The nucleotidase/phosphatase SAL1 is a negative regulator of drought tolerance in Arabidopsis

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Received 8 September 2008; revised 1 December 2008; accepted 8 December 2008; published online 28 January 2009.

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Summary

An *Arabidopsis thaliana* drought-tolerant mutant, *altered expression of APX2 (alx8)*, has constitutively increased abscisic acid (ABA) content, increased expression of genes responsive to high light stress and is reported to be drought tolerant. We have identified *alx8* as a mutation in SAL1, an enzyme that can dephosphorylate dinucleotide phosphates or inositol phosphates. Previously identified mutations in SAL1, including *fiery (fry1-1)*, were reported as being more sensitive to drought imposed by detachment of rosettes. Here we demonstrate that *alx8*, *fry1-1* and a T-DNA insertional knockout allele all have markedly increased resistance to drought when water is withheld from soil-grown intact plants. Microarray analysis revealed constitutively altered expression of more than 1800 genes in both *alx8* and *fry1-1*. The up-regulated genes included some characterized stress response genes, but few are inducible by ABA. Metabolomic analysis revealed that both mutants exhibit a similar, dramatic reprogramming of metabolism, including increased levels of the polyamine putrescine implicated in stress tolerance, and the accumulation of a number of unknown, potential osmoprotectant carbohydrate derivatives. Under well-watered conditions, there was no substantial difference between *alx8* and Col-0 in biomass at maturity; plant water use efficiency (WUE) as measured by carbon isotope discrimination; or stomatal index, morphology or aperture. Thus, SAL1 acts as a negative regulator of predominantly ABA-independent and also ABA-dependent stress response pathways, such that its inactivation results in altered osmoprotectants, higher leaf relative water content and maintenance of viable tissues during prolonged water stress.

Keywords: drought, water use efficiency, SAL1, Arabidopsis, signal transduction, phosphoinositides (PI).

Introduction

Abiotic stresses including drought, temperature extremes, salinity, excessive light and combinations thereof are major limiting factors in crop yields in many parts of the world (Boyer, 1982; Araus *et al.*, 2002). Plants can respond to drought by three different mechanisms: (i) escape, where the plant stops growth and promotes flowering to produce seed before death; (ii) avoidance, where the stomata are

closed to prevent water loss or water uptake is increased (Jones, 2007); and (iii) tolerance or acclimation, which we define as the ability of a plant to subsist and grow in conditions of limited water under which wild-type plants cannot. In agriculture, tolerance is the better target as it can allow the maintenance of yield. Tolerance involves the accumulation of antioxidants, osmolytes and chaperones that protect cell

components and changes in the root-to-shoot ratio to maximize water absorption (Sharp and Davies, 1985; Reddy *et al.*, 2004).

To date, much of the research on *Arabidopsis thaliana* (*Arabidopsis*) has focused on the molecular response to sudden changes in osmotic potential or osmotic shock, largely initiating a stress avoidance response that is measured over a period of hours. Multiple signaling pathways are activated in response to osmotic shock (reviewed in Shinozaki and Yamaguchi-Shinozaki, 2007). Slower, and arguably more physiologically relevant, soil-based experiments occur over 1–2 weeks and initiate some avoidance responses, but also activate tolerance responses (Kawaguchi *et al.*, 2004; Verslues *et al.*, 2006). However, soil-based experiments require controls to ensure that all plants are exposed to the same stress (Verslues *et al.*, 2006). While many components of these stress response pathways have been isolated, the exact order, interactions and outcomes of the stress networks in physiologically relevant conditions are unknown.

Osmotic stress pathways are typically clustered into ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki, 2005). One osmotic stress-induced pathway involves the activation of the plasma membrane-localized phospholipase C (PLC) and results in stomatal closure and expression of stress-responsive genes (Takahashi *et al.*, 2001). Phospholipase C cleaves the membrane phospholipid phosphatidylinositol (4,5) bisphosphate (PIP₂) to produce soluble inositol 1,4,5-triphosphate (IP₃) and membrane-bound diacylglycerol (DAG) (Hirayama *et al.*, 1995; Drobak and Watkins, 2000; DeWald *et al.*, 2001; Otterhag *et al.*, 2001). The increase in abundance of IP₃ in response to osmotic stress is rapid and transient in nature, occurring within a few seconds (Takahashi *et al.*, 2001). Accumulation of IP₃ then results in further signal transduction, due to a release of Ca²⁺ from intracellular stores and activation of K⁺ ion channels, resulting in stomatal closure in guard cells (Drobak and Ferguson, 1985; Blatt *et al.*, 1990; Gilroy *et al.*, 1990). Inositol 1,4,5-triphosphate can also be phosphorylated by a number of IP₃ kinases to produce IP₆, or phytic acid (Ongusaha *et al.*, 1998; Stevenson-Paulik *et al.*, 2002; Xia *et al.*, 2003), which is 100 times more potent as an activator of stomatal closure (Lemtiri-Chlieh *et al.*, 2000).

The levels of inositol phosphate molecules are regulated both by synthesis from PIP₂ (Munnik *et al.*, 1998) and catalysis by phosphatases, including 5-phosphatases (5Pases) and inositol polyphosphate 1-phosphatases, which hydrolyze the 5' position phosphate and 1' position phosphate, respectively (Gillaspy *et al.*, 1995; Quintero *et al.*, 1996; Berdy *et al.*, 2001). SAL1 is an inositol polyphosphate 1-phosphatase that was originally isolated from *Arabidopsis* for its ability to complement a salt-sensitive yeast strain (Quintero *et al.*, 1996). The protein is bifunctional, as it also exhibits 3',(2'),5'-bisphosphate nucleotide phosphatase

activity, being highly specific for 3'-polyadenosine 5'-phosphate (PAP) (Quintero *et al.*, 1996; Gil-Mascarell *et al.*, 1999). Recently, SAL1 was shown to be a repressor of post-transcriptional gene silencing (PTGS), as it degrades the exoribonuclease inhibitor PAP (Dichtl *et al.*, 1997; Gy *et al.*, 2007). The *fry1-1* (*fiery*) mutant allele of *SAL1* has higher basal levels of IP₃ and increased expression of some stress response genes, but paradoxically it is reported to have increased sensitivity to salt, cold and drought stress (Xiong *et al.*, 2001).

The *Arabidopsis* mutant *alx8* was isolated in a screen for elevated expression of the antioxidant enzyme ascorbate peroxidase 2 (*APX2*) under low light (LL) and high light (HL) (Rossel *et al.*, 2004). The *alx8* mutant also had increased tolerance to drought and altered leaf morphology (Rossel *et al.*, 2006). Positional cloning identified *alx8* as a mutation in the *SAL1* gene. Here we resolve aspects of the *SAL1* paradox by demonstrating that intact soil-grown *fry1-1* and *alx8* are drought tolerant. We investigate the processes by which SAL1 may impact on stress signaling pathways and propose that the acquired drought tolerance in *alx8* plants could be due partially to an increase in some sugars, osmoprotectants and antioxidants and up-regulation of stress-inducible genes.

Results

alx8 contains a point mutation in inositol polyphosphate 1-phosphatase, SAL1

alx8 (Col-0) was crossed with Landsberg *erecta*. The F₁ was wild-type in leaf phenotype, development and *APX2* expression (data not shown), confirming *alx8* as recessive. Coarse mapping of 53 mutant F₂ individuals with 22 primers indicated that *alx8* was linked to chromosome five (Figure 1a). Fine mapping of 4300 F₂ individuals was undertaken with two markers, MUB3 and MMI9, flanking the 617-kb region. Genotyping and phenotyping of recombinants eliminated all but seven genes on the MBM17 bacterial artificial chromosome (BAC), one of which was *SAL1* (At5g63980; *FRY1/HOS2*). A line with a T-DNA insertion in *SAL1*, *salk_020882*, phenocopied *alx8* and all F₁ progeny of *alx8* × *salk_020882* had altered leaf morphology and delayed development.

