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Protein Repair L-Isoaspartyl Methyltransferase1 Is Involved in Both Seed Longevity and Germination Vigor in *Arabidopsis* [W]

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The formation of abnormal amino acid residues is a major source of spontaneous age-related protein damage in cells. The protein L-isoaspartyl methyltransferase (PIMT) combats protein misfolding resulting from L-isoaspartyl formation by catalyzing the conversion of abnormal L-isoaspartyl residues to their normal L-aspartyl forms. In this way, the PIMT repair enzyme system contributes to longevity and survival in bacterial and animal kingdoms. Despite the discovery of PIMT activity in plants two decades ago, the role of this enzyme during plant stress adaptation and in seed longevity remains undefined. In this work, we have isolated *Arabidopsis thaliana* lines exhibiting altered expression of *PIMT1*, one of the two genes encoding the PIMT enzyme in *Arabidopsis*. PIMT1 overaccumulation reduced the accumulation of L-isoaspartyl residues in seed proteins and increased both seed longevity and germination vigor. Conversely, reduced PIMT1 accumulation was associated with an increase in the accumulation of L-isoaspartyl residues in the proteome of freshly harvested dry mature seeds, thus leading to heightened sensitivity to aging treatments and loss of seed vigor under stressful germination conditions. These data implicate PIMT1 as a major endogenous factor that limits abnormal L-isoaspartyl accumulation in seed proteins, thereby improving seed traits such as longevity and vigor. The PIMT repair pathway likely works in concert with other anti-aging pathways to actively eliminate deleterious protein products, thus enabling successful seedling establishment and strengthening plant proliferation in natural environments.

INTRODUCTION

Accumulation of spontaneous covalent damage in proteins is a main cause of aging (reviewed in Clarke, 2003). Proteolysis of altered proteins and their replacement by de novo synthesis generally occurs in all living systems to limit their deleterious effects in cells and tissues (Dalling, 1987; Stadtman et al., 1992). However, some types of age-related protein damage could be reversibly repaired at a lower energetic cost (Reissner and Aswad, 2003). The protein L-isoaspartyl (D-aspartyl) *O*-methyltransferase (EC 2.1.1.77; PIMT) is a repair enzyme whose function is to limit protein damage induced by accumulation of abnormal isoaspartyl (isoAsp) residues from spontaneous chemical degradation of asparaginyl (Asn) and aspartyl (Asp) residues (Lowenson and Clarke, 1992). Deamidation of Asn residues or dehydration of Asp residues leads to the formation of an unstable succinimidyl ring (Figure 1) that spontaneously hydrolyzes at either carbonyl group to generate a mixture of Asp and abnormal isoAsp residues (Geiger and Clarke, 1987; Aswad et al., 2000). The β-linkage distinguishing the isoAsp form introduces a kink into the peptide backbone that is generally detrimental for protein folding and activity (Mamula et al., 1999; Esposito et al., 2000). Such misfolding can be repaired by the ability of PIMT to specifically recognize isoAsp residues in polypeptides and to catalyze the first step that leads to their conversion to the Asp form. In this reaction, the isoAsp residue is transiently methylesterified at the α-carboxyl group (Figure 1) through an *S*-adenosyl-L-methionine (AdoMet)-dependent reaction (Aswad et al., 2000). Owing to these properties and since isoAsp formation represents a major source of spontaneous covalent damage that afflicts proteins under physiological conditions (reviewed in Clarke, 2003), it is surmised that PIMT plays a major role in repairing altered isoAsp-containing proteins in vivo (Doyle et al., 2003; Reissner and Aswad, 2003; Lanthier and Desrosiers, 2004).

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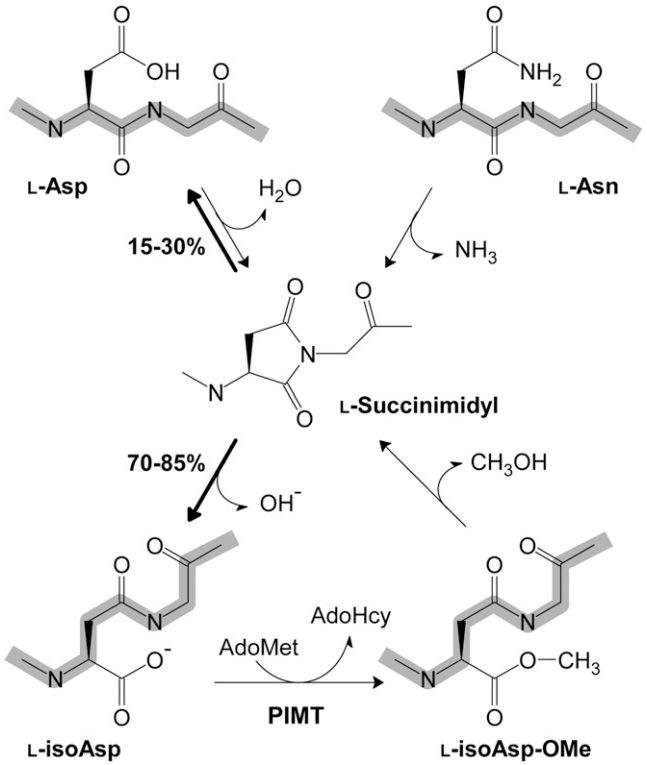


Figure 1. Mechanism of Spontaneous L-Isoaspartyl Formation and Enzymatic Repair by PIMT.

Deamidation of Asn (top right) and isomerization of Asp (top left) lead to the formation of an unstable succinimidyl ring that is spontaneously hydrolyzed to generate a mixture of Asp (15 to 30%) and of abnormal isoAsp (70 to 85%) residues at physiological pH. The peptide backbone is shown in gray to emphasize the kink in the polypeptide chain that occurs in the isoAsp bond and the linear nature of the chain in the normal Asp bond. PIMT catalyzes the first step of the conversion of isoAsp into Asp. The formed L-isoAsp-OMe spontaneously gives rise to Asp (15 to 30%) and isoAsp (70 to 85%). Thus, several enzymatic cycles are needed to fully repair proteins. AdoHcy, *S*-adenosyl homocystein; L-Asp, L-aspartyl; L-Asn, L-asparaginyl; L-isoAsp, L-isoaspartyl; L-isoAsp-OMe, L-isoaspartyl-*O*-methylester.

PIMT is a highly conserved enzyme that has been described in a wide range of organisms, including gram-negative bacteria, plants, invertebrates, such as nematodes, and vertebrates, including humans (Kagan et al., 1997b). Furthermore, in *Escherichia coli*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice (Reissner and Aswad, 2003; Shimizu et al., 2005), there is genetic evidence that PIMT plays a protective role in vivo to overcome environmental stress in aging tissues. Overaccumulation of PIMT in *E. coli* (Kindrachuk et al., 2003) and in *Drosophila* (Chavous et al., 2001) is associated with phenotypes of high heat-shock survival and extended lifespan under high temperature conditions, respectively. The role of PIMT as an aging-related repair enzyme is also supported by the characterization of PIMT-deficient mutants, which exhibit a reduced dauer phase survival in *C. elegans* (Kagan et al., 1997a), a higher sensitivity to oxidative stress in stationary *E. coli* cells (Visick et al., 1998a), and epileptic seizures in mice that severely limit their survival to only 12 weeks (Kim et al., 1997, 1999). In such PIMT-deficient backgrounds, isoAsp residues become overrepresented in proteins, indicating that in vivo PIMT maintains a low level of isoAsp in proteins, thus delaying aging, particularly in cells with low metabolic activity.

