evidence that ABA is required for low ψ<sub>w</sub>-induced Pro accumulation. Blocking ABA accumulation using either the ABA-deficient maize mutant *vp5* or fluridone dramatically reduced Pro deposition in low ψ<sub>w</sub>-treated primary roots (Ober and Sharp, 1994). In agreement with this, Strizhov *et al*., (1997) found that salt stress induction of the gene encoding the rate-limiting enzyme in stress-induced Pro biosynthesis, *P5C SYNTHASE1 (P5CS1)*, was inhibited in the ABA-deficient mutant *aba1* and in *abi1*. On the other hand, Savoure *et al*., (1997) have stated that expression of *P5CS1* was induced independently of ABA in response to salinity or low ψ<sub>w</sub>. Thus, the possible regulatory mechanisms controlling low ψ<sub>w</sub>-induced Pro accumulation include ABA-dependent and independent factors, as well as changes in ABA sensitivity.

The ABA-deficient mutant, *aba2-1*, was used to investigate the role of ABA in Pro accumulation and osmotic adjustment. It was observed that Pro accumulation requires ABA accumulation, yet ABA alone is not sufficient to induce the Pro content observed at low ψ<sub>w</sub>. Osmotic adjustment, by contrast, did not require wild-type ABA levels. To understand the mechanisms of ABA-dependent regulation of Pro accumulation further, low ψ<sub>w</sub>-induced ABA and Pro accumulation in *abi1-1*, *abi2-1*, *abi3*, *abi4*, and *abi5* were assayed and new phenotypes were found for several of these mutants. *abi1-1* and *abi2-1* have increased ABA accumulation indicating a role for these loci in controlling feedback regulation of ABA accumulation. *abi4* alters Pro accumulation in a sucrose-dependent manner indicating a role for ABI4 in connecting ABA and sugar signalling in the low ψ<sub>w</sub> response.

**Materials and methods**

**Plant material**

Seed stocks of the *abi* mutants were obtained from the *Arabidopsis* Biological Resource Center and the ABA-insensitive germination phenotype of each line was verified (Assmann *et al*., 2000). *aba2-1* was obtained from the same source and its genotype verified by its reduced growth and ABA accumulation at low ψ<sub>w</sub>. Data for each mutant was compared with that of the appropriate wild-type ecotype. Some experiments were performed with the Bensheim ecotype which has similar levels of Pro and ABA accumulation as Col (Verslues and Bray, 2004).

**Low ψ<sub>w</sub> and ABA treatments**

Low ψ<sub>w</sub> and ABA treatments were performed using vertically-positioned agar plates as previously described (van der Weele *et al*., 2000; Verslues and Bray, 2004). Seeds were plated on basal media (half-strength MS media with 2 mM MES buffer, pH 5.7, solidified with agar). Media were prepared without the addition of sucrose or other sugars unless otherwise noted. Prior to seed plating, the plates were overlain with a nylon mesh to facilitate transfer of seedlings to a new plate. After stratification for 3 d at 4 °C, plates were transferred to a growth room (23 °C, 16 h light period, 150–180 μE cm<sup>−2</sup> min<sup>−1</sup> light intensity) and placed vertically inside a humidified plexiglass chamber to prevent the plates from drying and to allow the seedlings to grow along the surface of the agar. After 3 or 4 d of growth,
Regulation of ABA and Pro accumulation at low water potential

Measurements of relative water content (RWC) and osmotic potential ($\psi_w$), and calculation of osmotic adjustment were performed as described previously (Verslues and Bray, 2004). Seedling solute content was quantified by homogenizing whole seedlings and measuring $\psi_c$ of the cell sap using a vapour pressure osmometer. Seedling RWC was measured by removing seedlings from the agar plate using the nylon mesh, briefly blotting to remove excess liquid and weighing. Seedlings were then incubated in ice-cold water for 2–3 h to rehydrate, blotted, reweighed, and dried overnight at 65°C. Dry weight was recorded, the weight of the nylon mesh subtracted from all measurements, and RWC calculated as: (fresh weight–dry weight)/(hydrated weight–dry weight)×100. $\psi_c$ at 100% RWC ($\psi_{100}$) was calculated by multiplying the RWC by the $\psi_c$.

