Seed Dehydration and the Establishment of Desiccation Tolerance During Seed Maturation is Altered in the Arabidopsis thaliana Mutant atem6-1

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The end of orthodox seed development is typified by a developmentally regulated period of dehydration leading to the loss of bulk water from the entire structure. When dehydration occurs, the cytoplasm condenses and intracellular components become more crowded, providing an environment amenable to numerous undesirable interactions that can lead to protein aggregation, denaturation and organelle–cell membrane fusion. Acquisition of desiccation tolerance, or the ability to withstand these very low water potentials and consequent molecular crowding, has been correlated with the accumulation of various protective compounds including proteins and sugars. Among these are the late embryogenesis abundant (LEA) proteins, a diverse class of highly abundant, heat-stable proteins that accumulate late in embryo maturation coincident with the acquisition of desiccation tolerance. Previous work led us to hypothesize that the protein ATEM6, one of the two Arabidopsis thaliana group 1 LEA proteins, is involved in regulating the rate at which water is lost from the maturing embryo; homozygous atem6-1 mutants display premature dehydration of seeds at the distal end of the silique. Here we demonstrate that rehydrated, mature seeds from atem6-1 mutant plants lose more water during subsequent air drying than wild-type seeds, consistent with a role for ATEM6 protein in water binding/loss during embryo maturation. In addition, and possibly as a result of premature dehydration, mutant seeds along the entire length of the silique acquire desiccation tolerance earlier than their wild-type counterparts. We further demonstrate precocious, and perhaps elevated, expression of the other A. thaliana group 1 LEA protein, ATEM1, that may compensate for loss or ATEM6 expression. However, this observation could also be consistent with acceleration of the entire normal maturation program in atem6-1 mutant embryos. Interestingly, ATEM6 protein does not appear to be required in mature seeds for viability or efficient germination.

Keywords: Arabidopsis • Dehydration • Desiccation tolerance • Germination • LEA protein • Seed maturation.

Abbreviations: ANOVA, analysis of variance; daf, days after flowering; GFP, green fluorescent protein; HSP, heat shock protein; LEA, late embryogenesis abundant; LSD, least significant difference; MS, Murashige and Skoog; PEG, polyethylene glycol; PVDF, polyvinylidene fluoride.

Introduction

Plant embryo development can conveniently be divided into two main phases, embryogenesis proper and maturation. During embryogenesis proper, important developmental cues lead to morphogenetic events that result in specification of the basic body plan and establishment of all the tissue types that will be found in the mature seed. This is followed by a period of maturation during which the body plan is elaborated, and storage compounds, that will serve as an energy source for the germinating seedling prior to photosynthetic competence, are accumulated. It is also during the later stages of the maturation phase that
the embryo acquires desiccation tolerance and undergoes a developmentally programmed dehydration event leading to dormancy and a quiescent state.

During desiccation, mature orthodox seeds reach levels of 5–10% water and can frequently be dried further to 1–5% water with little or no loss of viability. When dehydration occurs, the cytoplasm condenses and the intracellular components become more crowded. These conditions provide an environment for numerous undesirable interactions that can lead to protein aggregation and denaturation as well as organelle–cell membrane fusion (Hoekstra et al. 2001). Acquisition of desiccation tolerance, or the ability to withstand these very low water potentials and subsequent molecular crowding, has been correlated with the accumulation of various protective compounds including proteins (Bartels et al. 1988, Vertucci and Farrant 1995, Black et al. 1999) and sugars (Sun et al. 1994, Black et al. 1999, Buitink et al. 2000).

As the embryo prepares for desiccation, there is a shift in carbohydrate accumulation from simple to more complex sugars. Sucrose, raffinose and stachyose are present at low levels early in embryo development, but accumulate to much higher levels just prior to desiccation (Ooms et al. 1992). The ability of sugars to form superviscous fluids, particularly the non-reducing sugars found in mature seeds, was the original foundation for the prediction that intracellular glasses contribute to desiccation tolerance in plants (Burke 1986, Williams and Leopold 1989). More recently, numerous studies have evaluated the properties of model glasses as well as the glassy matrix in anhydrobiotic plants and seeds (Koster 1991, Leopold et al. 1994, Leprince et al. 1995, Wolkers et al. 1998). The overall conclusion of these studies is that carbohydrates cannot be the only molecules involved in vitrification (Ooms et al. 1992). Because many proteins are also abundant in the cytoplasm, it is likely that they also play a role in glass formation (reviewed in Buitink and Leprince 2004).