A wild-type copy of the *SAL1* gene and promoter, pCAM-SAL1, was used to complement *alx8* and wild-type plants. For *alx8*, two individual T₁ transformants with wild-type leaf morphology were identified. Their progeny segregated for kanamycin resistance and leaf phenotype with over 100 T₂ complemented individuals having wild-type leaf morphology, timing of flowering, and development (Table 1). Wild-type plants transformed with this construct showed no change in leaf morphology, *APX2* expression or development (Table 1).

Figure 1. Mapping and identification of the *alx8* null mutation in *SAL1*.

(a) Mapping of *alx8* identified a region on MBM17 that included *SAL1*. Bold indicates the positions of markers and italics the number of recombinants.

(b) Location of mutations in the *SAL1* gene. Numbers show the position of amino acid change and black boxes are exons.

(c) Abundance of SAL1 protein in soluble and total leaf protein extracts (20 µg) compared with recombinant SAL1 protein (5 ng) using anti-SAL1 antibodies. Lower panel, loading controls of the large subunit of Rubisco. Two plants were analyzed for each genotype.

Sequencing of the *alx8* gene, 2 kb of promoter and 3' untranslated region (UTR) from 10 mutant plants revealed a single nucleotide polymorphism of guanine to adenine at the 1226th base pair of the At5g63980.1 genomic sequence (TAIR accession: 4010730406) (Figure 1b). The progeny of the fourth generation of backcrossed *alx8* were also screened for the point mutation using derived cleaved amplified polymorphic sequence (Neff *et al.*, 1998) markers to confirm the leaf phenotype was co-inherited with the point mutation and not another locus.

The *alx8* mutation results in an amino acid change of glycine to aspartic acid at the 217th amino acid of the SAL1 protein (TAIR accession: 4010745380). *In silico* protein modeling against the known structure of the yeast homolog of SAL1, known as HAL2, showed that the amino acid substitution in *alx8* localizes in a conserved internal β -sheet of unknown function.

The SAL1 protein is absent in *alx8* and *fry1-1*

The recombinant protein for the wild-type *SAL1* gene (rSAL1) and the mutated *alx8* gene (rALX8) were produced as fusions to polyhistidine-tagged ubiquitin in *Escherichia coli*. Their calculated masses (~50 kDa) corresponded to the expected sizes based on the amino acid sequence plus the

Table 1 Complementation of *alx8* and the wild type with *SAL1*

Genotype	Leaf shape	Flowering time	Development
Col-0	+/+	+/+	+/+
Col-0+ <i>pCAM-SAL1</i>	+/+	+/+	+/+
<i>alx8</i>	Altered	Delayed	Delayed
<i>alx8</i> + <i>pCAM-SAL1</i>	+/+	+/+	+/+

+/+ indicates that the phenotype is indistinguishable from wild type. For *alx8*+ *pCAM-SAL1* 145 plants from two independent transformations were analyzed. For Col-0+ *pCAM-SAL1* plants from 11 independent transformations were analyzed.

tag. Although many different induction conditions were attempted, only the rSAL1 fusion protein was successfully purified in the soluble fraction as one band (Figure S1 in Supporting Information). This protein was assayed for phosphatase activity against PAP as described (Murguía *et al.*, 1995). Recombinant SAL1 fusion protein showed similar PAP phosphatase enzymatic activity ($16.6 \pm \text{SE } 3.65 \mu\text{mol PAP h}^{-1} (\text{mg protein})^{-1}$, $n = 3$) to that previously reported (Xiong *et al.*, 2001). The authentic protein obtained after cleavage of the tag was used to generate antibodies.

Western blot analysis was performed to determine the abundance of the SAL1 protein in wild-type and *SAL1*

mutant plants. A single 37-kDa band, whose size corresponded to that of the authentic recombinant protein rSAL1, was detected in the soluble protein extract from leaves of Col-0 and C24, but not in *alx8* or *fry1-1* leaves (Figure 1c). Additionally, SAL1 protein was not present in a urea-based total protein extract of *alx8* but was in Col-0 (Figure 1c).

Drought tolerance of plants with alx8, salk_020882 and fry1 mutations in SAL1

alx8 has previously been shown to be drought tolerant in soil-based experiments (Rossel *et al.*, 2006) whereas *fry1-1* was reported as being drought sensitive in response to osmotic shock (Xiong *et al.*, 2001). Hence, we tested *fry1-1* and *salk_020882* for soil-based drought tolerance by withholding water from mature vegetative plants for 18 days. In three independent experiments with at least five plants per experiment *fry1-1*, *salk_020882* and *alx8* were more drought tolerant than the respective C24 and Col-0 wild-types (Figure 2a). To quantify the extent of the tolerance, a measure of plant viability using chlorophyll fluorescence was undertaken as described (Woo *et al.*, 2008). The results demonstrate that *alx8* plants survive drought for 40–50% longer than Col-0 (Table 2).

In a different experiment, leaf relative water content (RWC) was measured after 12 days of drought (Figure 2b). As expected, the leaf RWC of *alx8* did not change significantly during drought treatment, while the leaf RWC of the wild-type decreased significantly. Leaf water potential, or the chemical potential of water divided by the partial molar volume, was calculated for the same plants using a thermocouple psychrometer (Figure 2c). The higher water potential of *alx8* plants grown under drought conditions correlated with the maintained turgor while water-stressed wild-type plants had a significantly lower leaf water potential.

To ensure that all plants were being exposed to the same water stress, the relative water content of the soil was calculated. There was no significant difference in the water content of the soil between genotypes throughout the experiment (Figure 2d), indicating that water was lost at similar rates from the soil whether via plant transpiration or evaporation. Moreover, approximately 80% of the water was lost in the first week of drought, resulting in values of soil water potential lower than –800 kPa for the remainder of the treatment (Figure 2e).

The drought tolerance of *SAL1* mutants was tested at different developmental stages and ages to ensure it was not a result of the developmental delay or smaller size of the plants (Table 3, Figure 3). *alx8* was more drought tolerant than Col-0 wild-type at 2, 4, 6 and 8 weeks old. *alx8* was also more drought tolerant than Col-0 wild-type when: both plant types had six leaves; both were mature and vegetative; and both were just starting to flower (Table 2, Figure 3a,b). When the plants were just commencing to flower all lines were of roughly the same rosette area and size, yet after 9 days of withholding water, *alx8* and *salk_020882* were both more turgid and green than wild-type (Figure 3a), although pots lost water at similar rates (Figure 3c). Similarly, *alx8* and *fry1-1* plants grown to similar vegetative stages were more drought tolerant than wild-type (Figure 3b). *alx8* is also more drought tolerant than Col-0 when it has two plants per pot versus one for Col-0 (Figure S2) (Rossel *et al.*, 2006). Finally, Col-0 plants of varying sizes are equally susceptible to drought (Figure 3d).

Water loss of detached rosettes was measured for the four genotypes to determine whether leaf and rosette shape alters the rate of water loss, or whether stomatal control (the initial phase of water loss) or cuticular evapotranspiration (the second phase of water loss) were altered. No significant difference was detected between the four lines in either phase (Figure 3e), which is similar to earlier observations for *fry1* (Xiong *et al.*, 2001).

As both *alx8* and *fry1-1* lack SAL1 protein, the difference in their reported stress sensitivity is surprising. To see if this variation is due to differing ecotype or the nature of the imposed stresses, we repeated the polyethylene glycol (PEG) osmotic shock experiment (Xiong *et al.*, 2001). *alx8* and *fry1* seedlings were more tolerant to PEG-6000-induced osmotic stress than their respective wild types in three separate experiments (Figures 3f and S3).

Molecular effects of the alx8 mutation

Global expression of *SAL1* mutants was measured to investigate the extent of change in mRNA, in addition to the six genes reported to change in *fry1* and *alx8* (Xiong *et al.*, 2001; Rossel *et al.*, 2006). Of the approximately 24 000 gene products quantified by microarray, 1414 were significantly up-regulated and 1033 down-regulated more than two-fold in *alx8* leaf tissue relative to their expression in Col-0 wild type ($P \leq 0.05$, $q \leq 0.1$; Figure 4a, Table S1). In *fry1-1* under

Figure 2. Loss-of-function mutations in SAL1 result in drought tolerance.