Data reported for these assays represent the combined mean of at least two independent experiments with two or three samples collected in each experiment. Statistically significant differences were determined by standard two-tailed t-test with P-values as noted in the text or figure legends.

Results

ABA accumulation is required to induce Pro accumulation at low $\psi_w$

Pro and ABA both accumulate in 3-d-old Arabidopsis seedlings when exposed to a constant, reproducible low $\psi_w$ stress using a PEG-infused agar plate system (van der Weele et al., 2000; Verslues and Bray, 2004); although the pattern of accumulation of these two metabolites differs over a 96-h experimental time-course (Fig. 1). To begin to determine the relationship between low $\psi_w$-induced Pro and ABA accumulation, Pro and ABA contents of the ABA-deficient mutant, aba2-1, were compared with wild type after the transfer of seedlings to either $\psi_w$ treatment. For ABA quantification, seedlings were removed from the agar plate using the nylon mesh and twice rinsed (each rinse <10 s) with a NaCl solution of the same concentration of ABA to both the basal media before the addition of PEG and to the PEG solution overlaid on the agar media.

Quantification of Pro, ABA and osmotic adjustment

Pro was assayed on water-extracted seedlings using the ninhydrin assay of Bates et al. (1973). Samples consisted of 10–30 seedlings depending on the experimental treatment. ABA was assayed by radioimmunoassay (Bray and Beachy, 1985). Seedling samples for ABA analysis consisted of 20–100 seedlings (15–200 mg of tissue) depending on the experimental treatment. For ABA quantification, seedlings were removed from the agar plate using the nylon mesh and twice rinsed (each rinse <10 s) with a NaCl solution of the same $\psi_w$ as the agar to remove any PEG or exogenous ABA that may interfere with the assay of seedling internal ABA. Seedlings were then blotted and weighed.

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decline with longer exposure to stress (Cowan et al., 1997). *aba2-1* had less than a 2-fold increase in ABA content in response to the 96 h low $\psi_w$ treatment and lacked the initial peak in ABA content observed at 8 h in the wild type (Fig. 1B).

These results indicate that, despite the different temporal pattern of ABA and Pro accumulation, ABA accumulation was required for the high level of low $\psi_w$-induced Pro accumulation of the wild type. In this experimental system, the partial Pro accumulation that occurred in the first 8 h of low $\psi_w$ treatment was not dependent on an increase in seedling ABA content, while the Pro accumulation occurring in the following 72–96 h required the elevated ABA content of the wild type. A portion of the Pro accumulation could be attributed to a decrease in seedling water content after transfer to low $\psi_w$. Seedling water content decreased as much as 40% in the first 8 h after transfer to $-1.2$ MPa and recovered to 15–20% below the unstressed level by 96 h (data not shown; Verslues and Bray, 2004). However, the level of Pro accumulation that occurred was larger than could be explained by decreased water content alone.

Confirmation that ABA deficiency caused the reduced Pro accumulation in *aba2-1* was obtained by applying exogenous ABA at $-1.2$ MPa to restore the ABA content of *aba2-1* to the wild-type level. For seedlings exposed to $-1.2$ MPa the Pro content of *aba2-1* in the presence of 0.5 $\mu$M S(+)-ABA was not significantly different from the wild type exposed only to the $-1.2$ MPa treatment (Fig. 2; an asterisk indicates a significant difference from the wild type at 0 $\mu$M ABA). Thus, the decreased Pro accumulation of *aba2-1* was complemented by the addition of ABA, indicating that ABA is required for Pro accumulation in this system.

**Exogenous ABA at high $\psi_w$ does not reproduce the low $\psi_w$ induction of Pro accumulation**

Reduced Pro accumulation in the ABA-deficient mutant, *aba2-1*, demonstrated that ABA accumulation was necessary to achieve a high steady-state level of Pro after a 96 h low $\psi_w$ treatment. Yet, the question remains whether ABA is sufficient to induce the high level of Pro accumulation that occurs at low $\psi_w$. To address this question, Pro and ABA contents were measured after seedlings were transferred to plates of a range of ABA concentrations at high $\psi_w$, or to PEG-infused plates of a range of $\psi_w$.