Two classes of proteins most likely to make a major contribution to cellular stability in mature seeds include the small heat shock proteins (HSPs) and the late embryogenesis abundant (LEA) proteins. Small HSPs, that accumulate late in seed development (Wehmeyer et al. 1996, Wehmeyer and Vierling 2000), may help minimize the aggregation effects of cytoplasm condensation (Feder and Hofmann 1999) by acting as molecular chaperones and thereby contribute to stabilization of a glassy state. LEA proteins are a diverse class of highly abundant, heat-stable proteins that accumulate late in embryo maturation and during the developmentally regulated period of dehydration at the end of seed development. Accumulation of LEA proteins has been correlated with the acquisition of desiccation tolerance (Bartels et al. 1988), and some members also accumulate in pollen, another anhydrobiotic structure in plants. In addition, as LEA proteins have also been shown to abrogate protein aggregation in vitro (Goyal et al. 2005), they may function in vivo in a manner similar to that of the small HSPs. It is likely, therefore, that aqueous glass formation involves at least oligosaccharides, HSPs and LEAs, together maintaining cellular stasis and avoiding the damaging consequences of dehydration (reviewed in Hoekstra et al. 2001).

LEA expression is induced by the stress-related phytohormone ABA, and may be activated by either endogenous or exogenously applied ABA. While LEA proteins are mainly active during seed development, many LEA genes are also inducible in response to water stress in vegetative and reproductive structures (Berge et al. 1989, Morris et al. 1990, Bostock and Quatrano 1992, Wilhelm and Thomashow 1993, Bies et al. 1998). Although individual LEA proteins have been shown to represent up to 4% of total cellular protein (Roberts et al. 1993), the transcripts and proteins rapidly degrade upon imbibition (Roberts et al. 1993, Bies et al. 1998). It is therefore possible that some LEAs have a dual role during the plant life cycle, and function as a storage protein during germination as well as in desiccation tolerance during seed development.

The D-19 family (Baker et al. 1988), or group 1 LEAs, were first identified in wheat by Cuming and Lane (1979). Since then, group 1 LEAs have been identified in many other plants including Arabidopsis thaliana (Gaubier et al. 1993). This group of proteins is typified by a highly conserved 20 amino acid signature motif that, although present only once in many of the group 1 LEAs, may be tandemly repeated up to four times (Espelund et al. 1992, Stacy and Aalen 1998, Cuming 1999). Unlike some of the other, more broadly expressed LEAs, group 1 proteins are expressed exclusively in embryonic tissues, and are not inducible in adult tissues, even in response to ABA (Bies et al. 1998).

Arabidopsis thaliana has two endogenous group 1 LEA genes, ATEM1 and ATEM6 (Gaubier et al. 1993). ATEM1 expression precedes expression of ATEM6 by approximately 2 d, though mRNA and protein remain abundant for both through maturation and into dry, mature seed. These two genes encode very similar proteins that differ primarily in the number of repeats in the conserved 20 amino acid signature motif (four copies in ATEM1 vs. one copy in ATEM6). The expression domain of ATEM1 is limited mainly to the provascular tissues and the root tip, although there is also some expression in the shoot meristem and vasculature of the cotyledons (Vicient et al. 2000). ATEM6 has a much larger expression domain, comprising essentially the entire embryo with particularly strong expression in the shoot meristem and provascular tissue.

Previous work in this lab identified an insertional mutation in the ATEM6 gene of Arabidopsis (Manfre et al. 2006). The T-DNA insertion was present 2 bp 3’ of the TAA stop codon, effectively separating the 3’-flanking region from the remainder of the gene by >7 kb. This mutant, atem6-1,
contains no detectable ATEM6 protein in homozygous mutant seed. The mutant also displays premature dehydration and maturation of seeds at the distal ends of siliques at a time coincident with normal ATEM6 expression, suggesting that this protein may function to buffer the rate at which water is lost from the seed. Introduction of a wild-type gene into *atem6-1* mutant plants (*ATEM6-C*) restores the wild-type phenotype.

To investigate further the in planta function of ATEM6 protein during seed development, we have evaluated the effect of loss of ATEM6 expression on water loss rates in mature seeds. In addition, we have characterized the germination capabilities of both mature and immature *atem6-1* mutant seeds. We present evidence that ATEM6 protein is not required in mature seeds for viability and/or efficient germination. Further, we show that absence of ATEM6 results in accelerated acquisition of desiccation tolerance in mutant seeds that may be associated with changes in seed water loss.