In contrast with other systems, the expression of plant PIMT exhibits several distinctive features. In *Arabidopsis thaliana*, there are two *PIMT* genes, *PIMT1* and *PIMT2* (Mudgett and Clarke, 1996; Xu et al., 2004), whereas animal or bacterial genomes contain only one such gene. While *PIMT1* mRNA accumulates to relatively high levels in a range of tissues, *PIMT2* transcripts are detected at low levels and only during seed maturation (Xu et al., 2004). Furthermore, unlike the constitutive *PIMT* gene expression in animal tissues (reviewed in O'Connor, 2006), PIMT activity in plants appears to be under environmental and developmental regulation. For example, in winter wheat (*Triticum aestivum*), seedlings challenged with dehydration, salt stress, or abscisic acid (ABA) treatment (Mudgett and Clarke, 1994) and in germinated maize (*Zea mays*), seeds submitted to osmotic or salt stress (Thapar et al., 2001), PIMT activity is elevated, suggesting that this enzyme is important mainly under stressful conditions. Furthermore, in most plants, PIMT activity is confined to the seeds, suggesting that this enzyme activity is necessary for maintaining seed vigor (Mudgett and Clarke, 1993; Mudgett et al., 1997). Thus, polycentenarian sacred lotus (*Nelumbo nucifera*) seeds exhibit an exceptionally high PIMT activity, presumably accounting for their astonishing longevity (Shen-Miller et al., 1995). Also, a low vigor 17-year-old barley (*Hordeum vulgare* cv Himalaya) seedlot was shown to contain both a high isoAsp amount and a very low PIMT enzyme activity (Mudgett et al., 1997). These data raised the hypothesis that protein damage is particularly detrimental for seed longevity and correlatively that a limitation of isoAsp accumulation in the seed proteome contributes to seed vigor and longevity (Mudgett and Clarke, 1993; Kester et al., 1997; Mudgett et al., 1997). Consistent with this, aged barley seeds were shown to contain increased levels of isoAsp residues in proteins (Mudgett et al., 1997).

The above findings suggest that PIMT activity contributes to seed viability in plants. Here, we examine the physiological consequences of altering PIMT accumulation in *Arabidopsis* seeds. We report the isolation and characterization of a T-DNA insertion in the 5′-region upstream of the *PIMT1* coding sequence. This mutation induces an increased accumulation of PIMT1 in freshly harvested dry mature seeds, leading to a reduced accumulation of aberrant isoAsp residues in proteins. Furthermore, this mutant displays enhanced seed longevity and increased germination efficiency under abiotic stress conditions. A phenotypic analysis of transgenic lines overaccumulating or underaccumulating the PIMT1 enzyme in *Arabidopsis* seeds confirmed our main finding that the PIMT repair enzyme limits in planta the accumulation of deleterious isoAsp residues in seed proteins and hence contributes to seed longevity and germination vigor.

RESULTS

Isolation and Molecular Characterization of a *PIMT1* T-DNA Insertion Line

To investigate the physiological role of PIMT1, we first screened collections of T-DNA insertion mutants of *Arabidopsis* (the Salk Institute Genomic Analysis Laboratory, SIGnAL *Arabidopsis* Gene Mapping Tool, http://signal.salk.edu/cgi-bin/tdnaexpress; The Arabidopsis Information Resource [TAIR], http://www.arabidospis.org). By this approach, no mutation in the coding sequence of the *PIMT1* gene (AT3G48330) could be detected. However, the use of a PCR screening assay with specific primers for T-DNA borders and the *PIMT1* locus (AT3G48330) allowed isolating a mutant (line DRR122) from the INRA-Versailles collection. This line contains a T-DNA insertion in the 5′ part of the *PIMT1* gene (Figure 2A), and we named the corresponding allele *pimt1-1*. Sequence analyses of the *pimt1-1* allele, which is in the Wassilewskija genetic background, identified a single T-DNA insertion in direct orientation located 187 bp upstream of the annotated translation start codon with exact left and right T-DNA borders and a deletion of 13 bp in the flanking region of the right border (Figure 2B). This 13-bp deletion encompasses the predicted MT1a ABA-responsive element (ABRE) that was previously suggested to be in the *Arabidopsis PIMT1* gene (Mudgett and Clarke, 1996) and that exhibits features of the *Em1a* ABRE motif of the wheat *Em* promoter (Marcotte et al., 1989). The self-progeny of the hemizygous T1 plants segregated the kanamycin resistance marker with a 3:1 segregation ratio (153 kanamycin resistant:47 kanamycin sensitive; $\chi^2 = 0.24$) testifying to a single Mendelian locus for the T-DNA insertion. DNA gel blot analysis using a T-DNA fragment and *PIMT1*-specific probes confirmed the occurrence of a single T-DNA insertion at the *PIMT1* loci in the DRR122 line (see Supplemental Figure 1 online). The T3 generation of homozygous DRR122 line was used throughout in the following study.

PIMT1 expression was assessed by RT-PCR in freshly harvested dry seeds and in 5-d-old seedlings grown either on water or on water for 4 d and then further incubated for 1 d on 100 μM ABA, both in wild-type and *pimt1-1* backgrounds (Figure 2C). *PIMT1* mRNA accumulation was high in the dry mature seeds of the *pimt1-1* mutant, while it was barely detectable in corresponding wild-type seeds. Furthermore, although *PIMT1* expression was very similar in 5-d-old wild-type and *pimt1-1* mutant seedlings grown on water alone (Figure 2C), ABA treatment led to a threefold increase of *PIMT1* expression in wild-type seedlings but not in *pimt1-1* mutant seedlings, which in the latter case remained at water control levels. However, germination of wild-type and *pimt1-1* seeds was similarly affected by exogenous ABA applied in a range of physiological concentrations (e.g., 0 to 3 μM that did not completely inhibit germination) (see Supplemental Figure 2 online).

The Higher PIMT1 Amount in *pimt1-1* Seeds Correlates with a Lower in Vivo IsoAsp Accumulation

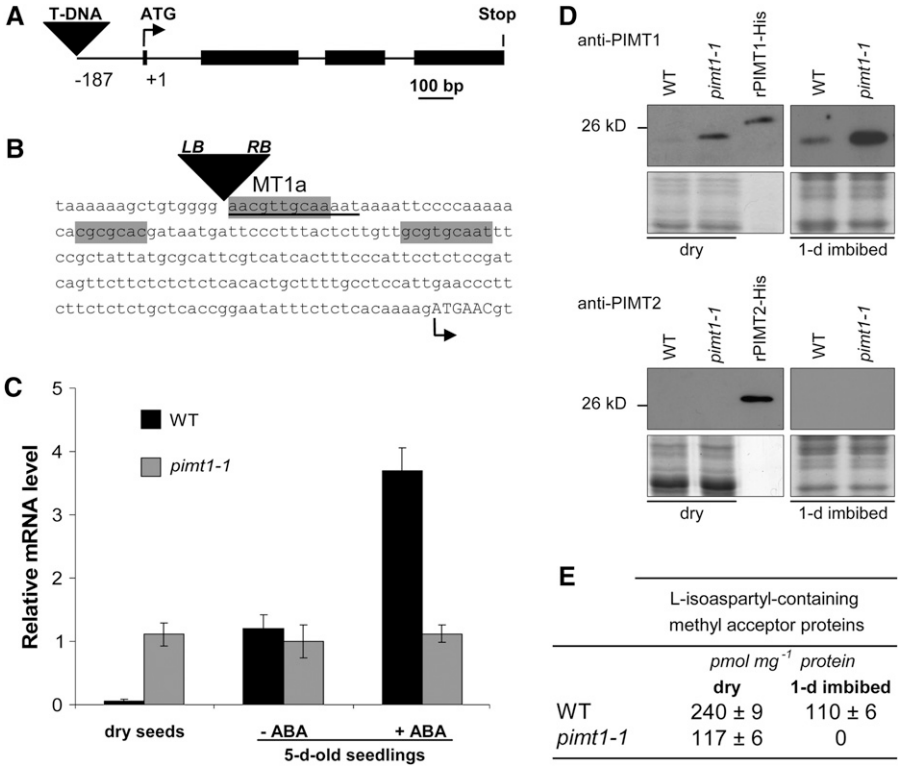
Based on the proposed role of PIMT in seed longevity (Shen-Miller et al., 1995; Mudgett et al., 1997), the accumulation of the PIMT1 enzyme and of isoAsp residues in seed proteins was compared in wild-type and *pimt1-1* backgrounds (Figures 2D and 2E). PIMT1 protein was detected only at very low levels in freshly harvested wild-type dry mature seeds (Figure 2D). In marked contrast, this level was substantially increased in the mutant background (Figure 2D). Thus, the different levels of *PIMT1* mRNA in wild-type and *pimt1-1* mutant seeds (Figure 2C) correlated well with respective protein levels. Since the *Arabidopsis* genome contains two *PIMT* genes, namely, AT3G48330 and AT5G50240, encoding PIMT1 and PIMT2, respectively (Mudgett and Clarke, 1996; Xu et al., 2004), it was important to distinguish between these two proteins in protein gel blotting experiments. To this end, in addition to the PIMT1-specific antibodies, we prepared PIMT2-specific antibodies (see Supplemental Figure 3 online). Figure 2D clearly shows that PIMT2 protein was not detected in dry mature seed samples, neither from wild-type nor from *pimt1-1* mutant backgrounds.