ABA treatment of seedlings at high $\psi_w$ ($-0.25$ MPa) caused a small (3.5-fold) increase in seedling Pro content despite a large increase in the internal ABA content of the seedlings (Fig. 3A). When these data were plotted as Pro content versus log internal seedling ABA content (Fig. 3B), there was a linear ($r^2=0.95$) relationship. It has previously been observed that application of concentrations as high as 100 $\mu$M ABA have little additional effect on Pro accumulation (Verslues and Bray, 2004). Thus, the range of ABA treatments examined here are within the range [the linear portion of a sigmoidal dose–response curve (Weyers et al., 1995)] where change in exogenous ABA concentration should have the greatest effect on seedling Pro content. These data suggest that in unstressed seedlings the sensitivity to ABA with respect to Pro accumulation is low.

By contrast, Pro and ABA content increased proportionally to decreasing $\psi_w$ over the range of $-0.25$ MPa to $-1.7$ MPa (Fig. 3C). The plot of seedling Pro content versus log internal seedling ABA content at low $\psi_w$ (Fig. 3D) is consistent with an increased sensitivity to ABA in low $\psi_w$-treated seedlings with respect to Pro accumulation. The response to ABA at low water potential could be explained by factors that act downstream of ABA and amplify the ABA signal; alternatively, ABA-independent factors that act directly to stimulate Pro accumulation at low $\psi_w$ without interacting with ABA may also exist.

**ABA accumulation is not required for osmotic adjustment**

Osmotic adjustment is an accumulation of solutes in response to low $\psi_w$ that decreases cellular osmotic potential.
and functions to prevent cellular water loss (Zhang et al., 1999). Osmotic adjustment in wild-type and aba2-1 seedlings was quantified to determine the involvement of ABA in osmotic adjustment. The fact that transpiration is minimal in this experimental system is especially advantageous in that osmotic adjustment can be quantified in response to a constant severity of low \( \psi_w \) without the confounding effects of increased transpirational water loss in the aba2-1 mutant. Seedling RWC and \( \psi_s \) were measured over a range of agar \( \psi_w \) and these values were used to calculate \( \psi_{s100} \) (Babu et al., 1999; Verslues and Bray, 2004). The RWC (Fig. 4A) and \( \psi_s \) (Fig. 4B) of aba2-1 and wild type was similar in seedlings exposed to a range of agar \( \psi_w \). Thus, \( \psi_{s100} \) did not differ between the mutant and wild type (Fig. 4C). This was verified by fitting regression lines to the \( \psi_{s100} \) data for each genotype. Statistical comparison of the regression lines (by F-test) revealed no significant difference between the mutant and wild type and a single line was fitted to the data of both genotypes (\( \psi_{s100}=0.30 \); \( \psi_w=-0.43; r^2=0.92 \)). The level of osmotic adjustment (131 mM MPa\(^{-1}\)) was calculated from the slope of the line and was similar to previous measurements for the ecotype Bensheim (150 mM MPa\(^{-1}\); Verslues and Bray, 2004). Thus, in aba2-1, wild-type levels of osmotic adjustment can occur without wild-type levels of ABA accumulation.

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abi1-1, abi2-1 and abi4 have altered ABA or Pro accumulation in response to low \( \psi_w \) or exogenous ABA