### Results

#### Seed water loss and water content assay

Earlier work in this lab documented evidence of accelerated seed dehydration and maturation (as evidenced by accumulation of seed coat pigmentation) of *atem6-1* seeds at the distal end of the siliques (Manfre et al. 2006). Due to the hydrophilic nature of ATEM6 protein and the fact that it is expressed throughout maturing wild-type embryos, we postulated that ATEM6 protein might function to influence the rate at which water is lost during seed development.

To test this hypothesis, water loss rates for fully hydrated, mature seeds from *atem6-1* and wild-type plants were determined. In addition, we assessed the water content of seeds at different stages of treatment. Seeds were uniformly dried and five replicates of 100 seeds each were re-hydrated overnight at 4°C with sterile water. The seeds were then placed in an aluminum pan and residual water removed by centrifugation (see Materials and Methods for details). Water loss from the fully hydrated seeds was monitored by weighing on a microbalance, with readings taken every minute for 90 min.

As can be seen in Fig. 1A, weight loss by drying is a biphasic process for both genotypes. Initial weight loss is relatively rapid, and more than two-thirds of the total loss during the 90 min assay is achieved in the first 20 min. The rate of weight loss is essentially identical for both genotypes during this time, and we interpret this phase of drying to be due to the loss of water that was absorbed by the outermost layers of the seed. This is followed by a significantly slower rate of weight loss that we attribute to water that is more tightly bound, most probably water that has been taken up by the embryo during the overnight imbibition period. Less than 0.20% of the total weight loss occurs during the final 10 min of the assay, suggesting that any remaining water is very tightly bound.

![Fig. 1 Water loss and water content assays. (A) Average weight loss by slow drying for five replicate samples of *atem6-1* mutants seeds (open circles) and wild-type seeds (open squares). The data are expressed as mg H₂O lost per mg dry weight. (B) Average seed water content of *atem6-1* mutants seeds (gray bars) and wild-type seeds (black bars) before and after overnight hydration and after slow drying for 90 min. The data are expressed as mg H₂O mg⁻¹ dry weight. Error bars in both panels represent the standard deviation.](https://academic.oup.com/pcp/article-abstract/50/2/243/1858755/243-253)

Interestingly, and despite no difference in mature seed water content before or after treatment in an LiCl hygrostat (Fig. 1B, Initial and LiCl, respectively), *atem6-1* seeds take up more water than wild-type seeds (Fig. 1B, Hydrated).
On drying, both genotypes lose >97% of their water content during the assay period, but mutant seeds lose more water than wild-type seeds. Only part of this difference can be accounted for by the increase in water uptake, and atem6-1 mutant seeds reach a significantly lower water content than wild-type seeds (P < 0.001).

### Polyethylene glycol germination assay

The visible phenotype of atem6-1 seeds, premature dehydration and maturation, is coincident with the time during which wild-type embryos express ATEM6 protein. Despite the lack of detectable ATEM6 protein, seeds from homozygous mutant plants are not only viable but germinate readily under standard conditions. However, germination conditions in the laboratory are usually near optimal, and any fitness cost associated with the atem6-1 lesion may not be easily observed. To determine if atem6-1 seeds are compromised for germination under non-optimal conditions, germination rates of wild-type and atem6-1 seeds were assessed on media of decreasing water potential.

The high molecular weight solute polyethylene glycol (PEG) 8000 was chosen to reduce the water potential of the media based on experiments described by van der Weele et al. (2000) and Penfield et al. (2001). Because autoclaving changes the size of the PEG polymer, all solutions were filter sterilized before use. Five replicates of 100 seeds each were plated on Murashige and Skoog (MS) media containing different levels of PEG, stratified for 3 d at 4°C, and germination was scored after 5 d. The data in Fig. 2 represent the average percentage germination. All seeds, regardless of genotype, displayed approximately 100% germination at 0, 5 and 10% PEG concentrations. The rates of germination drop to approximately 55–75% at 15% PEG and to <25% at the 20% PEG concentration. Although germination was significantly suppressed at 20% PEG, there was no statistically significant difference between germination rates for wild-type and atem6-1 mutant seed (P = 0.08) at this or any other level of PEG. These data demonstrate that loss of ATEM6 in atem6-1 homozygous seed produces no difference in mature seed viability or germination as compared with wild-type seeds under the conditions tested.