These specific antibodies allowed the demonstration that the accumulation of PIMT1 protein sharply raised after 1 d of imbibition, both in wild-type and *pimt1-1* mutant seeds (Figure 2D). All together these results indicate that in *Arabidopsis* dry mature seeds and during early germination the major part of PIMT activity originates from *PIMT1* gene expression. Measurement of extractable PIMT activity from freshly harvested dry mature seeds of the wild type and *pimt1-1* mutant (see the legend to Figure 2D) allowed the determination of an enzyme-specific activity of 0.36 and of 1.25 pmol min^{-1} mg^{-1} protein, respectively. The higher extractable PIMT activity in *pimt1-1* mutant seeds fits well with the higher PIMT1 protein accumulation in these seeds (Figure 2D) and suggests that the stock of PIMT enzyme identified in the mature seeds could be readily functional during germination.

The accumulation of isoAsp residues was measured in freshly harvested dry mature and early imbibed seeds of both the wild-type and *pimt1-1* backgrounds (Figure 2E). In wild-type dry and 1-d imbibed seeds (Figure 2E), isoAsp content was substantially higher (240 and 96 pmol mg^{-1} protein, respectively) than in corresponding *pimt1-1* seeds (117 and ~0 pmol mg^{-1} protein, respectively). Also, it appears clearly that for both the wild-type and mutant seeds, the initial level of isoAsp identified in the dry mature state decreased markedly following seed imbibition. These findings are consistent with the hypothesis that PIMT1 prevents in vivo isoAsp accumulation in the seed proteome by a repair mechanism, presumably during seed maturation (cf. wild-type and *pimt1-1* isoAsp levels in dry mature seeds) and at early stages of seed germination (cf. isoAsp levels between dry and imbibed seeds for both wild-type and *pimt1-1* seeds).

Seeds of the *pimt1-1* Mutant Are More Resistant to Aging Treatments

To further document the role of PIMT1 in seeds, both wild-type and *pimt1-1* mutant seeds were submitted to a storage treatment at 40°C that has been previously described as the controlled deterioration treatment (Bentsink et al., 2000; Groot et al., 2000; Tesnier et al., 2002; Rajjou et al., 2008). This aging test is widely used for estimating the storage potential of seedlots



	L-isoaspartyl-containing methyl acceptor proteins	
	pmol mg^{-1} protein	
	dry	**1-d imbibed**
WT	240 ± 9	110 ± 6
pimt1-1	117 ± 6	0

Figure 2. Characterization of the *pimt1-1* Mutant Allele.

(A) Schematic representation of the *PIMT1* locus. The arrow indicates the translational start site, black rectangles represent exons, and introns are indicated between exons. The black triangle indicates the position of the T-DNA insertion 187 bp upstream of the ATG initiation codon.

(B) *PIMT1* sequence flanking the T-DNA insertion. The black triangle marks the T-DNA insertion site. LB and RB indicate the left and right borders of the T-DNA insert, respectively. The sequence deletion in *PIMT1* caused by the insertion of the T-DNA is underlined. The three putative ABRE motifs previously described by Mudgett and Clarke (1996) are highlighted in gray, and the first motif that encompasses the 13-bp deleted sequence is called MT1a (Mudgett and Clarke, 1996).

(C) RT-PCR comparison of *PIMT1* transcript accumulation in the wild type (black) and *pimt1-1* mutant (gray) freshly harvested dry mature seeds and in 5-d-old seedlings cultivated on water (−ABA) or on water for 4 d and transferred the fourth day to water containing 100 μM ABA for an additional day (+ABA). Means ± SD are shown (n = 3).

(D) PIMT1 and PIMT2 protein accumulation levels were compared in wild-type and *pimt1-1* dry mature seeds and corresponding 1 d imbibed seeds. Proteins (30 μg) were separated by SDS-PAGE and immunodetected with polyclonal antibodies specifically raised against PIMT1 or PIMT2. Recombinant rPIMT1-His or rPIMT2-His proteins (30 ng) were used as controls. Parts of the Coomassie blue–stained gels are presented below the protein gel blot as loading controls. Total protein extracts of wild-type and *pimt1-1* dry seeds display a PIMT enzyme activity of 0.36 ± 0.05 pmol min^{-1} mg^{-1} protein and 1.2 ± 0.09 pmol min^{-1} mg^{-1} protein, respectively.

(E) Quantitation of L-isoaspartyl-containing methyl-accepting substrates in wild-type and *pimt1-1* dry mature seeds and corresponding 1 d imbibed seeds. Values are from three repetitions each using 75 mg of seeds (mean ± SD).

(Delouche and Baskin, 1973) for all species with orthodox storage behavior (Kruse, 1999). Both temperature of the storage conditions and seed moisture content are key factors of seed deterioration and viability loss during storage (Roberts, 1972; Priestley, 1986; Roberts and Ellis, 1989; McDonald, 1999; Bradford et al., 1993). Accordingly, two storage conditions at 40°C were used that differed by the relative humidity (RH) at which treatments were conducted. Namely, these two conditions allowed seeds to equilibrate at 40°C to ~15 to 20% or 8% water content, respectively. With the storage treatment conducted at 40°C and 15 to 20% water content, a loss in seed vigor occurred after 4 and 6 d of treatment for the wild-type and the *pimt1-1* mutant seeds, respectively (Figure 3A). This behavior is similar to that described with dry mature seeds from various *Arabidopsis* accessions (Bentsink et al., 2000; Tesnier et al., 2002; Clerkx et al., 2004; Sattler et al., 2004; Devaiah et al., 2007; Rajjou et al., 2008; Wohlbach et al., 2008). After 8 and 10 d of storage at 40°C and 15 to 20% water content, the germination percentages monitored 4 d after sowing reached 52 and 25%, respectively, for the wild-type seeds compared with 80 and 50%, respectively, for the *pimt1-1* mutant seeds. These data indicate that the *pimt1-1* seeds are significantly more resistant to a loss in seed vigor imposed by the storage treatment than the wild-type seeds.

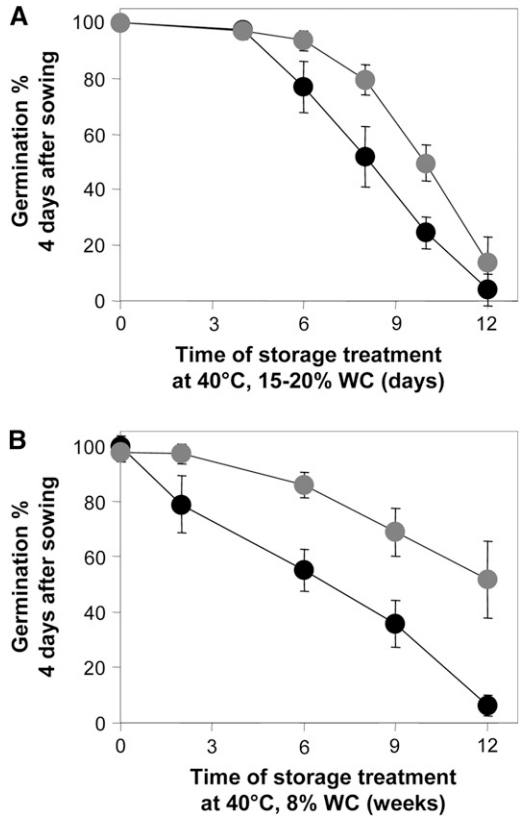


Figure 3. Sensitivity of *pimt1-1* Mutant Seeds to Storage Treatments Conducted at 40°C and 15 to 20% or 8% Water Content.