- (a) Drought tolerance of 4-week old soil-grown plants. Pictures are representative of the five plants per line of a thrice-repeated experiment.
 (b) Leaf relative water content (RWC) and (c) leaf water potential of leaves in non-stressed conditions and after 12 days of drought. Columns are mean $\pm$ standard deviation (SD) of at least four plants. The experiment was repeated twice with similar results.
 (d) Pot water content (g water/g dry mass) during drought treatment. Data are the mean $\pm$ SD for five pots, each consisting of a plant, soil and a thin plastic pot. The experiment was repeated three times.
 (e) Soil water potential at different soil water contents (SWC).

Table 2 Viability of *alx8* and wild-type during drought

Genotype	<i>n</i>	Viability (days)	<i>P</i> -value
Col-0	15	13 ± 3.0	
<i>alx8</i>	8	19 ± 1.1	<0.00001

Viability of plants during drought was assayed by measuring F_v/F_m . The day on which F_v/F_m is <33% of the controls, the plant is deemed unviable and will not recover if rewatered (Woo *et al.*, 2008).

Table 3 Drought tolerance at various ages and stages of development

Age (weeks)	Tolerant (yes/no)	Replicates
2	Yes (<i>alx8</i>)	15
4	Yes (<i>alx8</i> , <i>salk_020882</i> , <i>fry1-1</i>)	15, 10, 10
6	Yes (<i>alx8</i>)	11
8	Yes (<i>alx8</i> , <i>salk_020822</i>)	5, 5
Developmental stage		
Six leaves	Yes (<i>alx8</i>)	4
Mature vegetative	Yes (<i>alx8</i> , <i>fry1-1</i>)	27, 5
Flowering	Yes (<i>alx8</i> , <i>salk_020882</i> , no (<i>fry1-1</i>))	5, 5, 5

Plants grown to the same chronological age or the same developmental stage were subjected to drought as described in Figure 2 and scored as more tolerant than wild type (yes) or not different from wild type (no). The number of replicates for each mutant tested at a given stage is indicated.

well-watered conditions 1099 genes were significantly up-regulated and 745 down-regulated more than two-fold relative to C24 wild type ($P \leq 0.05$, $q \leq 0.1$; Table S2). *APX2*, *ZAT10* and *RAP2.6* are up-regulated in both the arrays and quantitative reverse transcription-PCR (qRT-PCR) (Rossel *et al.*, 2006). The 3.1-fold change in *RAP2.6* in the arrays was not significant due to variability between biological replicates (Table S1, Xiong *et al.*, 2001). The absence of any change in the arrays for *NCED3*, *GST6*, *APX1*, *RD29A* and *DREB2A* in *alx8* under well-watered conditions was also consistent with published results (Rossel *et al.*, 2006). The lack of induction of *RD29A* was confirmed by qRT-PCR (Figure 4b). Significantly, neither *SAL1* nor any of the five *SAL1* homologs were down-regulated in the arrays. One, *SAL3* (At5g63990), was up-regulated 16-fold; this was confirmed by qRT-PCR where a four-fold up-regulation was observed (SI in Table S3). Quantitative RT-PCRs for *SAL1* and *SAL2* (At5g64000) transcripts in *alx8* were marginally higher than wild-type, but this was not statistically significant (SI in Table S3).

A number of stress response genes were up-regulated in *alx8*, including: transcription factors such as *ZAT10*, *ZAT12*, *MYC2*, *RAP2.6* and *HB6*; several early light inducible proteins, *ELIPs*; an aquaporin *TIP5;1*; stress signaling kinase *SnRK2.2*; stress-inducible proteins *VSP1* and *VSP2*; and antioxidant enzymes *APX2*, *CSD1* and *CSD2* (Table 4).

Genes whose expression was up-regulated more than 25-fold were analyzed for co-expression in arrays stored in public databases using Genevestigator (Figure 4c). There was some correlation with drought, wounding, heat and cold arrays. Twelve per cent of the up-regulated genes in *alx8* were ABA-inducible, but a similar number were down-regulated by ABA. There was no strong correlation with other hormone treatments, except possibly with jasmonic acid treatment.

Role of *SAL1* in stomatal morphology and dynamics

Previously, *alx8* was shown to have lower stomatal conductance relative to wild-type as measured by gas exchange (Rossel *et al.*, 2006). This was not due to altered stomatal density, size and/or morphology of the *alx8* stomata. The stomata in *alx8* were normal in appearance and of a similar size to the wild type (Figure 5a). The stomata were not clustered as seen in mutants with altered stomatal development, neither were they located in a pit, which would decrease conductance by increasing resistance due to the boundary layer affect. The stomatal index of the abaxial and adaxial surface of *alx8* and wild-type leaves was calculated using epidermal peels (Figure 5b). No difference in stomatal index on the abaxial surface was found and there was a small, but significant, decrease on the adaxial surface of *alx8* leaves ($P < 0.01$, d.f. = 48). This was confirmed by calculations of the stomatal index from scanning electron microscopy images of the abaxial surface of independently grown plants (data not shown). The stomatal aperture, as measured by epidermal peels under non-stressed conditions at four time-points during the day, was not significantly different between *alx8* and wild type at all time-points and on both leaf surfaces (Figure 5c,d). The carbon-13/carbon-12 isotope ratio ($\delta^{13}C_p$) of leaf tissue of *alx8* and Col-0 wild type was compared as an indication of water use efficiency (WUE) over the lifetime of the plant. This ratio is inversely proportional to WUE and it is dependent on both the rate of carbon fixation and stomatal conductance (Farquhar and Richards, 1984). Under well-watered conditions it was found that there was no difference in $\delta^{13}C_p$ between the wild type and *alx8* (Figure 5e).

Plant morphology, growth and development

The leaves of *alx8*, *fry1-1* and *salk_020882* were shorter and rounder than wild-type leaves, with more lobed edges; the surface was often undulated and the petioles were shorter (Figure 6a). There were changes to the internal structure of the leaf including disorganized vascular bundles, altered cell shape, less defined palisade structure, smaller chloroplasts (Figure 6b) and thicker leaves (Figure 6b,c). There was no visible difference in the cuticle thickness or structure in *alx8* compared with wild type (Figures 5a and 6b). Although

Figure 3. Impact of size, development and morphology on drought tolerance.

(a) Drought tolerance of Col-0, *alx8* and *salk_020882* at the same developmental stage (commencement of flowering) (see Table 2).
 (b) Drought tolerance of plants at the same vegetative stage of development (mature green): *alx8* and *fry1-1* were 8 weeks old, Col-0 was 4 weeks old (see Table 2).
 (c) Pot weights for (a) were measured and plotted as a percentage of the original weight; the average $\pm$ SD of five of each line is plotted.
 (d) Rosette area of a range of 30-day old Col-0 plants grown in two separate experiments (Exp. 1 and 2) were plotted against drought viability by the method described in Table 2.
 (e) Dehydration of detached rosettes. Data are the mean $\pm$ SD for four biological replicates. The experiment was repeated three times.
 (f) Ion leakage of 10-day old seedlings after treatment with increasing polyethylene glycol (PEG) concentration. The values are the mean SE of three different experiments.

delayed in development and appearing smaller due to changes in leaf and rosette shape, *alx8* plants can accumulate similar rosette fresh and dry mass to the wild type by 8 weeks of growth (Table 5).

Metabolomics and starch accumulation

The metabolic profiles of *alx8* and *fry1-1* were compared with those of their respective wild types under well-watered conditions by gas chromatography-mass spectrometry (GC-MS). All four lines had different profiles as indicated by principal components analysis (PCA), with a degree of overlap between C24 and Col-0 (Figure 7). Both mutants were clearly separated from their respective wild types by the first principal component (PC1; accounting for 47.2% of

total variance) representing the largest class separation observable by PCA. Interestingly, *alx8* and *fry1-1* were clearly separated by the second principal component (PC2; accounting for 25.1% of total variance) while the two wild types were not.

There were significant increases in the levels of the polyamine putrescine in *alx8* and *fry1-1* (Table 6). This correlated with an increase in expression of the rate-limiting polyamine biosynthesis gene *arginine carboxylase* (*ADC2*) (6.43-fold) and a decrease in expression of the enzyme that converts spermidine to spermine, *ACL5* (–3.90-fold). There was no significant difference in the proline abundance in *alx8* relative to Col-0 despite an increase in the proline biosynthesis gene, *pyrroline-5-carboxylate reductase* (3.61-fold). In both *SAL1* mutants there were changes in the

Figure 4. Effect of mutations in *SAL1* on transcription.