Analysis of Pro and ABA contents of wild type indicated a role of ABA signalling in low \( \psi_w \)-induced Pro accumulation, thus Pro and ABA accumulation were assayed in five ABA-insensitive (abi) mutants to determine if these mutants are altered in Pro accumulation. Since the five abi mutants are in three different Arabidopsis ecotypes (Ler, Col, and WS) and the different ecotypes have different Pro contents, each mutant was compared to its wild type. Ler had the highest Pro content at both \(-0.25 \) and \(-1.2 \) MPa followed by WS and Col (\( P <0.04 \); Fig. 5A). WS had significantly higher ABA content than Ler and Col (\( P <0.04 \)) at both \(-0.25 \) and \(-1.2 \) MPa (Fig. 5B). Only one of the abi mutants, abi1-1, had a significant reduction in low \( \psi_w \)-induced Pro accumulation (Fig. 5A); although, the 28% reduction was not as great as the 80% reduction of the ABA-deficient mutant, aba2-1. abi1-1 seedlings also accumulated less Pro than wild type in response to 2 \( \mu M \) \( S(+) \)-ABA.
Surprisingly, \textit{abi1-1} seedlings had 2–4-fold higher ABA content than \textit{Ler} in response to both low $\psi_w$ (Fig. 5B) and exogenous ABA at high $\psi_w$ (Fig. 5D). \textit{abi2-1} seedlings also had higher ABA contents. Both \textit{abi1-1} and \textit{abi2-1} have an approximately 4–5-fold reduction in Pro content per unit of ABA content at low $\psi_w$ compared with the wild type (Table 1).

Of the other ABA-insensitive mutants, only \textit{abi4} had significantly lower Pro content (31% lower) than its wild type in response to exogenous ABA at high $\psi_w$. However, \textit{abi4} had higher Pro content than the wild type after the low $\psi_w$ treatment (Fig. 5A). The ABA content of \textit{abi4} did not differ from the wild type (Fig. 5B, D) resulting in a greater Pro content per unit of ABA at low $\psi_w$ than the wild type (Table 1). No effect on Pro or ABA accumulation was seen in \textit{abi3} and \textit{abi5} (Fig. 5) relative to their parental ecotypes. This is consistent with the conclusions of Brady \textit{et al.} (2003) who placed \textit{abi3} and \textit{abi5} on a different branch of ABA signalling pathways than \textit{abi4} and downstream of \textit{abi1-1} and \textit{abi2-1}.

\textit{abi1-1} and \textit{abi2-1} have altered ABA homeostasis

The increased ABA accumulation in the \textit{abi1-1} and \textit{abi2-1} mutants indicates that ABI1 and ABI2 are regulators of ABA content as well as ABA responses. A time-course of ABA accumulation in \textit{abi1-1} and \textit{abi2-1} after transfer to low $\psi_w$ and the response of \textit{abi1-1} and \textit{abi2-1} to combined ABA and low $\psi_w$ treatments was also quantified. In both \textit{abi1-1} and \textit{abi2-1}, the peak ABA content that occurred approximately 8 h after transfer to low $\psi_w$ was 1.5–2-fold higher than the wild type \textit{Ler} (Fig. 6A). ABA content in the mutants then declined, following a similar pattern as \textit{Ler}, but remained higher than the wild type at the end of the 96 h time-course.

Treatment of \textit{Ler} seedlings with 0.5 or 2 $\mu$M ABA at −1.2 MPa increased seedling ABA content up to 2-fold, whereas \textit{abi1-1} and \textit{abi2-1} seedlings had an approximately 4-fold increase (Fig. 6B). This is similar to the 2–4-fold greater ABA content of \textit{abi1-1} and \textit{abi2-1} when 2.0 $\mu$M exogenous ABA was applied to unstressed seedlings (Fig. 5D). The increased ABA content of \textit{abi1-1} and \textit{abi2-1} was not dependent on the source of ABA; both exogenous ABA and ABA synthesized in response to low $\psi_w$ accumulated to a greater extent in \textit{abi1-1} and \textit{abi2-1} than the wild type. The increased ABA accumulation of \textit{abi1-1} and \textit{abi2-1} was also not affected by low $\psi_w$; ABA applied at either high or low $\psi_w$ caused a similar increase in the ABA content of \textit{abi1-1} and \textit{abi2-1} relative to the wild type. The simplest explanation for these data is that \textit{abi1} and \textit{abi2} have a decreased rate of ABA turnover, caused by a decrease in ABA catalolism and/or conjugation. However, these experiments cannot directly determine the metabolic mechanisms involved in the increased ABA content of the \textit{abi1-1} and \textit{abi2-1} mutants.