Wild-type (black) and *pimt1-1* (gray) dry mature seeds were submitted to a storage treatment performed for 12 d either at 15 to 20% (40°C, 75% RH) or 8% (40°C, 35% RH) water content (WC). Germination percentages from these seed samples were measured 4 d after sowing. Values are from four repetitions of 100 seeds (4× 100) (mean ± SD).

(A) Germination performance of seed samples submitted to the high-RH storage treatment.

(B) Germination performance of seed samples submitted to the low-RH storage treatment.

To further document this finding, the storage treatment at 40°C was also conducted at a lower water content of 8% (Figure 3B) that is equivalent to the seed water content in *Arabidopsis* dry mature seeds measured at room temperature (Baud et al., 2002). There was a lower decrease of seed vigor throughout this storage than during the corresponding treatment performed at the higher 15 to 20% seed water content (Figures 3A and 3B), indicating that this protocol imposed a milder aging condition. Thus, the germination percentages of wild-type seeds, as monitored 4 d after sowing (Figure 3B), decreased only from 96% (control seeds) to 79, 55, 36, and 6% after 2, 6, 9, and 12 weeks of aging, respectively. In marked contrast, under the same experimental conditions, the germination potential of the *pimt1-1* mutant seeds remained unaffected during the two first weeks of the mild aging treatment and then steadily decreased to 86, 69, and 52% after 6, 9, and 12 weeks of aging, respectively. Again, it is clear that the *pimt1-1* mutant seeds are more resistant to aging than the wild-type seeds. These data also document that the initial levels of accumulation of PIMT and isoAsp in mature seed are determinants of seed longevity potential.

IsoAsp-Related Damage in Seed Proteins Accumulates during Aging

It is surmised that no biochemical activity can proceed in seeds at such a low water content as the 8% used for conducting the mild aging treatment (Clark, 2004), especially in the case of the PIMT enzyme-catalyzed reaction for which diffusional constraints of the reactants might be important as both the enzyme and its substrates are high molecular mass molecules. To verify that is indeed the case for PIMT activity, the accumulation of the PIMT1 protein and the level of isoAsp residues were quantitated both in wild-type and in *pimt1-1* dry mature seeds submitted to the 8% water content storage treatment at 40°C for 0, 6, and 12 weeks (Figure 4). Protein gel blotting experiments disclosed a constant pattern of PIMT1 protein accumulation (Figure 4A) during the time course of storage, similar to the level initially observed in freshly harvested dry mature seeds (Figure 2D). On the other hand, despite this constancy in PIMT1 protein accumulation, the levels of isoAsp residues in proteins increased markedly and at about similar rates during the progress of the storage treatment performed with the wild-type and *pimt1-1* seeds (Figure 4B). Altogether these results suggest that PIMT1 repair probably does not occur during seed storage at such low water content.

Enhanced Seed Longevity Is a Characteristic Feature of PIMT1 Overaccumulating Lines

Because only a single mutant allele of *PIMT1* was presently identified, it was necessary to confirm the *pimt1-1* phenotype by additional means. To this end, transgenic *Arabidopsis* lines with altered *PIMT1* expression were generated by plant transformation (Bechtold and Pelletier, 1998), using a *35S:PIMT1* transcriptional fusion. Three independent transgenic lines (called O1, O2, and O3) overaccumulated PIMT1 both in leaves (see Supplemental Figure 4A online) and in freshly harvested dry mature seeds (Figure 5A). By protein gel blotting, we first verified that PIMT2 regulation was not affected in these transgenic lines (Figure 5B). Then the amount of isoAsp residues accumulated in freshly harvested dry mature seeds of the wild type and the transgenic lines was measured (Figure 5C). Compared with a content of 289 pmol mg^{-1} protein for the wild-type control, the O1, O2, and O3 lines displayed respective contents of 214, 203, and 226 pmol mg^{-1} protein. Therefore, in these lines, a lower extent of isoAsp accumulation (Figure 5C) was associated with a higher accumulation of PIMT1 (Figure 5A), strongly suggesting a higher in vivo PIMT1 repair activity in these PIMT1 overaccumulating lines during seed maturation.

In our standard growing conditions, the O1, O2, and O3 transgenic lines were phenotypically indistinguishable from wild-type plants all during plant development (see Supplemental Figure 5 online). Seed phenotypes were investigated more closely by submitting the seeds from these transgenic lines to

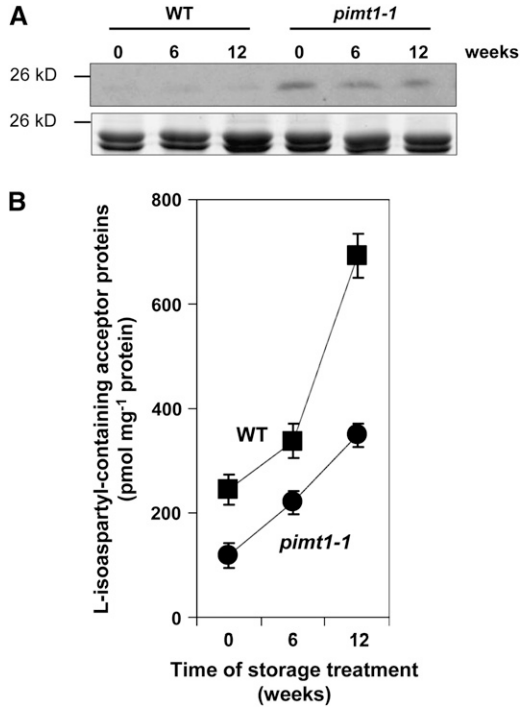


Figure 4. PIMT1 and IsoAsp Contents throughout Storage at 8% Water Content.

Wild-type and *pimt1-1* dry mature seeds were submitted for 0, 6, and 12 weeks to a storage treatment conducted at 40°C and 35% RH yielding a seed water content of 8%, similar to the water content of the dry mature seeds. PIMT1 accumulation levels were estimated by protein gel blotting following SDS-PAGE of protein extracts (30 μg). Quantitation of L-isoaspartyl–containing methyl-accepting substrates was also performed.
(A) PIMT1 accumulation during storage treatment at 8% water content. Parts of the Coomassie blue–stained gels are presented below the protein gel blots as loading controls.
(B) Quantitation of L-isoaspartyl–containing methyl-accepting substrates. Values are from three repetitions each using 100 mg of seeds (mean ± SD).

the storage treatment at 40°C and 15 to 20% seed water content. Remarkably, imposing this treatment for 6 d decreased the germination potential of the O1, O2, and O3 seeds only twofold compared with an almost total loss of seed vigor for the wild-type seeds (Figure 5D). As shown by the tetrazolium salt vital stain (Debeaujon et al., 2000), wild-type seed batches submitted to 5 d of storage treatment (Figure 5E) exhibited a higher extent of seed mortality. This was illustrated by the detection of a substantially higher number of uncolored embryos in the wild-type than in the O1, O2, and O3 transgenic lines. The difference in seed viability is consistent with the observed deleterious effect of the storage treatment on seed germination performance (Figure 5D) and strengthens the idea that the decrease in germination performance during aging is representative of a survival behavior. Altogether these data strongly support the existence of a relationship between PIMT1 overaccumulation in the freshly harvested dry mature seeds and seed survival potential as observed when comparing the *pimt1-1* line with wild-type seeds (Figures 2D and 3).