(a) Overlap of significant gene expression changes (>two-fold, $P \leq 0.05$, $q \leq 0.1$) between *alx8* and *fry1-1*.

(b) Relative mRNA abundance of *RD29A* in *alx8* and *fry1-1*. Each column is the mean $\pm$ SD of three biological replicates. The experiment was repeated twice with similar results.

(c) Heat map of genes up-regulated 25-fold or more in *alx8* compared with their regulation in response to abiotic stress and hormone treatments.

abundance of a large number of sugars. These included decreased levels of fructose, galactose, glucose, cellobiose and a large number of unknown sugars and sugar derivatives. There was also a dramatic accumulation of unknown sugars and sugar derivatives that were present at very low levels in the wild type (Table 6). Also striking were strong decreases in the organic acids citrate, isocitrate, fumarate and malate and strong increases in a number of metabolites of unknown class (Table 6).

Transmission electron microscopy of wild-type and *alx8* leaves revealed that *alx8* chloroplasts lack transitory starch granules (Figure 8a). Iodine staining confirmed the relative reduction in starch accumulation in *alx8* and *fry1* compared with their respective wild types in both evening and morning samples (Figure 8b). The decrease in starch correlates with an increase in expression of two β -amylases, *BM1* (9.53-fold) and *BM8* (2.31-fold), in *alx8*.

Discussion

SAL1 is a negative regulator of drought tolerance

This study shows that the nucleotidase/phosphatase *SAL1* is a negative regulator of drought response networks that alter the transcriptome, metabolome and morphology of the rosette. Three different *SAL1* mutations all confer drought tolerance, thereby resolving the apparent paradox of *SAL1*; namely, *SAL1* is a negative regulator of stress signaling, but the *fry1-1* mutant did not show enhanced tolerance to the abiotic stresses tested (Xiong *et al.*, 2001). Here we demon-

strate that *SAL1* does negatively regulate transcriptional and metabolic pathways and *SAL1* knockouts have enhanced tolerance to drought.

The paradox did not reflect gain-of-function mutations as *alx8* and *fry1-1* lack detectable *SAL1* protein (Figure 1c) and *fry1-1* has reduced enzymatic activity on both substrates of *SAL1* (Xiong *et al.*, 2001). The *alx8* mutation, which occurs in a conserved domain of unknown function, inhibits accumulation of *SAL1* protein (Figure 1c), but does not inhibit *SAL1* transcription. The mutated *ALX8* protein produced in *E. coli* could not be recovered in the soluble fraction. It is not clear whether a translational blockage or a lack of protein stability in plants accounts for the absence of *SAL1* in *alx8* plants. Significantly, both alleles lack detectable *SAL1* protein in leaves.

The contrasting reports of drought sensitivity of *fry1-1* may in part reflect the different conditions used to impose water stress and as a consequence provide insight into the role of *SAL1*. Here intact, mature plants in soil are subjected to a gradual decrease in water availability over a period of 1–2 weeks. In the study where *fry1-1* was shown to be drought sensitive (Xiong *et al.*, 2001) leaves were detached or the seedlings were grown on media and exposed to mannitol or PEG-6000 to induce osmotic shock. Treatments with high concentrations of mannitol may result in plasmolysis of the cell rather than cytorrhysis (Munns, 2002; Verslues *et al.*, 2006) and toxicity (Hohl and Schopfer, 1991; Verslues *et al.*, 1998) leading to effects not related to water stress. With respect to PEG-6000, we observed decreased electrolyte leakage in *SAL1* mutants in response to PEG (Figures 3

Table 4 Changes in gene expression in *alk8* relative to Col-0

Gene ID	Gene name	Description	Stress induction ^a	Fold change	P-value	q-value
Stress response genes						
AT5G24770, AT5G24780	Vegetative storage proteins 1 and 2	VSP1/VSP2	ABA, JA, S, D, W	214.84	0.000	0.000
AT5G02490	Heat shock cognate 70 kDa protein 2	HSP70-2	H	17.40 ^b	0.002	0.005
AT3G09640	Ascorbate peroxidase 2	APX2	ABA, HL, O	9.30 ^b	0.003	0.006
AT4G14690	Early light inducible protein 2	ELIP2	C, H, OS, S	8.86 ^b	0.005	0.007
AT3G22370	Alternative oxidase 1A	AOX1A	ABA, C, OS, S	8.24	0.001	0.003
AT1G32640	Basic helix-loop-helix transcription factor	MYC2	D, W, JA, ABA	8.18	0.010	0.010
AT5G59820	Zinc finger protein 12	ZAT12	C, H, O, W, S	7.93	0.000	0.000
AT3G47440	Tonoplast intrinsic protein	TIP5;1	ABA, S(-)	7.76 ^b	0.000	0.000
AT1G52890	NAC domain containing protein	ANAC019	ABA, D, S	6.29 ^b	0.000	0.000
AT1G27730	Zinc finger protein 10	ZAT10	HL, C, S, D, ABA	5.00 ^b	0.028	0.017
AT1G08830	Copper/zinc superoxide dismutase 1	CSD1	H(-), ABA(-)	3.62	0.012	0.011
AT3G02480	ABA-responsive protein related	CSD2	C, S, H, D, ABA	3.61 ^b	0.033	0.019
AT2G28190	Copper/zinc superoxide dismutase 2	CSD2	ABA(-)	3.37	0.024	0.016
AT3G50500	SNF1-related protein kinase 2.2	SnRK2.2	O, S, ABA	3.19	0.000	0.000
AT5G14800	Pyrroline-5-carboxylate reductase	P5CR	H, S	2.83	0.001	0.003
AT3G22840	Early light-inducible protein	ELIP	C, S, OS, H, ABA	2.68	0.011	0.010
AT3G22840	Early light-inducible protein 1	ELIP1	C, H, OS, S	2.68	0.011	0.010
AT2G22430	HD-zip transcription factor	HB6	ABA, C, D, H(-)	-2.16	0.007	0.008
AT5G25610	Response to dehydration 22	RD22	S, OS	-3.73	0.001	0.003
AT4G19170	9- <i>cis</i> -epoxycarotenoid dioxygenase, putative	NCED4	H	-4.20	0.000	0.000
Starch metabolism and osmoprotectant synthesis						
AT4G15210	Beta-amylase 1	BMV1	W, D, C(-)	9.53	0.033	0.019
AT4G34710	Arginine decarboxylase 2	ADC2	S, OS, H(-), C, ABA	6.43	0.010	0.010
AT4G22590	Trehalose-6-phosphate phosphatase, putative		S, H(-), C	4.36	0.008	0.009
AT4G17090	Chloroplastic beta-amylase	BMV 8	C, OS(-)	2.31	0.003	0.006
AT5G19530	Spermine synthase	ACL5	S(-), H	-3.90 ^b	0.007	0.008
AT1G70290	Trehalose-6-phosphate synthase, putative		C(-)	-4.16	0.009	0.009

Selected up-regulated genes (2-fold, $P \leq 0.05$, $q \leq 0.1$). Values are the average of three biological replicates run on separate Affymetrix ATH1 chips.

^aStress inductions are from the literature or Genevestigator.

^bFold change compared to A call (below background level).

ABA, abscisic acid; JA, jasmonic acid; S, salt; D, water deprivation; W, wounding; HL, high light; C, cold; H, heat; O, oxidative; OS, osmotic; LH, low humidity; BR, brassinosteroids; GA, gibberellic acid.

Figure 5. Stomatal morphology and dynamics. (a) Representative images of stomatal morphology of the leaf abaxial surface. (b) The stomatal index was calculated from epidermal peels using five fields of view from leaves of five plants. Columns are means $\pm$ SD. (c), (d) Stomatal aperture under well-watered growth conditions as measured by epidermal leaf peel over a 9-h period. Values are the mean $\pm$ SD of four peels from each of three leaves from each of five plants. (e) Water use efficiency in *alx8* and *Col-0* of well-watered plants as measured by the carbon-13 isotope ratio ($\delta^{13}\text{C}$). Values are the mean $\pm$ SE of three replicate experiments of three biological replicates each.