### Acquisition of desiccation tolerance (ADT) assay

The results presented above suggest that atem6-1 seeds acquire essentially full desiccation tolerance even in the absence of detectable ATEM6 protein. In fact, the visible phenotype of premature dehydration and maturation of seeds, that is often limited to the first 4–5 seeds at the distal end of the silique, suggests that the entire maturation program may be altered in mutant seeds. If this is the case, mutant seeds might be predicted to acquire desiccation tolerance at a time different from the wild type. To examine this, and to determine if there is a difference between plant-proximal and plant-distal seeds during the maturation process, the four proximal-most and four distal-most seeds from either side of the replum of wild-type and atem6-1 plants were dissected from siliques at 7, 10, 13 and 16 days after flowering (daf). These seeds were dried in a hygrostat for 2 d and subsequently rehydrated. Germination was scored over 10 d as no further germination was observed after 10 d post-rehydration. Some seeds were also plated directly (without drying) on germination medium to assess fresh germination rates.

Seeds placed directly on germination medium after dissection (7, 10, 13 and 16 daf) displayed no difference in germination rates between the two genotypes (data not shown). There was, however, a marked difference in germination for the dried seeds. Fig. 3A and B show germination rates for seeds dissected 10 daf, plant-proximal and plant-distal, respectively. As can be clearly seen, atem6-1 seeds germinate much more readily than wild-type seeds in both the proximal and distal positions, although location does appear to have an effect. These differences are no longer seen for seeds dissected at 13 daf when virtually all seeds for both genotypes germinate over 10 d after rehydration (Fig. 3C, D). Essentially complete germination is also obtained for seeds dissected from the plant-proximal end of siliques 16 daf (Fig. 3E). Interestingly, seeds dissected from the plant-distal end of siliques 16 daf show decreased germination rates for both genotypes (Fig. 3F). The reason for this is not clear. No seeds dissected 7 daf germinated after drying, regardless of genotype.

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**Fig. 2** PEG germination assay. Average percentage germination for five replicate plates of atem6-1 mutants seeds (gray bars) and wild-type seeds (black bars) on various concentrations of PEG. Error bars represent the standard deviation.
A fully fit three-way analysis of variance (ANOVA) was performed to evaluate these differences in germination more critically (Table 1). This analysis found that all three factors of the model (genotype, seed position and daf) explain significant proportions of the variation in percent germination. A least significant difference (LSD) post hoc test found that the atem6-1 genotype displayed significantly higher germination ($t > 0.315, P < 0.001$) than either wild-type or ATEM6-C seeds. However, a significant genotype by daf interaction ($F_{4,162} = 13.702, P < 0.001$) indicates that this difference was only observed at 10 daf (LSD post hoc test for 10 daf: $t > 0.514, P < 0.001$). Interestingly, seeds from the proximal end of siliques were found to have higher germination rates than distal seeds ($F_{1,162} = 12.737, P < 0.001$), with the greatest differences at 10 and 16 daf.

**Developmental progression**

The results presented above suggested that atem6-1 seeds acquire essentially full desiccation tolerance even in the absence of detectable ATEM6 protein and at a time earlier than the wild-type. If this is the case, it should be possible to demonstrate that other gene products associated with desiccation tolerance also accumulate precociously. While the details of this process are still poorly understood, one
gene product that could be immediately assessed was the other group 1 LEA protein, ATEM1. The polyclonal anti-wheat Em antibody already in hand (Manfre et al. 2006) recognizes both ATEM6 and ATEM1, and allowed examination of ATEM1 expression in developing wild-type and atem6-1 seeds.

In order to compare the temporal expression pattern of ATEM1 in wild-type and atem6-1 mutant seeds, the four plant-proximal and plant-distal seeds from each side of the central replum were harvested from siliques at some of the same time points used for the ADT assay (10, 13 and 16 daf). Approximately 40 seeds were used for extraction and 35 µg of total soluble protein was electrophoresed on SDS–polyacrylamide gels with mature wild-type seed protein extracts (both proximal and distal) as controls. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane for detection of ATEM proteins using the polyclonal anti-wheat Em antibody.

In wild-type seeds, ATEM1 and ATEM6 proteins both became detectable in plant-proximal and plant-distal seeds at approximately 16 daf and remained detectable through the mature seed stage (Fig. 4A). In atem6-1 seeds, however, ATEM1 protein became detectable at 13 daf (Fig. 4B), consistent with a general acceleration of the maturation process. Detection of ATEM1 protein in both plant-proximal and plant-distal seeds is also consistent with the observance of accelerated desiccation tolerance in seeds at both positions. While these immunoblots do not represent a true quantitative assessment of group 1 LEA expression, the level of ATEM1 protein in atem6-1 seeds appears to be significantly higher than in wild-type seeds.