Transgenic Plants Underaccumulating PIMT1 Are Adversely Affected in Seed Viability

Among the kanamycin-resistant T1 transgenic lines that were analyzed by protein gel blotting, three independent lines (called U1, U2, and U3) displayed reduced levels of PIMT1 protein accumulation in their seeds compared with wild-type control seeds (see Supplemental Figure 4B online). We presume that the observed underaccumulation identified in these U1, U2, and U3 lines did not originate from feedback regulatory processes, but rather from cosuppression effects that are triggered by the viral cauliflower mosaic virus promoter (Vaucheret et al., 1998). This lower level of PIMT1 accumulation was confirmed in freshly harvested dry mature seeds from the T2 generation of these independent lines (Figure 6A). Using the PIMT2-specific antibodies, we first verified that PIMT1 underaccumulation in the seeds of these transgenic lines was not compensated by an overaccumulation of the PIMT2 enzyme (Figure 6B). Then, the isoAsp content of seed proteins was determined in freshly harvested dry mature seeds from the U1, U2, and U3 transgenic lines. This content reached 599, 865, and 816 pmol mg^{-1} protein, respectively (Figure 6C), which is substantially higher than the wild-type control level (260 pmol isoAsp mg^{-1} protein).

The capacity of seed survival was evaluated using the storage treatment at 40°C and 15 to 20% seed water content applied to the T3 progenies of the U1, U2, and U3 transgenic lines (Figure 6D). Clearly seeds from all these three lines displayed much higher susceptibility toward storage than the wild-type seeds. The use of the tetrazolium assay displayed a severely depressed viability of aged seeds from the PIMT1-underaccumulating transgenic lines (Figure 6E).

PIMT1 Confers the Ability to the Seeds to Overcome Abiotic Stress Limiting Germination

The presence of PIMT1 in the dry and 1-d imbibed freshly harvested mature seeds (Figures 2C and 2D) also suggests a role for this enzyme during the germination process. To explore the latter possibility, dry mature seeds from wild-type, *pimt1-1* mutant, and all transgenic lines that over- or underaccumulate PIMT1 were challenged with a salt stress or an osmotic stress during germination (Figure 7). It is clear that both *pimt1-1* mutant seeds and seeds from the O1, O2, and O3 PIMT1-overaccumulating lines were much more tolerant to salt stress (100 mM NaCl) or osmotic stress (300 or 400 mM mannitol) than control seeds (Figures 7A and 7B). Conversely, the germination of seeds from the U1, U2, and U3 PIMT1-underaccumulating lines proved much more sensitive to these stresses than was the germination of the wild-type seeds (Figure 7C). These results provide evidence that genetic alteration of *PIMT1* expression modifies tolerance to NaCl or mannitol stress in germinating *Arabidopsis* seeds.

DISCUSSION

PIMT repair enzyme activity is widespread in all living systems. Studies in prokaryote and in animal organisms have documented its protective role against aging or stressful conditions (Kim et al.,

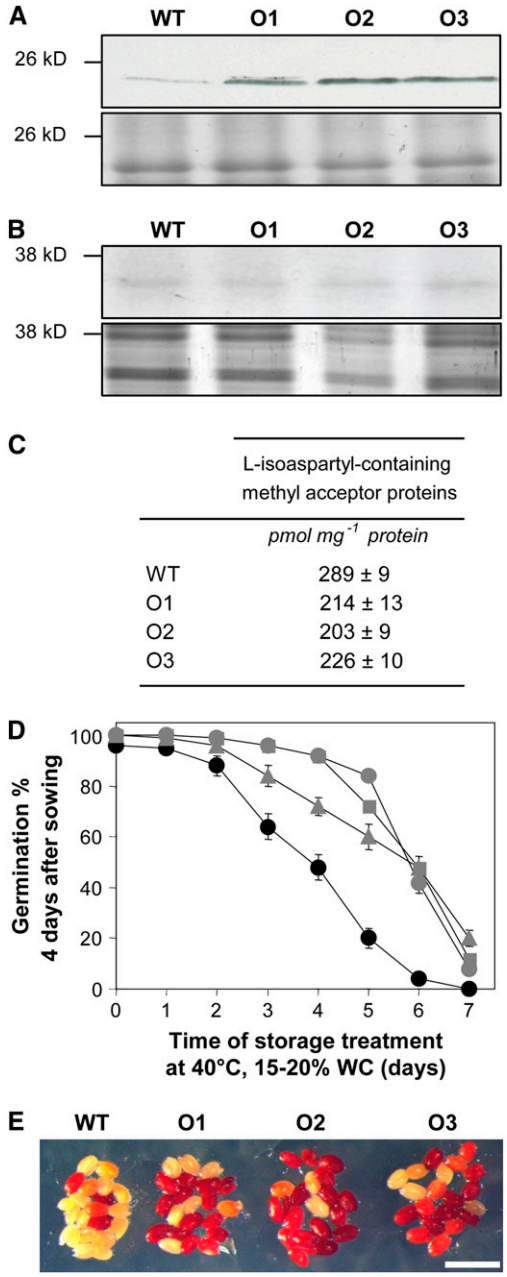


Figure 5. Characterization of PIMT1 Overaccumulating Lines.

PIMT1 and PIMT2 accumulation levels were compared in dry mature seeds or green siliques, respectively, for wild-type and three independent PIMT1 overaccumulating (O1, O2, and O3) lines. Proteins (30 μg) were separated by SDS-PAGE and immunodetected with polyclonal antibodies raised specifically against PIMT1 or PIMT2. Parts of the Coomassie blue–stained gels are presented below the protein gel blots as loading controls. PIMT2 accumulation was analyzed in green siliques because Xu et al. (2004) described the transient accumulation of *PIMT2* transcript during *Arabidopsis* seed formation. Our detection of the PIMT2 protein is in agreement with these data and documents a constant accumulation of this protein independently of that of PIMT1.

(A) Quantitation of PIMT1 accumulation levels.

(B) Quantitation of PIMT2 accumulation levels.

1997, 1999; Visick et al., 1998b; Yamamoto et al., 1998; Chavous et al., 2001; reviewed in Clarke, 2003). In plants, PIMT activity is thought to be involved in response to abiotic stress (Mudgett and Clarke, 1994; Kester et al., 1997; Mudgett et al., 1997; Thapar et al., 2001; Xu et al., 2004), but its role remains to be clearly established, especially in seeds. In this work, we have isolated *Arabidopsis* mutant lines exhibiting varying levels of PIMT and investigated the consequences of such a genetic modulation on several seed characteristics, including isoAsp accumulation in proteins, seed longevity, and seedling establishment under salt or osmotic stressful conditions. The *Arabidopsis* genome contains two genes encoding PIMT enzymes (Mudgett and Clarke, 1996; Xu et al., 2004). Since *PIMT2* is only weakly expressed in seeds compared with *PIMT1* (Figure 2D) (Xu et al., 2004), we have focused our study on *PIMT1* using a *pimt1-1* T-DNA insertion line affected in *PIMT1* regulation and transgenic lines over- or underaccumulating the PIMT1 protein.

PIMT1 Contains a Functional ABA-Responsive *cis*-Regulatory Element

The regulation of PIMT expression in plants by the stress-responsive plant hormone ABA has been previously invoked to account for observations showing that accumulation of this enzyme can be induced at the RNA or enzyme activity level under numerous abiotic stress conditions and by exogenous ABA application (Mudgett and Clarke, 1994; Thapar et al., 2001; Xu et al., 2004). The presently observed ABA-induced expression of *PIMT1* in wild-type seedlings (Figure 2C) is in general agreement with previous studies in *Arabidopsis* (Mudgett and Clarke, 1996; Xu et al., 2004), wheat (Mudgett and Clarke, 1994), and maize (Thapar et al., 2001) seedlings. Compared with the wild type, *PIMT1* upregulation by ABA in seedlings was lost in the *pimt1-1* homozygous line (Figure 2C) that is characterized by a 13-bp deletion of the MT1a ABRE consensus sequence (Mudgett and Clarke, 1996) located 187 bp upstream of the annotated translation start codon (Figure 2B). Our data provide experimental evidence that the postulated MT1a ABRE (Figure 2B) (Mudgett and Clarke, 1996) corresponds to a functional ABA-responsive regulatory sequence required for *PIMT1* ABA responsiveness in *Arabidopsis*. However, further work is needed to

(C) Quantitation of L-isoaspartyl–containing methyl-accepting substrates in dry mature seeds. Values are from three repetitions each using 75 mg of seeds (mean ± SD).