Figure 6. Leaf morphology of SAL1 mutants. (a) Leaf morphology. (b) Representative cross-sections of embedded leaves stained with Toluidine blue. (c) Leaf thickness. Columns are the mean $\pm$ SE of multiple readings of nine leaves. The P -value of an unpaired t -test is shown.

and S3), which is the opposite result to that reported by Xiong *et al.* (2001). Regardless of the differing results for electrolyte leakage in response to PEG, the drought tolerance in soil was highly significant (Figures 2 and 3). In this context it is worth noting that only 27 of 806 up-regulated

genes were co-regulated in three water stress experiments using mannitol, filter paper or soil water deficit (Bray, 2004). Consequently, it is important to consider the nature of the imposed stress when evaluating the role of components of stress signaling networks.

Table 5 Fresh and dry rosette weights

Genotype	Fresh weight [SD] (g)	Dry weight [SD] (g)
Col-0	0.92 [0.42]	0.07 [0.03]
<i>alx8</i>	0.78 [0.27]	0.06 [0.02]

Whole rosettes of 8-week-old plants were measured for their fresh and dry weight. Eight plants from each genotype were analyzed. Differences between genotypes were not significant by an unpaired *t*-test.

Figure 7. Principal components analysis (PCA) of metabolite profiles of *alx8*, *fry1*, C24 and Col-0.

The abundance of over 150 metabolites in at least four biological replicates of each ecotype was used to calculate the PCA. The percentage of total variance accounted for by principal components 1 and 2 (PC1 and PC2) is plotted.

SAL1 mutants have higher leaf relative water content during drought but not constitutively increased water use efficiency

To investigate the mechanisms of drought tolerance we explored the physiological and morphological changes in *alx8* and *fry1-1*. The rate of detached rosette dehydration was the same for *alx8* and wild type (Figure 2), as was reported for *fry1* (Xiong *et al.*, 2001) and similar to reports of another drought tolerant mutant over-expressing the transcription factor, AREB1ΔQT (Fujita *et al.*, 2005). This suggests that the morphological changes such as different rosette shape, leaf shape and thickness in *alx8* (Figure 6) are not required for its drought tolerance.

The *alx8* plants were able to maintain constitutively higher RWC and leaf water potential than the wild type during drought at identical stress conditions, as indicated by similar pot water contents (Figure 2d). This is strong evidence that *alx8* plants are not avoiding drought by taking up less water,

rather they are able to maintain cell turgor at low soil water content for a longer period than the wild-type.

The drought tolerance of *alx8* is not dependent on developmental stage or the size of the plants (Table 3 and Figure 3): that is, equivalently sized *alx8* plants are more tolerant than Col-0 (Figure 3a,b). Finally, both SAL1 mutants lost fewer electrolytes than the wild types under high PEG concentration, suggesting there was a reduction of cellular damage under dehydration stress (Verslues *et al.*, 2006) (Figure 3f). Although this response was unexpected based on previous results (Xiong *et al.*, 2001), the reduced electrolyte leakage of young SAL1 mutants is consistent with all the soil-based experiments which show prolonged survival under water deficit conditions in soil.

Water use efficiency is the ratio of photosynthate produced to water used (Kirda *et al.*, 1992; Masle *et al.*, 2005) and is influenced by the relative rates of photosynthesis and transpiration; thus WUE need not indicate drought tolerance or vice versa (Martin *et al.*, 1999). Gas exchange measurements indicated that *alx8* had increased WUE during a short-term light response curve (Rossel *et al.*, 2006). However, carbon isotope ratio determination indicated that WUE of well-watered *alx8* and wild-type plants is similar (Figure 5e). The difference is possibly due to altered stomatal response times (data not shown) in the light response experiment (Rossel *et al.*, 2006), which are not significant over the lifespan of the plant. Alternatively, more complex interactions between stomatal conductance and SAL1 may explain the differences. For example, there may be secondary variable effects of SAL1 on phytic acid or ABA-response pathways that alter stomatal conductance. With respect to this, genes encoding three isoforms of phospholipase D (PLD, *At4g11830*, *At1g52570*, *At4g38560*), which catalyzes the production of phosphatidic acid, thereby promoting closure, are up-regulated up to six-fold in *alx8* (Table S1). Furthermore, the addition of putrescine can increase ABA in polyamine mutants (Cuevas *et al.*, 2008). Further investigation into the role of SAL1 in stomatal signaling is required.

SAL1 regulates the metabolome of Arabidopsis, including the production of osmoprotectants

Increased polyamine levels due to over-expression of biosynthetic enzymes have previously been shown to induce tolerance to a range of abiotic stresses, including drought (Kurepa *et al.*, 1998; Kasukabe *et al.*, 2004). The level of the polyamine, putrescine, was 15.2-fold (*P*-value = 0.008) higher in *alx8* leaves than in Col-0 leaves. Correspondingly, *alx8* has a higher expression of the polyamine biosynthetic enzyme ADC2, which is normally up-regulated in response to osmotic stress (Perez-Amador *et al.*, 2002; Urano *et al.*, 2003). Constitutively high putrescine levels have been reported in a drought-tolerant wheat variety and in oxidative

Table 6 Characteristic metabolic profiles of *SAL1* mutants

Metabolite class	Metabolite name	<i>alx8</i> versus Col-0		<i>fry1-1</i> versus C24	
		Fold difference	<i>P</i> -value	Fold difference	<i>P</i> -value
Organic acids	Citrate	<0.01	<0.001	0.09	0.009
	Fumarate	0.03	0.003	0.08	0.001
	Isocitrate	0.05	0.014	0.03	0.004
	Malate	0.08	<0.001	0.21	0.021
Polyamines	Putrescine	15.2	0.008	5.23	0.001
Carbohydrates	[Unknown disaccharide] RI = 2869	0.01	<0.001	0.01	<0.001
	Glucose	0.03	<0.001	0.09	<0.001
	[Unknown disaccharide] RI = 1946.9	0.04	0.001	0.11	<0.001
	[Unknown sugar] RI = 2116.9	0.05	<0.001	0.19	0.001
	[Unknown sugar] RI = 2122.6	0.05	<0.001	0.16	0.001
	Cellobiose	0.06	0.047	0.06	0.007
	Galactose	0.11	0.001	0.09	<0.001
	[Raffinose, putative ^a] RI = 3335.7	0.14	0.002	0.04	0.002
	Fructose	0.16	<0.001	0.29	0.002
	[Unknown disaccharide] RI = 2730.9	0.16	0.044	0.3	0.035
	[Unknown monosaccharide] RI = 1777.7	0.32	0.002	0.14	0.001
	[Galactinol, putative ^a] RI = 2963	0.41	0.007	0.07	0.003
	[Unknown sugar] RI = 2094.1	0.45	0.012	0.66	0.041
	[Unknown putative disaccharide] RI = 2836.1	0.45	0.009	0.22	<0.001
	[Unknown disaccharide] RI = 2534.4	5.63	<0.001	5.97	0.001
	[Unknown disaccharide] RI = 2544.4	13.92	<0.001	5.39	0.002
	[Unknown putative disaccharide] RI = 2808.4	50.86	<0.001	22.62	0.002
	[Unknown putative disaccharide] RI = 2923.4	75.11	0.001	26.13	0.018
	[Unknown putative disaccharide] RI = 2898.4	155.75	<0.001	227.13	0.001
	[Unknown sugar] RI = 2149.9	168.5	<0.001	60.67	0.001
	[Unknown probable disaccharide] RI = 2748.9	313.1	<0.001	136.59	<0.001
	[Unknown putative disaccharide] RI = 2997.5	2767.48	<0.001	5684.57	<0.001
	[Unknown putative disaccharide] RI = 2915.4	4624.26	<0.001	456.97	0.001
Unknown class	[Unknown] RI = 2656.2	13.33	<0.001	34.94	0.026
	[Unknown] RI = 3116.0	778.55	<0.001	92.18	<0.001
	[Unknown] RI = 2656.2	1027.05	<0.001	1100.47	0.006
	[Unknown possibly indole related] RI = 1404.6	99.82	0.002	26.54	0.005
	[Unknown related to tryptamine] RI = 1505.1	16.38	0.003	32.48	0.001
	[Unknown possibly indole related] RI = 1389.1	200.04	<0.001	104.69	0.002

Fold differences and *P*-values (*n* = 5) are shown for the major (most intense) metabolite differences that were common to both *alx8* and *fry1-1*. Unknown metabolites (enclosed in square brackets) were annotated based on the similarity of their mass spectra to reference spectra in the NIST05 mass spectral library. Retention indices (RI) are given for unknown metabolites.