\textit{ABI4} is involved in the interaction of ABA, sugar and low $\psi_w$ in regulating Pro accumulation

ABA has been implicated in the ability of \textit{Arabidopsis} seedlings to respond to signals generated by sugars and the \textit{abi4} mutant is insensitive to sugar with respect to seedling development (Laby \textit{et al.}, 2000; Arenas-Huertaro \textit{et al.}, 2000; Rook \textit{et al.}, 2001). If sugars also influence Pro
metabolism, as has been previously proposed (Hellmann et al., 2000), this may provide an explanation for the altered Pro accumulation of \( \text{abi4} \). Pro contents of \( \text{abi4} \) seedlings and its wild type, Col, were quantified after treatment with a range of sucrose contents applied at low \( w_w \) (Fig. 7A). Seedlings were germinated and grown on sucrose-free media prior to transfer to \( /C255 \) 1.2 MPa PEG-infused plates containing sucrose. The addition of sucrose increased low \( w_w \)-induced Pro accumulation for both genotypes and the Pro content of \( \text{abi4} \) was greater than wild type at all sucrose treatments except at 3% sucrose, the highest concentration tested. Because \( \text{abi4} \) had a higher Pro content than the wild type in the absence of sucrose, the relative stimulation of Pro accumulation by sucrose was greater in the wild type than in \( \text{abi4} \).

In the absence of low \( w_w \), the sucrose treatment did not affect steady-state Pro accumulation in either Col or \( \text{abi4} \) (Fig. 7B). As reported in Fig. 5B, exogenous ABA promoted Pro accumulation and this response was partially blocked in \( \text{abi4} \). Addition of 0.5% sucrose to the media blocked ABA-induced Pro accumulation in the wild type and, to a lesser extent, in \( \text{abi4} \) (Fig. 7B). The combined results indicate that the wild type ABI4 gene product is a negative regulator of low \( w_w \)-induced Pro accumulation whose effect is decreased by high levels of sugar.

### Discussion

ABA is known to be an important regulator of low \( w_w \) responses, but not all ABA-dependent responses utilize the same signalling intermediates or are affected in the same manner by changes in ABA content (Zhu, 2002). The importance of ABA-dependent and ABA-independent signalling pathways varies among different low \( w_w \) responses. Evidence is presented here that ABA accumulation and changes in ABA sensitivity are important for the regulation of Pro accumulation, but not osmotic adjustment. The
ABA-insensitive mutants, abi1-1, abi2-1, and abi4, have altered ABA and Pro accumulation. This extends the range of phenotypes known to be regulated by these important ABA-signalling loci. A simplified model integrating these results is presented in Fig. 8.

**ABA accumulation and response**

The regulation of ABA content is complex, involving ABA synthesis as well as ABA catabolism and conjugation (Zeevaart, 1999). Under stress, it has been observed that ABA content is correlated with the degree to which the stress alters plant water status as measured by changes in turgor and RWC (Pierce and Raschke, 1980; Verslues and Bray, 2004; Zhang and Davies, 1987). Thus, multiple aspects of ABA metabolism may be involved in a homeostasis mechanism preventing excess ABA accumulation and matching ABA content to the type and severity of the stress to which the plant is exposed. The increased ABA accumulation of abi1-1 and abi2-1 indicates that the ABI1 and ABI2 protein phosphatase 2Cs are involved in ABA homeostasis. The most straightforward explanation accounting for the increased ABA content of abi1-1 and abi2-1 in response to both low ψw and exogenous ABA is that these loci are involved in a feedback mechanism that regulates the turnover of ABA (Fig. 8). However,
Regulation of ABA and Pro accumulation at low water potential