(D) Germination performance of the seed samples. Dry mature seeds from wild-type (black circles) and three independent PIMT1 overaccumulating lines (O1 [gray squares], O2 [gray circles], and O3 [gray triangles]) were submitted to a storage treatment conducted at 15 to 20% water content (WC; 40°C, 82% RH) for 7 d. Then, the germination percentages of these seeds were monitored 4 d after sowing. Values are from four repetitions of 100 seeds (4× 100) (mean ± SD).

(E) Viability of the seed samples. Wild-type, O1, O2, and O3 dry mature seeds were submitted to a storage treatment conducted for 5 d at 15 to 20% water content (40°C, 82% RH). Seed viability was estimated using tetrazolium staining according to the protocol described by Wharton (1955). Dark-red staining indicates that seeds are viable; light-pink staining indicates reduced seed viability. Bar = 1 mm.

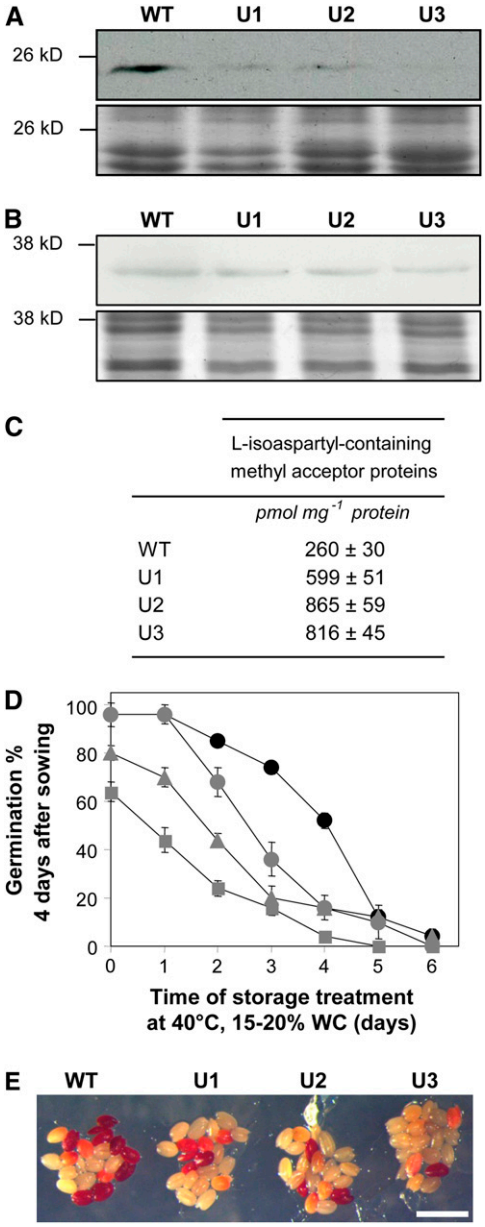


Figure 6. Characterization of PIMT1 Underaccumulating Lines.

PIMT1 and PIMT2 accumulation levels were compared in dry mature seeds or green siliques, respectively, for wild-type and three independent PIMT1 underaccumulating lines (U1, U2, and U3). Proteins (30 μg) were separated by SDS-PAGE and immunodetected with polyclonal antibodies raised specifically against PIMT1 or PIMT2. Parts of the Coomassie blue–stained gels are presented below the protein gel blots as loading controls.

(A) Quantitation of PIMT1 accumulation levels.

(B) Quantitation of PIMT2 accumulation levels.

(C) Quantitation of L-isoaspartyl–containing methyl-accepting substrates in dry mature seeds. Values are from three repetitions each using 75 mg of seeds (mean ± SD).

(D) Germination performance of the seed samples. Dry mature seeds from wild-type (black circles) and three independent PIMT1 underaccumulating lines (U1 [gray circles], U2 [gray triangles], and U3 [gray squares]) were submitted to a storage treatment conducted at 15 to 20% water content (WC, 40°C, 82% RH) for 6 d. The germination percentages of these seeds were monitored 4 d after sowing. Values are from four repetitions of 100 seeds (4× 100) (mean ± SD).

(E) Viability of the seed samples. Dry mature seeds of wild-type, U1, U2, and U3 lines were submitted to a storage treatment conducted at 15 to 20% water content (40°C, 82% RH) for 4 d. Seed viability was then estimated using tetrazolium staining (Wharton, 1955). Dark-red staining indicates that seeds are viable; light-pink staining indicates reduced seed viability. Bar = 1 mm.

better characterize the physiological significance of the identified *PIMT1* transcriptional regulation. Germination of nondormant *pimt1-1* and wild-type dry mature seeds displayed a comparable sensitivity to exogenous ABA (see Supplemental Figure 2 online), which was very similar to that reported for nondormant seeds of the Wassilewskija accession (Papi et al., 2000), indicating that ABA regulation of *PIMT1* does not influence ABA sensitivity of seed germination. ABA is a well known central regulating factor of desiccation tolerance, and the ABA induction of PIMT1 repair enzyme could be beneficial under severe water stress conditions, as occur notably during the desiccation phase at the end of the seed maturation program (Gutierrez et al., 2007).

The PIMT Repair Enzyme Is Required in Seeds to Limit Protein Damage by IsoAsp Accumulation

IsoAsp formation is both a major source of deleterious protein alteration that occurs in physiological conditions and one of the few spontaneous protein alterations that are biochemically reversible (reviewed in Clarke, 2003). In mammals, as in prokaryotes, the toxicity of this protein damage is generally buffered by dilution through cell division in growing tissues or eliminated by proteolysis (Fujii et al., 1994; Tarcsa et al., 2000; Lowenson et al., 2001; Athmer et al., 2002). The present measurements of isoAsp content in seed proteins from *Arabidopsis* lines altered in PIMT1 expression indicate that isoAsp accumulation is regulated through PIMT repair activity. Thus, in the *pimt1-1* mutant, as in the O1, O2, and O3 transgenic lines, a lower extent of isoAsp accumulation in the dry mature seeds (Figures 2E and 5C) was associated with a higher accumulation of PIMT1 (Figures 2D and 5A), a finding strongly suggesting a higher in vivo PIMT1 repair activity in these PIMT1 overaccumulating lines. Correlatively, the dramatically higher isoAsp accumulation in the dry mature seeds of the U1, U2, and U3 transgenic lines (Figure 6C) underaccumulating PIMT1 compared with the wild type (Figure 6A) is in excellent agreement with a role of PIMT1 in limiting isoAsp accumulation in seeds during their maturation on the mother plant. Furthermore, the contrasting levels of isoAsp residues encountered in seed proteins of the *Arabidopsis* lines that under- or overaccumulated PIMT1 strongly suggest a mechanism in which at least a substantial part of the damaged seed proteins are repaired via a PIMT1-mediated process. In agreement with this, *E. coli* and mice PIMT-deficient mutants exhibit an increased accumulation of isoAsp-containing damaged proteins (Kim et al., 1997; Visick et al., 1998b).