^aPeaks for these metabolites were putatively identified by comparison with quadrupole reference spectra in the 'Q_MSRI_ID' library available from http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_msri.html but were not verified by comparison with authentic standards.

stress-tolerant variety of the weed *Conyza bonariensis* (Ye *et al.*, 1997). Also, levels of putrescine in the stress-sensitive *Arabidopsis* mutant *adc2-1* are only 25% of wild type (Urano *et al.*, 2004). Although the exact role of putrescine in tolerance remains uncertain, it has been suggested to function as a direct or indirect antioxidant defense (Ye *et al.*, 1997) and as a regulator of spermine and spermidine synthesis during drought (these provide an antisenesescence effect at the whole-plant level), resulting in phenotypically normal plants (Capell *et al.*, 2004). It would be of interest to decrease the production of putrescine in the *alx8* background by crossing with the *adc2-1* mutant or by treatment with the ADC inhibitor difluoromethylarginine (Xiong *et al.*, 2006) in order

to determine the contribution of putrescine and other polyamines to the drought tolerance of *alx8* and *fry1-1*.

Levels of a number of sugars are also altered in *alx8* leaf tissue, including large increases in a number of unidentified sugars (Table 6). These changes are inversely correlated with decreased accumulation of transitory starch in the chloroplasts of *alx8* (Figure 8a,b). Sugars may play an important role in the osmotic stress response as osmoprotectants, protecting macromolecules and preventing membrane fusion (Bartels and Sunkar, 2005). The accumulation of sugars and osmoprotectants in *alx8* and *fry1-1* is likely to be a contributing factor to the higher water content and viability of *SAL1* mutant leaves during drought. The reduced

Figure 8. Accumulation of starch in *SAL1* mutants compared with wild-type. (a) Starch granules in chloroplasts as imaged by transmission electron microscopy. Images are representative of those taken from three plants per line harvested at the same time of day. Cy, cytoplasm; Cp, chloroplast; V, vacuole; St, starch granule. (b) Starch content of leaves detected by iodine staining at the start of the day (08:30 am) and the end of day (20:30 pm).

loss of electrolytes observed in *SAL1* mutants during PEG treatment (Figure 3) also suggests a reduction of cellular damage under dehydration stress (Verslues *et al.*, 2006). Osmoprotectants may limit membrane damage, or alternatively the accumulation of sugars could alter water potential, resulting in reduced water flow and electrolyte leakage.

With regard to the changes in sugar-related osmoprotectants, one of the more interesting findings was that the most highly studied of the sugar-based osmoprotectants were either unaltered or decreased in concentration in *alx8* compared with the wild type. Specifically, trehalose, the accumulation of which has been shown to confer drought tolerance in transgenic *Arabidopsis* (Karim *et al.*, 2007) and rice (Garg *et al.*, 2002) plants, was unaltered in *alx8* or *fry1-1*.

Similarly, no changes were seen in the metabolite signal tentatively matched to *myo*-inositol (matched on the basis of mass-spectral similarity, abundance and approximate retention index match to MPIMP library, data not shown), a metabolite also implicated in osmoprotection and the biosynthesis of other osmoprotectants (Loewus and Murthy, 2000). *Myo*-inositol can be produced by the dephosphorylation of L-*myo*-inositol 1-phosphate (Loewus and Loewus, 1983). At least two *myo*-inositol 1-phosphate phosphatases (At1g31190 and At4g38690) were down-regulated in *alx8*. Perhaps more surprising was that metabolite and transcript data suggested a transcriptional down-regulation of the biosynthesis of raffinose family oligosaccharides (RFOs) – oligogalactosides of sucrose which have been implicated in desiccation tolerance of seeds (Taji *et al.*, 2002b) and resistance to drought, cold and other stresses (Taji *et al.*, 2002a; Nishizawa *et al.*, 2008). Galactinol synthase (GolS) catalyzes the rate-limiting step in RFO biosynthesis: the synthesis of galactinol (the donor of galactosyl units for RFO extension) from UDP-glucose and *myo*-inositol (Karner *et al.*, 2004). The drought-inducible isoform of GolS in *Arabidopsis*, AtGolS2 (At1g56600) (Taji *et al.*, 2002b), was down-regulated 28- and 57-fold in *alx8* and *fry1-1*, respectively. These decreases in AtGolS2 transcript abundance were correlated with 59% and 93% decreases in galactinol and 86% and 96% decreases in raffinose in *alx8* and *fry1-1*, respectively. Thus, the *alx8* drought-tolerant phenotype is likely to be independent of the production of galactinol and RFOs.

While we could find no evidence for increases in the most highly studied osmoprotectants, major accumulation was seen in a number of sugar-related compounds that could not be identified, even tentatively, by matching against public mass-spectral databases. These were the metabolites annotated as [Unknown Putative Disaccharide] eluting with retention indices of 2997.5 and 2915.4 (Table 6). Mass-spectral searching revealed a high degree of mass-spectral similarity to trehalose, suggesting that they may be unknown structural analogs of trehalose with similar properties conferring drought tolerance. Further structural elucidation work using complementary analytical technologies will therefore be required to determine the structures of these metabolites and, together with more targeted genetic experiments, may lead to the identification of novel osmoprotectant-producing pathways in *Arabidopsis*.

SAL1 regulates ABA-independent and ABA-dependent drought stress signaling pathways

The absence of *SAL1* resulted in up-regulation of more than 1000 genes and down-regulation of 500–1000 genes under non-stressed growth conditions compared with wild-type plants. This role as a regulator of gene expression in both stress and development is reinforced by its negative role in

post-transcriptional gene silencing (Gy *et al.*, 2007) and the altered morphology and flowering time of *SAL1* mutants. Just 7.6% of the 2447 genes differentially regulated in *alx8* are classified as stress response genes and most of these are not inducible by ABA, which suggests not all stress response pathways are activated in *alx8* under non-stressed growth conditions. For example, *APX2*, *RAP2.6*, *sHSP* and *DREB2A* are induced between 2- and 20-fold in non-stressed *alx8* leaves, but high light stress (HL) results in 25- to 700-fold induction of the same genes (Rossel *et al.*, 2006), demonstrating that other pathways that regulate these genes are stress-inducible and act synergistically with the *SAL1* pathway. Second, the expression of *RD22* is up-regulated by ABA (Yamaguchi-Shinozaki *et al.*, 1992) and this induction is partially dependent on the transcription factor *MYC2* (Abe *et al.*, 1997, 2003). However, in *alx8* despite an 8.2-fold increase in *MYC2* mRNA, *RD22* is down-regulated 3.7-fold. Likewise, there is no significant induction of *ADH1* or *KIN2/COR6.6*, which are ABA-responsive and *MYC2*-regulated (Abe *et al.*, 2003). This indicates a need for the interaction of multiple pathways in addition to the ABA-independent pathways constitutively activated in *alx8* and *fry1* for full activation of the stress response.

Of the known stress responsive transcription factors a small subset are significantly up-regulated in *alx8* under non-stressed conditions, including two zinc finger transcription factors, *ZAT10* and *ZAT12*. Both *ZAT10* and *ZAT12* are involved in the abiotic stress response pathways such as drought and high light (Rossel *et al.*, 2002, 2007; Sakamoto *et al.*, 2004; Davletova *et al.*, 2005). Overexpression of *ZAT10* induces expression of *APX2* and 18% of the genes up-regulated in HL (Rossel *et al.*, 2007). Correspondingly, 24.6% of the genes up-regulated by HL (Rossel *et al.*, 2007) are also up-regulated in *alx8*, further emphasizing the overlap between drought and light stress response networks (Kilian *et al.*, 2007). High levels of expression of *SAL1* in the vascular tissue (Xiong *et al.*, 2001) suggest a role in the transduction of signals during drought stress. This localization correlates with HL-induced H_2O_2 production, *APX2* and *ZAT10* expression, and systemic acquired acclimation in the bundle sheath (Karpinski *et al.*, 1997, 1999; Fryer *et al.*, 2003; Rossel *et al.*, 2007).