To assess further the expression of ATEM1 in the atem6-1 background, wild-type and atem6-1 mutant plants were transformed with an ATEM1–green fluorescent protein (GFP) fusion construct. This construct contains the entire ATEM1 gene including the promoter (with the 5′-untranslated region), coding region (with intron) and 3′ control sequences, into which a GFP coding region (Chiu et al. 1996) has been inserted 5′ of the ATEM1 stop codon. Therefore, it should be possible to obtain information about both the temporal and spatial pattern of ATEM1 expression. Developing seeds were dissected from siliques at 12, 13, 14 and 15 daf, as described for the ADT assay, and seed coats were removed to facilitate GFP imaging.

GFP fluorescence is not detectable in either wild-type or mutant embryos until 13 daf. However, while spatial expression at this stage does not appear to be significantly altered, fluorescence intensity is noticeably higher in atem6-1 mutant embryos than in the wild type (compare Fig. 5B and F). The difference in expression levels between the wild type and atem6-1 is even more dramatic in embryos 14 daf and there appears to be noticeable expansion of the expression domain in atem6-1 embryos. This observation is consistent with elevated levels of ATEM1 protein seen in atem6-1 seeds in Fig. 4. Interestingly, there is also a consistent difference in expression level between distal and proximal embryos within the two genotypes in which distal embryos have greater

### Table 1 ANOVA, effect of genotype, seed position and daf on germination in the ADT assay

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^DF, degrees of freedom.

Fig. 4 Immunoblot analysis of wild-type and atem6-1 seed proteins during seed development. ATEM proteins were detected with a polyclonal anti-wheat Em protein antibody that detects both ATEM1 and ATEM6. (A) Wild-type; (B) atem6-1. Lanes 1, 10 daf proximal; 2, 10 daf distal; 3, 13 daf proximal; 4, 13 daf distal; 5, 16 daf proximal; 6, 16 daf distal; 7, mature seed proximal; 8, mature seed distal; 9, wild-type mature seed control.
fluorescence than proximal embryos. By 15 daf, distal atem6-1 embryos have lost most if not all of their chlorophyll (as evidenced by a reduction in autofluorescence) and begun to dehydrate, whereas wild-type embryos still display some chlorophyll autofluorescence and are fully hydrated.

**Discussion**

Acquisition of desiccation tolerance is a tightly regulated process that occurs during the maturation phase of seed development. Seeds activate a variety of genes and metabolic pathways during this time, and the accumulation of sugars as well as specific groups of proteins has been correlated with maximal desiccation tolerance (Bartels et al. 1988). We recently identified a mutant (atem6-1) that no longer expresses ATEM6, one of the two group 1 LEA proteins in Arabidopsis. The phenotype of this mutant, that includes premature dehydration of plant-distal seeds, led us to suggest that ATEM6 protein may be involved in regulating the rate at which water is lost from the maturing seed (Manfre et al. 2006).

To assess the potential role of ATEM6 protein in seed water loss in a more direct way, we measured the rate and extent of weight loss for re-hydrated mature seeds after removal of excess water. The results of this assay provided a biphasic curve in which drying during the initial 20 min was very rapid, and both the rate and amount of water lost from wild-type and atem6-1 seeds was indistinguishable. During development of the Arabidopsis seed, a hydrophilic polysaccharide (mucilage) is deposited in the outer cell layer of the seed coat (Windsor et al. 2000). This epidermal layer of mature seeds hydrates very rapidly on imbibition and forms a hydrogel around the entire seed that, in isolation (i.e. a non-soil environment), might be predicted to lose water readily to the atmosphere. We attribute this rapid early phase of drying to the loss of water that was absorbed by the mucilage.

Weight loss after 20 min is much slower and we attribute this phase of drying to water that is more tightly bound by the mature seeds, potentially representing water absorbed by the embryo. Notably, the amount of water lost by atem6-1 seeds is greater than that by the wild type beginning at approximately 25 min into the assay. This difference is statistically significant after 35 min of drying and remains so for the duration of the assay. Seeds for both genotypes lost <10 ng of weight (<0.20% of the total weight loss) during the final 10 min, suggesting that water remaining after 90 min is very tightly bound. An intriguing observation of this experiment was that atem6-1 seeds appear to take up significantly more water during imbibition than wild-type seeds (Fig. 1B, Hydrated, \( P = 0.04 \)). The additional water present in hydrated atem6-1 seeds only partially accounts for the difference in water lost between wild-type and mutant seeds in the assay, and atem6-1 seeds reach a statistically significantly lower water content than wild-type seeds (\( P < 0.001 \)). Certainly the mechanism(s) by which seeds lose water during normal maturation on the plant will be somewhat different from this assay. However, this result is consistent with the phenotype of atem6-1 mutant plants and is also consistent with a role for ATEM6 protein in embryonic water binding.