Interaction of low $\psi_w$, Pro, ABA, and sugar

The role of ABA in regulating Pro accumulation has been a matter of some confusion and debate (Hare et al., 1998, 1999; Ober and Sharp, 1994; Savoure et al., 1997; Strizhov et al., 1997). The results presented here demonstrate that while ABA accumulation is required for Pro accumulation, ABA accumulation alone is not sufficient to elicit the levels of Pro accumulation observed at low $\psi_w$. For both wild-type and $aba2$-1 seedlings, application of 2.0 $\mu$M ABA at $-1.2$ MPa increased seedling ABA content by more than 2-fold compared with wild-type seedlings subjected to $-1.2$ MPa alone (Fig. 2B), however, this exogenous ABA treatment did not significantly increase the Pro content (Fig. 2A). This may indicate the existence of additional mechanisms of Pro homeostasis that maintain seedling Pro content at a level appropriate to the stress severity, even if ABA content is increased to a higher than normal level. Alternatively, the perception or response to ABA at $-1.2$ MPa may already be saturated by the wild-type level of ABA. However, more severe stress treatments ($-1.7$ MPa, Fig. 3C) did lead to increased accumulation of both ABA and Pro. Thus, both of these regulatory factors, severity of low $\psi_w$ stress and ABA, must increase in tandem to increase Pro accumulation further.

Sugars also affect Pro accumulation. This is perhaps not surprising; a major change in metabolism such as increased Pro synthesis would probably be responsive to the levels of substrate and reductant needed for Pro synthesis. Several studies have found that sugars can stimulate Pro accumulation (Pesci, 1993; Stewart et al., 1966). However, recent data indicate a more complex interaction. Hellmann et al. (2000) found that the reduced sugar resposnel ($rsr1$) mutant, which was originally isolated as being impaired in sugar sensing, is also hypersensitive to exogenous Pro. The Pro hypersensitivity of $rsr1$ can be alleviated by the addition of glucose or sucrose to the media (Hellmann et al., 2000). This indicates that Pro metabolism may respond directly to sugar-sensing mechanisms. In addition, several mutants originally identified as ABA-deficient ($aba1$, $aba2$, and $aba3$) or ABA-insensitive ($abi4$ and $abi5$) are also sugar-insensitive (Arenas-Huertero et al., 2000; Laby et al., 2000; Rook et al., 2001). The increased Pro accumulation observed in $abi4$ in this study indicates that $ABI4$ may be part of a mechanism to restrict Pro accumulation when carbohydrate status is low (Fig. 8). Although sugar can influence the level of Pro accumulation, sugar cannot substitute for ABA in inducing Pro

Additional experiments that directly examine the rate of ABA turnover in $abi1$ and $abi2$ will be needed to determine which step of ABA metabolism is affected by $ABI1$ and $ABI2$. This uncertainty is indicated in Fig. 8 by the dashed line connecting $ABI1$ and $ABI2$ feedback regulation to ABA degradation/conjugation. Since $abi1$-1 and $abi2$-1 are both dominant alleles and recessive alleles of these genes are hypersensitive to ABA (Merlot et al., 2001), it can be hypothesized that recessive alleles of $ABI1$ and $ABI2$ would have a reduced level of low $\psi_w$-induced ABA accumulation indicating that $ABI1$ and $ABI2$ are negative regulators of ABA catabolism or conjugation (or positive regulators of ABA synthesis).

Further understanding of feedback regulation by ABA and the rates of ABA catabolism and turnover, as well as the possible role of $ABI1$ and $ABI2$ in controlling these processes, are necessary to understand the regulation of ABA accumulation by low $\psi_w$. Half-life values of exogenously applied ABA range from less than 1 h in the maize root apex (Ribaut et al., 1996) to 3–10 h in cell cultures and leaves (Balsevich et al., 1994; Zeevaart and Creelman, 1988). A limited number of studies comparing ABA catabolism in turgid and wilted leaves have either found no difference (Cornish and Zeevaart, 1984; Murphy, 1984) or decreased ABA catabolism in stressed leaves (Cowan and Railton, 1987). Impaired ABA degradation leading to increased ABA accumulation has previously been observed in $pew1$, a phytochrome-chromophore-deficient mutant of tomato (Kraepiel et al., 1994). Several investigators have suggested that ABA accumulation, application of exogenous ABA, or transgenic approaches to increase ABA synthesis stimulate ABA catabolism (Cutler and Krochko, 1999; Qin and Zeevaart, 2002; Xiong and Zhu, 2003; Zeevaart, 1999).