Drought induces an increase in ABA, and there is typically a corresponding increase in expression of the rate-limiting ABA biosynthetic enzymes *NCED3* and *NCED1* (Iuchi *et al.*, 2001). However, these genes are not up-regulated in *alx8*, nor is there down-regulation of genes involved in ABA catabolism; in fact, the catabolic enzymes *CYP707A1* and *CYP707A3* are up-regulated (Figure S4). Hence, the increase of ABA content in *alx8* (Rossel *et al.*, 2006) does not appear to be transcriptionally regulated. Interesting questions that remain to be answered are, as mentioned above: how does ABA change during drought in *alx8* and how does *SAL1* regulate stomatal signaling?

The majority of the up-regulated (66%) and down-regulated (53%) genes in *fry1-1* were co-expressed in *alx8* compared with wild-type. However, there were still differences in gene expression and metabolic profile between *alx8* and *fry1-1*, which is interesting given they are both loss-of-function mutations in the same gene. *C24* and *Col-0* had unique, although similar, metabolite profiles and thus it is possible that ecotype differences subtly alter the role of *SAL1* and the effects of its loss in each ecotype.

What are the substrate(s) of SAL1 in vivo?

SAL1 has been proposed to function in PI metabolism based on its high sequence homology with yeast inositol phosphatases, *MET22/HAL2* (Glaser *et al.*, 1993) and previous enzymatic assays of recombinant proteins (Murguía *et al.*, 1995; Quintero *et al.*, 1996; Xiong *et al.*, 2001). *SAL1* has been reported to dephosphorylate PAP, implicating it in sulfate metabolism and post-transcriptional gene silencing (Xiong *et al.*, 2001; Gy *et al.*, 2007). Furthermore, any actual role for IP_3 in abiotic stress signaling in plants has been debated (Perera *et al.*, 2008). Thus, a consideration of the possible substrate(s) for *SAL1* *in vivo* and how the substrate(s) affect overall cellular metabolism is needed.

Mature *fry1-1* rosette plants have higher basal levels of IP_3 compared with wild-type plants and an altered IP_3 response upon ABA treatment (Xiong *et al.*, 2001). However, the activity of *SAL1* against IP_3 and $Ins(1,4)P$ is 4% and 34%, respectively, compared with that against PAP and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) (Murguía *et al.*, 1995; Quintero *et al.*, 1996; Xiong *et al.*, 2001). This could be used to circumstantially argue against IP_3 being the primary substrate of *SAL1* *in vivo*. If that were the case, the reported increase in IP_3 could be a secondary effect of loss of *SAL1*. A proposal for this to occur via substrate feedback effects has been made (Guneseckera *et al.*, 2007). For example, *SAL1* could result in changes in other phosphatases, such as the 5Ptases. However, we did not observe any change in the transcript abundance in the microarrays for any of the 5Ptases. Alternatively, polyamines, specifically spermine and spermidine, increase the IP_3 pool in animal systems by stimulating PLC (Hedekov *et al.*, 1991; Spath *et al.*, 1991), PIP 5-kinase (that catalyses the production of the IP_3 precursor $Ins(4,5)P$) (Singh *et al.*, 1995) and decreasing the enzymatic activities of IP_3 5-phosphatase (Seyfred *et al.*, 1984). Additionally, as *alx8* plants have 15-fold higher levels of putrescine this could potentially alter PI pools.

3'-Phosphoadenosine-5'-phosphate and PAPS are involved in sulfate assimilation and transfer of sulfate groups (Saito, 2004). But *SAL1* may be dispensable for sulfate metabolism as *SAL1* mutants grow in hydroponics with sulfate as the only source of sulfur (data not shown).

3'-Phosphoadenosine-5'-phosphate inhibits exoribonuclease (XRN) activity (Dichtl *et al.*, 1997) and implicates SAL1 as a negative regulator of PTGS (Gy *et al.*, 2007). If this were the case, message levels of the target genes of XRN4 in *alx8* may be expected to be up-regulated. Two of 14 targets of XRN4 were found to be up-regulated in the microarrays in similar ways (two-fold) in both *alx8* and *xrn4-5* mutants (SI in Table S1; Souret *et al.*, 2004).

In summary, a careful and detailed evaluation of the synthesis and accumulation of PAP, PAPS and IP₃ and other potential substrates of SAL1 under a range of developmental and stress treatments in wild type, *alx8* and other relevant germplasm, such as other SAL homologs that have different relative specificities for PAP and IP (Gil-Mascarell *et al.*, 1999), is required to determine the actual *in vivo* substrate of SAL1 and which compound is regulating drought and high light-stress signaling.

Conclusions

The *SAL1* mutants *alx8* and *fry1* survive prolonged drought for 40–50% longer than wild-type plants. Whilst development is altered in these mutants this effect is temporary, as by the later stages of development the biomass of *alx8* and *fry1-1* rosettes is similar to their respective wild types. We hypothesize that the SAL1 protein has a key role in the negative regulation of pathways controlling morphological, physiological and molecular changes whose activation results in enhanced induction of stress networks. The evident health and growth of *alx8* leaves during drought is indicative of enhanced tolerance, rather than avoidance or escape responses. Under non-stressed conditions stress response genes such as the antioxidant *APX2* are constitutively up-regulated, amounts of osmoprotectants such as polyamines and sugars are elevated and ABA is accumulated. Thus, SAL1 negatively regulates drought tolerance.

Experimental procedures

Plant growth and drought stress

Arabidopsis plants were grown on metro mix soil (35% Canadian Peat Moss, 19% Perlite 500, 46% vermiculite, 1.5 g l⁻¹ lime), vernalized for 72–96 h and fertilized with 0.5 × Hoagland's solution once a fortnight (Hoaglands and Arnon, 1950). Growth conditions were either a light (150 µmol photons m⁻² sec⁻¹)/dark cycle of 12/12 h at 21 ± 2°C and 55% relative humidity or 8/16 h for Figures 3d, 6b–d and 7 or 16/8 h for Figure 3f. All seed was obtained from the Arabidopsis Biological Resource Center (ABRC) except *alx8* (Rossel *et al.*, 2004).

Drought treatments were applied to plants in soil with sufficient water by withholding further watering. Viability was determined by measuring leaf and soil relative water content, visual assessment, chlorophyll fluorescence and recovery after rewatering. See Appendix S1 for details.

Positional cloning and complementation

Linkage analysis was performed with the F₂ progeny of a cross between *alx8* and Landsberg *erecta* as described by Lukowitz *et al.* (2000). Further markers were designed around simple sequence length polymorphisms (SSLPs) and single-nucleotide polymorphisms (SNPs) found in the Cereon database (<http://www.arabidopsis.org/>; details available upon request). Derived cleaved amplified polymorphic sequences (dCAPS) primers were designed using dCAPS Finder 2.0 (<http://helix.wustl.edu/dcaps/dcaps.html>; Neff *et al.*, 2002). For sequencing, 600-bp fragments were amplified using Phusion Taq polymerase (Finnzyme, <http://www.neb.com/>), A-tailed and cloned into pGem T-easy (Promega, <http://www.promega.com/>) as per the manufacturer's instructions.

For complementation the T8H11 BAC was obtained from ABRC. *SAL1* and 1.7 kb of the upstream genomic sequence was digested from the BAC using *Pst*I, ligated into the binary vector pCAM2300 (Cambia, <http://www.cambia.org/>) and transformed into *Arabidopsis*.