Greater water uptake by atem6-1 seeds also suggests a role for ATEM6 during imbibition in which it may function to limit and/or buffer water uptake. It is relatively well established that intracellular glasses form during late maturation of angiosperm seeds and that these glasses are probably composed of sugar–protein mixtures (Wolkers and Hoekstra 1997, Buitink and Leprince 2004). In fact, the glasses may have a significant protein component (Wolkers et al. 1998, Buitink et al. 2000). Due to their abundance in the cytoplasm of embryonic cells during this time of seed development,
it is also likely that group 1 LEA proteins contribute directly to glass stability. In the absence of ATEM6 protein, the stability of the glass present in the embryo may be compromised such that more water can be absorbed. Much like it has been proposed to prevent a rapid efflux of water during maturation, ATEM6 may help prevent a rapid influx of water during imbibition that could be deleterious, particularly to membranous structures. Additional biophysical characterization ofatem6-1 mutant seeds may shed light on this possibility.

Considering that group 1 LEA protein structure and expression pattern are so highly conserved across essentially all angiosperms, monocot and dicot alike, and the Arabidopsis genome only encodes two group 1 LEAs, complete absence of one might be anticipated to be associated with a variety of effects. Despite this mutation, however, mature dry seeds fromatem6-1 plants appear fully viable and germinate readily under laboratory conditions. This result was somewhat unexpected. Further, even when testing germination under suboptimal conditions, there was no observable difference between wild-type andatem6-1 mutant seeds. Clearly, ATEM6 protein is not essential for seed viability or germination proficiency, at least under the conditions tested.

While the complexity of desiccation tolerance in seeds and the involvement of a number of different molecules may make it difficult to assess the contribution of any individual molecular species, the absence of ATEM6 protein inatem6-1 seeds clearly has an effect on seed maturation (Manfre et al. 2006). If ATEM6 makes an important and direct contribution to desiccation tolerance, then one could hypothesize that the acquisition of tolerance in the mutant might be delayed until later in seed maturation when other factors have accumulated that can compensate for the absence of ATEM6. In fact, our results demonstrate that desiccation tolerance inatem6-1 mutant seeds is acquired earlier than in the wild type, rather than later. Considering the potential role of ATEM6 protein in water binding/loss during maturation, absence of this protein could lead to lower seed water content at a time in development earlier than normal. This could be perceived as a stress by the plant or, alternatively, embryo water potential may serve as an endogenous cue during maturation. Either of these possibilities could then lead to a general acceleration of the normal maturation program to help ensure seed survival. Interestingly, there is also a difference in the acquisition of desiccation tolerance between plant-proximal and plant-distal seeds within a silique wherein plant-proximal seeds develop tolerance slightly earlier. However, this appears to be a general observation for both wild-type andatem6-1 mutant seeds and is, therefore, not related to the presence or absence of ATEM6 protein.

If there is a general acceleration of the maturation program, whether as the result of a perceived stress or of an endogenous cue, it should be possible to demonstrate premature accumulation of other molecular species involved in desiccation tolerance. Unfortunately, our understanding of the molecular mechanisms involved in this phenomenon is still limited. As a first approximation, we have examined expression of the other group 1 LEA protein, ATEM1, as this molecule is equally likely to contribute to desiccation tolerance and displays a similar temporal expression pattern in the embryo. Both immunoblot and GFP fusion analyses show that expression of ATEM1 is indeed detectable earlier inatem6-1 seeds than in wild-type seeds. In addition, accelerated expression is observed in both plant-proximal and plant-distal seeds, further supporting a mechanism that affects maturation in all mutant seeds, not just those in a plant-distal location. It is not yet known if expression of ATEM1 inatem6-1 mutant seeds directly compensates for the lack of ATEM6. Because the embryonic expression patterns of these proteins only partially overlap (Bies et al. 1998), this might be unlikely in the absence of extensive expansion of the ATEM1 expression domain. Our results with the ATEM1-GFP protein fusion, however, may support some expansion of the ATEM1 expression domain, but this possibility will need to be examined in a more critical analysis.