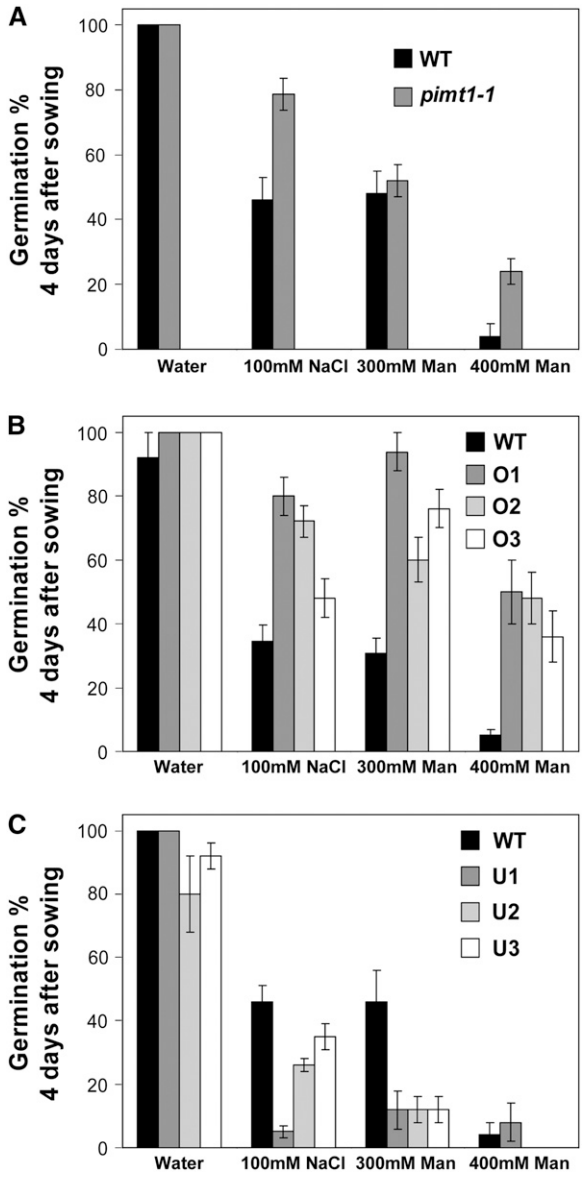


Figure 7. Sensitivity of Germination to NaCl or Mannitol.

The influence of 100 mM NaCl or 300 and 400 mM mannitol on germination percentages was examined using freshly harvested dry mature seed samples exhibiting varying accumulation of PIMT1. Germination percentages were monitored 4 d after sowing. Values are from four repetitions of 100 seeds (4× 100) (mean ± SD). Man, mannitol.
(A) Seeds from the *pimt1-1* mutant and respective wild-type controls.
(B) Seeds from the three independent PIMT1 overaccumulating lines O1, O2, and O3 and respective wild-type controls.
(C) Seeds from the three independent PIMT1 underaccumulating lines U1, U2, and U3 and respective wild-type controls.

PIMT1 Is Required for Seed Longevity

Seed longevity is a major trait for both ecological and agronomic aspects. Despite the major importance of this trait, very few studies have focused on identification of the mechanisms regulating it. Particularly, the molecular bases of the incredible seed longevity that is encountered in nature, as illustrated by the viability of multicentenarian sacred lotus seeds (Shen-Miller et al., 1995), remain unclear (Priestley, 1986; reviewed in McDonald, 1999; Clerkx et al., 2004). In orthodox seeds of a wide range of species, aging tests were developed for investigating longevity mechanisms and estimating the storage potential of seed (Delouche and Baskin, 1973). Oxidative reactions are responsible for aging of dry seeds (Hendry, 1993), but they are dependant on the seed water content and water strength of association with macromolecules (Kibinza et al., 2006). Some studies have highlighted the importance of the physicochemical properties of the low hydrated seeds (reviewed in Rajjou and Debeaujon, 2008) as determined by the presence of antioxidant metabolites (reviewed in Bailly, 2004; Bailly et al., 2008), for example, tocopherols (vitamin E) (Sattler et al., 2004), or through the formation of a glassy state of the cytoplasm of embryo cells that physically stabilizes cells against deterioration accompanying desiccation at the end of the seed maturation program (reviewed in Buitink and Leprince, 2004, 2008). Noticeably, water acts as a plasticizer of glasses (Walter, 1998) and influences both enzyme activity and cytoplasmic viscosity. Besides these nonenzymatic factors, other studies have highlighted the importance of enzymes, such as catalase, glutathione oxidase, or superoxide dismutase, that detoxify reactive oxygen species (reviewed in McDonald, 1999; Bailly, 2004) to limit lipid oxidation and oxidative damage to proteins and nucleic acids. These enzymatic detoxifying mechanisms are thought to occur in metabolically active seeds both during maturation and germination (Bailly, 2004).

The combined effects of moisture and temperature in a constant storage environment have been interpreted in terms of mathematical models allowing some predictions to be made about seed longevity under controlled environmental conditions (Ellis and Roberts, 1980). From these studies, optimum aging conditions and optimum methods for handling seed samples have been identified (Powell, 1995), giving rise to two different standardized seed storage protocols. One corresponds to the accelerated aging test and the other to the controlled deterioration test (Association of Official Seed Analysts, 1983; Hampton and Tekrony, 1995). In this study, we have used a seed storage treatment at 40°C and high RH, which corresponds to the controlled deterioration test of Tesnier et al. (2002) and that those authors adapted for *Arabidopsis* seeds. Accordingly, this aging condition is widely used by the seed community working on *Arabidopsis* (Bentsink et al., 2000; Groot et al., 2000; Tesnier et al., 2002; Clerkx et al., 2004; Sattler et al., 2004; Devaiah et al., 2007; Rajjou et al., 2008; Wohlbach et al., 2008). In agreement with previous findings suggesting the contribution of protein repair in seed longevity (Mudgett et al., 1997), we document a close relationship between PIMT1 accumulation in dry mature seeds (Figure 2D) and the seed resistance to aging as revealed by applying storage treatments under stressful conditions (Tesnier et al., 2002) to seeds of the *pimt1-1* line (Figure 3). In the analysis of the *pimt1-1* mutant, storage treatments were conducted independently at 15 to 20% and at 8% water content, the latter condition being characterized by a delay in viability loss (Bradford et al., 1993). This link between PIMT1 overaccumulation in dry mature seeds and increased seed longevity is further

supported by the varying sensitivity exhibited by dry mature seeds of the O1, O2, and O3 transgenic lines toward storage treatment (Figures 5A and 5D). Thus, our results raise the hypothesis that the extent of PIMT1 activity achieved during seed maturation regulates seed longevity. This contention is consistent with previous results obtained in *Drosophila* where an overexpression of PIMT results in an increased lifespan under mild heat shock conditions (Chavous et al., 2001). The ability of overexpression of protective proteins such as superoxide dismutase (Orr and Sohal, 1994) and heat shock proteins (Tatar et al., 1997; Kindrachuk et al., 2003; Prieto-Dapena et al., 2006) to extend lifespan has already been documented, lending further support to the hypothesis that cellular protective processes compete with aging (Knight, 2000). The ability of PIMT1 overaccumulation to increase seed longevity in *pimt1-1* (Figures 2D and 3) and in the O1, O2, and O3 lines (Figures 5A, 5D, and 5E) strengthens the idea that PIMT repair activity belongs to those protective functions limiting aging. Moreover, the strong phenotype of markedly higher susceptibility to loss of seed vigor during storage at 40°C and 15 to 20% seed water content of the U1, U2, and U3 lines underaccumulating PIMT1 (Figures 6A, 6D, and 6E) lend further support to the proposal that in *Arabidopsis* seeds PIMT1 contributes significantly to seed longevity. The varying isoAsp levels in seed proteins of the wild-type and *pimt1-1* mutant lines determined during storage treatment performed at 40°C and 8% water content (Figure 4B) fit well with a previous study in barley seeds (Mudgett et al., 1997). These authors showed that seed storage conducted for either several years at room temperature and low RH or for a short time at 40°C and high RH can trigger isoAsp accumulation and decrease seed viability, which led them to propose that a loss of PIMT repair activity efficiency in seeds results in enhancement of seed mortality. In our storage treatment, the 8% water content imposed on the seeds is similar to the water content of the dry mature *Arabidopsis* seeds stored at room temperature (Baud et al., 2002). Therefore, by inducing aging at 40°C in the low water condition of dry storage, we confirm here in *Arabidopsis* that elevated isoAsp-related protein damage in seed proteins is a main cause of seed aging. Moreover, the observed high and quite similar rates of isoAsp accumulation in wild-type and *pimt1-1* seeds during 6 weeks of storage at 40°C and 8% water content (Figure 4B) point out that despite a higher PIMT amount in the mutant seeds (Figure 4A), the PIMT1 enzyme is probably not active under such low water conditions. It is generally assumed that low water contents do not favor enzyme activity (Clark, 2004). In conclusion, our data demonstrate that the PIMT1 repair activity limits the level of isoAsp related damage in the proteome of mature seeds and thereby contributes to upgrading seed storability.