SAL1 protein analysis

To produce recombinant SAL1 protein, total RNA was extracted from Col-0 and *alx8* leaves using a Plant RNeasy Kit with on-column DNase digestion (Qiagen, <http://www.qiagen.com/>) and used for first-strand cDNA synthesis (SuperScript II, Invitrogen, <http://www.invitrogen.com/>). The complete *SAL1* coding sequence was amplified from the cloned Col-0 and *alx8* *SAL1* cDNAs with the primers *Sac*II-SAL1 F1 (5'-CTCCGCGGTGGTATGGCTTACGAGAAAGAGC-3') and *Eco*RI-SAL1 (5'-GCTCGAATTCTCAGAGAGCTGAGCTTTCTC-3'), then cloned into the pHUE vector (Baker *et al.*, 2005). Recombinant proteins were expressed in *E. coli* strain BL21(DE3) (Novagen, <http://www.emdbiosciences.com/>) after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified by affinity chromatography using His-Bind resin according to the manufacturer's instructions (Novagen). SAL1 protein without the tag was assayed for phosphatase activity against PAP (Murguia *et al.*, 1995). The anti-SAL1 rabbit polyclonal antibodies (IMVS, Adelaide, <http://www.imvs.sa.gov.au/>) were isolated from the IgG fraction by immunoaffinity purification. For western blots, 20 µg of leaf protein was resolved on a 4–12% gradient gel, electrotransferred to a nitrocellulose membrane, and probed with a 1:1000 dilution of purified anti-SAL1 antibodies. After washes with 0.05% (v/v) Tween PBS, blots were incubated with a 1:10 000 dilution of HRP-conjugated goat anti-rabbit IgG and developed using the SuperSignal West Femto Chemiluminescent detection kit (Pierce, <http://www.piercenet.com/>). See Appendix S1 for detailed protocols.

Gene expression analysis

Total RNA was extracted from mature green leaves from 5-week old soil grown plants 2 h after 'dawn' using the Plant RNeasy Kit as described. The quality and quantity of RNA was tested by gel electrophoresis, BioAnalyzer (Agilent, <http://www.chem.agilent.com/>) and/or spectrophotometry. For qRT-PCR 1 µg of RNA was reverse transcribed to make cDNA using T23V primer and SuperScriptII (Invitrogen). Two technical replicates for each of the two reverse transcription reactions were done using LightCycler480 SYBR Master (Roche, <http://www.roche-applied-science.com/>) and a Rotorgene3000 (Corbett Research, <http://www.corbettlifesciences.com/>). Results were analyzed by comparative quantification with

each set of replicates averaged and normalized against a reference gene (see Appendix S1, Czechowski *et al.*, 2005; Schmittgen and Zakrajsek, 2000) and the control (Warton *et al.*, 2004). Primers used are listed in Appendix S1.

Microarrays were performed and analyzed using Affymetrix GeneChip® Arabidopsis Genome ATH1 Arrays (Affymetrix, <http://www.affymetrix.com/>), MAS5 normalization, statistical analysis and false discovery rate correction as described (Rossel *et al.*, 2007). Three biological replicates were analyzed for each genotype. All microarray data have been deposited in the ArrayExpress database (<http://www.ebi.ac.uk/microarray-as/ae/>) under the accession E-MEXP-1495.

Microscopy

The abaxial surface of leaves was imaged by cryogenic scanning electron microscopy and thin cross-sections of resin-embedded leaf material were used for transmission electron microscopy and light microscopy. See Appendix S1 for details.

Metabolomics

Metabolites from whole leaves were extracted and derivatized using a method modified from Roessner-Tunali *et al.* (2003). Derivatized metabolite samples were analyzed on a GC/MSD system (Agilent Technologies). Data files were processed using in-house MetaMiner software to carry out all peak detection, quantification, library matching, normalization, statistical analysis and data visualization. Detailed methodology is presented in Appendix S1. Analyte abundance matrices were exported from MetaMiner and chemical artifacts, internal standards and metabolite signals that were not detected in at least 80% of the replicates from at least one sample class (genotype) were removed from the dataset prior to PCA. Principal components analysis was carried out with Avadis Prophetic (Version 4.3, Strand Life Sciences, <http://www.strandgenomics.com/>) software with mean centering and scaling of all variables to unit variance. The input data matrix is supplied in Appendix S2.

Morphological and physiological methods

Total water potential was measured using a custom-built thermocouple psychrometer (Morgan, 1991). Leaf disks were placed into equilibration chambers for 4 h at 22°C. A Peltier cooling current was passed through the thermocouple and the electromotive force (e.m.f.) read as a needle deflection in a microvoltmeter (HR 33 Dew Point, Wescor, <http://www.wescor.com/environmental/>). The leaf water potential (MPa) was calculated by interpolation of the e.m.f. to a standard curve. Soil water potential was measured using a pressure plate apparatus as in Klute (1986).

Rosette leaves from the same plants were excised, the fresh weight (F_w) recorded and incubated in water for at least 4 h at 4°C in the dark. The leaves were blotted and the turgid weight (T_w) measured. Finally, leaves were dried at 80°C overnight and weighed to determine the dry weight (D_w). The relative water content (RWC) was calculated as (Jones, 2007)

$$\text{RWC} = \frac{F_w - D_w}{T_w - D_w}$$

For Figure 3d, plants were cultivated under 8/16 h light (100 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$)/dark cycle at $21 \pm 2^\circ\text{C}$ and 55% relative humidity for 30 days before initiating drought treatment.

Rosette size estimates were derived from fluorescence data at day 0 of drought treatment, and were plotted against the eventual drought survival times determined for each specimen. Fluorescence measurements were obtained using a CF Imager (Technologica, <http://www.technologica.co.uk/>).

To calculate the ^{13}C isotope ratio ($\delta^{13}\text{C}$), leaves from 4–5-week-old plants were harvested, dried and ground. The amount of carbon present was then measured by mass spectrometry and the carbon isotope ratio was calculated as described in Farquhar and Richards (1984).

For epidermal leaf peels, impressions of the leaf surfaces of 5-week-old plants were made using surface activated dental putty (Coltene Whaledent, <http://www.coltenewhaledent.biz/>). Clear cellulose varnish was applied to the impressions, mounted onto slides and viewed through a light microscope (Leica DMLB, <http://www.leica-microsystems.com/>). The stomatal index (SI) was calculated as $\text{SI} = [S/(E + S)] \times 100$ where S is the number of stomata and E is the number of epidermal cells. For starch analysis, the chlorophyll was removed by boiling in ethanol and leaves were stained in Lugol's solution for 5 min [5% (g/v) iodine, 10% (g/v) potassium iodide], then destained for 1 h in water. A precision micrometer (Mitutoyo, <http://www.mitutoyo.com/>) was used to measure leaf thickness.

Acknowledgements

Research was supported by the ARC Centre for Plant Energy Biology (CE0561495) and a Meat and Livestock Australia Postgraduate Award (PBW). JAL is funded by a Royal Society Research Fellowship, KJF by a University of Sheffield Scholarship, AJC by a GRDC PhD Scholarship and AHM by an ARC Australian Professorial Fellow (DP0771156). We are grateful for the assistance of Jan Bart Rossel in identifying *alx8* and establishing our interest in it; David Deery, CSIRO, for soil water potential measurements; Lily Shen and Dr Cheng Huang at ANU Electron Microscopy Unit; Drs Rana Munns and Grant Cramer for discussions; Drs Rohan Baker (JCSMR, ANU) and Spencer Whitney (RSBS, ANU) for providing the pHUE vector; Drs Tony Condon and Richard Richards (Plant Industry, CSIRO) for help with leaf water potential measurements and Stephen Graham for assistance with protein analysis.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression and purification of rSAL1 and rALX8 recombinant proteins.

Figure S2. Drought tolerance of two *alx8* plants versus one Col-0 plant per pot.

Figure S3. Measurement of electrolyte leakage in response to osmotic shock.

Figure S4. Expression of abscisic acid biosynthesis and catabolism.

Table S1. Microarray analysis of gene expression changes in *alx8*.

Table S2. Microarray analysis of gene expression changes in *fry1-1*.

Table S3. Gene expression of *SAL1*, *SAL2* and *SAL3* in Col-0 and *alx8*.

Appendix S1. Detailed experimental procedures.

Appendix S2. Metabolite abundance matrix for principal components analysis (PCA).

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