This study provides evidence that the ATEM6 protein is involved in a mechanism(s) associated with water retention/loss during seed maturation. Whether ATEM6 is directly involved in desiccation tolerance per se is not clear at this time, but it is not required for viability or efficient germination under any conditions tested here. Absence of the protein leads to premature dehydration of seeds, particularly those at a plant-distal location within the silique, and is correlated with accelerated acquisition of desiccation tolerance in mutant seeds. In addition, expression of the other A. thaliana group 1 LEA protein that may also be involved in seed water relations, ATEM1, is similarly accelerated. While not conclusive proof, these observations are consistent with general acceleration of an otherwise normal maturation process inatem6-1 mutant seeds. It will be of great interest to examine the expression patterns of other gene products that may also be involved in the acquisition of full desiccation tolerance.

Materials and Methods

Plant materials

Wild-type A. thaliana plants were of the Columbia-0 ecotype. Theatem6-1 T-DNA insertion line has been previously described (Manfre et al. 2006).

Seed water loss and water content assay

Mature seeds from each genotype (100 seeds per replicate) were initially dried over a saturated LiCl solution...
dehydration of all samples. Seeds were rehydrated by saturating the filter papers with 0.5× MS medium. To minimize evaporation, each plate was wrapped with Parafilm M, then placed in a pan containing water-saturated 3MM paper and covered with a plastic dome lid to maintain humidity. Plates were incubated under a 16 h photoperiod at 24±2°C of white fluorescent light at 250 µmol m⁻² s⁻¹. Germination was scored by radicle emergence once a day for 10 d.

Statistical analyses
Data from the water loss assay and PEG germination assay were individually subjected to unpaired t-test analyses. Data from the ADT germination assay were analyzed using a fully fit three-way ANOVA and an LSD post hoc test using Systat software (Version 10, Systat, Point Richmond, CA, USA). The dependent variable for the three-way ANOVA, percentage germination, was subjected to arc-sine square root transformation to meet the assumption of normality of residuals.

Immunoblot analysis
The four plant-proximal and plant-distal seeds from each side of the central replum were harvested from wild-type andatem6-1 siliques at 10, 13 and 16 daf. Total soluble protein was isolated from approximately 40 seeds for each sample by grinding in a solution of 10 mM Tris–HCl (pH 8.3), 10 mM NaCl in a microcentrifuge tube. The tubes were vortexed well and centrifuged repeatedly for 2 min at maximum speed at 4°C until no solid material remained at the bottom of the tube. Extracts were then frozen at −20°C and put directly into the centrifuge to spin at maximum speed at 4°C for 30 min. The supernatant was transferred to a new tube and, heated to 80°C for 20 min followed by centrifugation at maximum speed at 4°C for 30 min. This supernatant was transferred to a fresh tube and protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA) at 100 V and 4°C for 30 min. The supernatant was transferred to a fresh tube and protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Extract volumes containing 35 µg of total soluble protein were brought to a final concentration of 25% trichloracetic acid, incubated on ice for 5 min and pelleted at 4°C in a microcentrifuge at maximum speed for 5 min. The supernatant was removed and the protein pellets were washed with 100% acetone and spun for another 5 min at 4°C. The acetone was removed, and the pellets dried slightly and resuspended in gel loading buffer. After resolution on denaturing 15% Tris-glycine gels, proteins were electro-transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA, USA) at 100 V and 4°C for 1 h. Blots were processed using a primary polyclonal rabbit anti-wheat Em antibody and a secondary alkaline phosphatase-conjugated goat anti-rabbit antibody. Chemiluminescent detection was performed using CSPD-ready-to-use (Roche Diagnostics, Indianapolis, IN, USA) and images captured on a Fuji LAS-1000plus (Fujifilm Life Science USA, Stamford, CT, USA).

PEG germination assay
PEG 8000 at a concentration of 50% (w/w) was sterilized by filtration through a 0.22 µm filter. MS medium (one packet of MS minimal salts (#M6899, Sigma, St Louis, MO, USA) supplemented with 1% sucrose, 0.8 mg l⁻¹ thiamine, 0.5 mg ml⁻¹ pyridoxine, 0.5 mg ml⁻¹ nicotinic acid and 50 mg l⁻¹ myo-inositol) was mixed with 50% PEG (w/w) and water to a final concentration of 0, 5, 10, 15 and 20% PEG and 0.25× MS. Mature seeds were uniformly dried by treatment in a hygrostat containing a saturated LiCl solution (relative humidity approximately 13%) for 2 d and subsequently surface sterilized using a vapor method (http://plantpath.wisc.edu/~afb/vapster.html). Five replicate plates with 100 seeds each on Whatman-1 filter paper saturated with 0.25× MS medium ± PEG in plastic Petri plates (7 cm diameter) were prepared. To minimize evaporation, each plate was wrapped with Parafilm M. The seeds were stratified at 4°C for 3 d and then grown at 24±2°C under white fluorescent light at 105°C overnight and weight loss measured for 90 min. Data were acquired using WinWedge® 32 Standard v. 3 software (TAL Technologies, Philadelphia, PA, USA). After slow drying for 90 min, the pans with seeds were placed in an oven at 105°C for 24 h and then reweighed. The weight of each pan was determined after removing the dry seeds and subtracted from all weights for that sample.