Fig. 8. Model of the interaction of low $\psi_w$, ABA, and sugar in the regulation of Pro and ABA accumulation. Perception of low $\psi_w$, via an unknown mechanism, induces signalling events which lead to increased ABA synthesis and accumulation, osmoregulatory changes, and changes in ABA response and/or other ABA-independent regulation (indicated by dashed lines). Bold up arrows indicate an increase in Pro or ABA content. $ABI1$ and $ABI2$ are regulators of ABA content via a feedback mechanism which may act on ABA catabolism or conjugation (indicated by the dashed line). $ABI4$ is a negative regulator of Pro accumulation at low $\psi_w$ and serves to connect Pro accumulation to sugar sensing.
accumulation at low \( \psi_w \). \textit{aba2-1}, a sugar-insensitive mutant, accumulates very low levels of Pro at low \( \psi_w \) because ABA accumulation is required for Pro accumulation. High levels of Pro accumulation can only be induced by the interaction of several signals including ABA, sugar, and osmoregulatory signals (Fig. 8; Verslues and Bray, 2004).

**Osmotic adjustment does not require wild-type levels of ABA accumulation**

Analysis of \textit{aba2-1} demonstrated that seedling osmotic adjustment was not dependent on ABA accumulation. This agrees with a previous characterization of the \textit{lwr2} mutant which has reduced osmotic adjustment, but is unaffected in ABA-responsive Pro accumulation or transpirational water loss (Verslues and Bray, 2004). Both the \textit{lwr2} and \textit{aba2-1} data demonstrate that osmoregulatory control of cellular solute and water content is distinct from the stomatal regulation of water loss and is controlled by different signalling mechanisms that do not require wild-type levels of ABA. Assaying osmotic adjustment under conditions of constant low \( \psi_w \) stress and minimal transpiration was essential to quantify the osmotic adjustment phenotype of \textit{aba2-1} accurately and to separate osmoregulatory responses from stomatal-regulated water loss.

LaRosa \textit{et al.} (1987) did observe increased osmotic adjustment in ABA-treated tobacco suspension cells exposed to salt stress. Most of the increase in solute content was caused by reducing sugars and Pro. One possible explanation is that ABA-stimulated accumulation of these compounds may prevent damage by NaCl toxicity and thus have a protective effect on metabolism which, in turn, allows further solute accumulation to occur. Whether or not ABA application can stimulate osmotic adjustment induced by non-ionic low \( \psi_w \), where NaCl toxicity is not a factor, is not known. LaRosa \textit{et al.} (1987) also observed a strong correlation between Pro content and cellular \( \psi_w \), suggesting an osmoregulatory component in controlling Pro accumulation.

The observation that wild-type levels of osmotic adjustment could occur despite the reduced Pro accumulation of \textit{aba2-1} raises the question of whether Pro accumulation is an essential component of osmotic adjustment. Pro is largely contained in the relatively small volume of the cytoplasm (Hare and Cress, 1997; Hare \textit{et al.}, 1998; Leigh \textit{et al.}, 1981) and within the cytoplasm can reach concentrations that are significant in osmotic adjustment. This has been directly demonstrated in studies of Pro accumulation in the unvacuolated cells of the maize root tip where Pro concentrations can reach as high as 120 mM (Ober and Sharp, 1994; Verslues and Sharp, 1999; Voetberg and Sharp, 1991). Thus, an inhibition of Pro accumulation, such as occurs in \textit{aba2-1}, may not have a large impact of the overall solute content of highly vacuolated tissues, but can have a large impact on the cytoplasmic solute content. In the case of \textit{aba2-1}, it is hypothesized that Pro normally accumulated in the cytoplasm as part of osmotic adjustment is replaced by other solutes such as K\(^+\). These solutes may be less effective than Pro at protecting cytoplasmic structure and function. Thus, promoting the accumulation of protective solutes in the cytoplasm, rather than simply promoting greater total solute accumulation, may be a function of ABA that increases cellular tolerance to low \( \psi_w \) and other stresses.

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