ADT germination assay
Developing seeds were dissected from siliques at four time points during development as determined by tagging individual flowers on the day of anthesis. To collect immature seeds, the siliques were opened from the base to the tip with Dumont forceps (#4 and #5). All dissections were done with sterile instruments in a laminar flow hood. Ten replicate plates for each data point were sown with 16 seeds onto water-saturated 2.0 cm diameter Whatman No. 1 filter paper in 2.5 cm diameter plastic Petri plates. The seeds were dried over saturated LiCl in a hygrostat for 2 d to ensure equal dehydration of all samples. Seeds were rehydrated by saturating the filter papers with 0.5× MS medium. To minimize evaporation, each plate was wrapped with Parafilm M, then placed in a pan containing water-saturated 3MM paper and covered with a plastic dome lid to maintain humidity. Plates were incubated under a 16 h photoperiod at 24±2°C of white fluorescent light at 250 µmol m⁻² s⁻¹. Germination was scored by radicle emergence once a day for 10 d.

Acquisition of desiccation tolerance

**Table 2** PCR primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATEM1-L</td>
<td>CTACACCTTTAACACCTTAATCCAAATC</td>
</tr>
<tr>
<td>ATEM1-R</td>
<td>TGAGATCTCTTTAAATCAGAAGTTGCTTGTG</td>
</tr>
<tr>
<td>ATEM1-Stul-L-C</td>
<td>GAATCTAAGACGATCTTTGCTGAAGTATT</td>
</tr>
<tr>
<td>ATEM1-Stul-L-NC</td>
<td>CTAAGGCTGTGTGGATTTCCTGACTCATC</td>
</tr>
<tr>
<td>ATEM1-Stul-R-C</td>
<td>AAGCGGTTAGACTTACAGCTACTAGTC</td>
</tr>
<tr>
<td>ATEM1-Stul-R-NC</td>
<td>CAAAAGTATACTTCTCCAACCTTTTATAATC</td>
</tr>
</tbody>
</table>

**GFP analyses**

A 3,007 bp fragment of genomic DNA containing the entire ATEM1 gene was PCR amplified using ATEM1-L and ATEM1-R primers (Table 2) and blunt-end cloned into pBluescript II KS+ at the Smal site to create pBM487. A unique Stul site was introduced at the ATEM1 stop codon for insertion of a GFP-coding region. Two PCR products were generated using the ATEM1-Stul-L-C/ATEM1-Stul-L-NC (548 bp) and ATEM1-Stul-R-C/ATEM1-Stul-R-NC (498 bp) primer pairs, digested with Stul and ligated together. The product was then digested with BglII–NdeI and the 818 bp fragment used to replace the corresponding fragment in pBM487 to create plasmid pBM498, and the Stul site verified by sequence analysis. A jellyfish GFP-coding region was isolated from the GFP(S65T) plasmid (Chiu et al. 1996) by digestion with Stul–PstI and the PstI site rendered blunt by treatment with the large Klonek fragment of Escherichia coli DNA polymerase I. This fragment was ligated into the Stul site of pBM498 to create pBM499 and verified by sequence analysis. The entire ATEM1-GFP fragment was transferred to pBI101 as an EcoRI–HindIII fragment to create pBM507, and verified by restriction enzyme digestion.

Plasmid pBM507 was introduced into Agrobacterium tumefaciens strain LBA4404 by heat shock (Chen et al. 1994) and the resulting strain used to transform both wild-type and Atem6-1 A. thaliana plants using a floral dip method (Clough and Bent 1998). Transformed seeds were selected on kanamycin (50 μg ml⁻¹) and verified by PCR. Developing seeds were dissected from siliques at 12, 13, 14 and 15 d after anthesis as described for the ADT assay. Seed coats were removed and images captured using a Nikon SMZ1500 dissecting scope with an X-Cite® light source and Q-Capture software (version 2.68.6, Q-Imaging, Burnaby BC, Canada). Magnification and exposure settings were held constant for all images.

**References**


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