PIMT1 Improves Germination and Seedling Establishment in Stress Conditions

Previous studies showed that PIMT accumulation and activity in seedlings are generally induced by water and salt stress (Mudgett and Clarke, 1994; Thapar et al., 2001; Xu et al., 2004). In agreement with this, the presently observed phenotypes of varying tolerance toward NaCl or mannitol stress (Figure 7) in germinating *Arabidopsis* seeds of the various mutant lines revealed a tight correlation between the accumulation level of PIMT1 in the dry mature seeds and seed germination vigor. Furthermore, measurements of extractable PIMT support the hypothesis that the PIMT1 enzyme present in the dry mature seeds could be functional as soon as the imbibed seeds will become metabolically active during early germination (Figure 2D). Also, the observed de novo PIMT1 protein accumulation during imbibition (Figure 2D) fits well with the described occurrence of PIMT1 transcript in 1 d hydrated *Arabidopsis* seeds (Xu et al., 2004). Mudgett and Clarke (1994) had also identified substantial levels of PIMT RNA and PIMT activity in germinating winter wheat. In *Arabidopsis*, the observed PIMT1 accumulation (Figure 2D) and the concomitant decreased level of isoAsp (Figure 2E) during imbibition support a model in which the PIMT repair pathway is an important regulating mechanism to reduce isoAsp-related damage at early stages of seed germination. Altogether these data strongly suggest that PIMT1 not only plays a role in seed longevity but also during seed germination. Because germination is considered a stressful event inducing important protein damage in the seeds (Job et al., 2005), the presently observed upaccumulation of PIMT1 in seeds following 1 d of imbibition should favor germination success and contribute to the physiological quality of the seeds. This is in agreement with previous data in *Arabidopsis* showing the importance of the proteins stored in the dry mature seeds and of de novo protein translation for the success of seed germination (Rajjou et al., 2004, 2008). However, how protein repair could regulate seed germination remains to be established. In this work, the quantitative measurements of isoAsp levels allowed global estimations of the extent of protein damage in dry and germinating seeds. However, it is possible that during imbibition, PIMT targets specific proteins that must be repaired for successful germination. Proteomic approaches will help address this question (Vigneswara et al., 2006; Zhu et al., 2006; Dinkins et al., 2008). Since water limitations in soil and salinity stress are frequently encountered today in agronomic conditions (Pasternak and De Malach, 1994; Flagella et al., 2002; Quadir and Oster, 2004), our results stress the paramount importance of this enzyme for plant propagation in nature and for determining crop yields. Consistent with our results, an induction of PIMT activity has been observed in aged tomato (*Solanum lycopersicum*) seeds submitted to an invigorating treatment known as priming (Kester et al., 1997) and in germinated maize seeds subjected to osmotic or salt stress (Thapar et al., 2001). All these results point out that protein repair could be critical for seed cell maintenance under stressful conditions. Our work showing a close link between seed longevity during dry storage and tolerance to salt and/or osmotic stress during germination is not without precedent. Clerkx et al. (2004) reported the colocalization of quantitative trait loci in *Arabidopsis* regulating both seed longevity and germination under salt stress. Interestingly, such genetic loci include reactive oxygen species scavenging enzymes as catalase, superoxide dismutase, or small heat shock proteins involved in preventing irreversible protein denaturation and modulating cellular redox state (Grene, 2002), suggesting the hypothesis that mechanisms limiting oxidative stress influence both seed longevity and seed vigor. In this context, several studies demonstrated that tocopherols are essential

antioxidants, which by limiting nonenzymatic lipid peroxidation regulate *Arabidopsis* seed longevity and germination potential (Grene, 2002; Sattler et al., 2004; Sattler et al., 2006; Maeda and DellaPenna, 2007). Noticeably in the context of this work, cytotoxic lipid peroxidation products are known to entail extensive damage to proteins (Winger et al., 2007). Our data provide insights showing that, besides protection afforded by antioxidant systems, protein repair also contributes substantially to seed longevity and germination vigor under stressful conditions.

This property is of both applied and fundamental interest. For example, the *PIMT1* gene could be used to monitor/improve invigoration seed pretreatments that improve crop yield by accelerating and synchronizing seed germination and plantlet establishment (Heydecker et al., 1973; Grappin et al., 2005). Expression levels of the *PIMT* genes can also serve as markers to determine which seedlots should be stored and, on the contrary, those that can be eliminated because they will not survive dry storage. Favorable alleles of such genes can also be introgressed in crops to improve yield through enhanced and superior seedling establishment.

The postgerminative part of the plant life cycle appeared unaltered, at least under our greenhouse conditions, by genetic modifications of *PIMT1* expression (see Supplemental Figure 5 online). This contrasts with the situation in mammal systems where evidence suggests a close link between PIMT functioning and aging throughout lifespan (Kim et al., 1997; Yamamoto et al., 1998; DeVry and Clarke, 1999; Huebscher et al., 1999; Lowenson et al., 2001; Ghandour et al., 2002). An absence of adverse phenotypes during postgerminative plant development strengthens the potential for PIMT1 modulation to be used to produce more vigorous seedlings.

METHODS

Plant Material, Growth Conditions, and Mutant Screening

Arabidopsis thaliana (Wassilewskija) plants were cultivated in a growth chamber with a 16-h fluorescent light (250 μmol m^{-2} s^{-1}) condition. Temperature was set at 28°C during the light period and at 23°C during the dark period. After 2 months of culture, plants were transferred to 25°C, 24-h light period, during 2 weeks prior to seed harvest. In all experiments, seeds of mutant lines and their corresponding wild-type control were produced in the same culture cycle.

For growth in vitro, seeds were sown on Petri dishes (CML) containing the germination medium (7 g/L agar HP 696 [Kalys] and 580 mg/L MES [Sigma-Aldrich], pH 5.7, solubilized in water). Growth conditions were 16 h of light (as above) and 8 h of darkness, with the temperature set at 25°C during the light cycle and at 20°C during the dark cycle. For segregation analysis, seeds were sown onto germination medium supplemented with 100 μg/mL of kanamycin or 50 μg/mL of hygromycin.

ABA Treatment of Seedlings

Seeds were germinated on strips of sterile nylon (Nycom 5 μm; Bio Technofix) placed on germination medium. Seeds were stratified at 4°C during 2 d. After 4 d of growth, seedlings were transferred for 1 d to new germination media containing no ABA or 100 μM ABA [(+)-*cis, trans*-ABA; Sigma-Aldrich]. Seedlings were then harvested and ground with a mortar and pestle in liquid nitrogen prior to RNA extraction.

Germination Assays

Four replicates of 100 seeds each were germinated in Petri dishes containing germination medium supplemented or not with ABA, NaCl, or mannitol, as indicated in the legends to figures. In standard conditions, Petri dishes were sealed with Parafilm (American National Can), and seeds were stratified at 4°C during 2 d and then incubated in a controlled culture room under a 16-h photoperiod at 25°C (light period)/20°C (dark period). Germination measurements were scored every day. In *Arabidopsis*, germination that ends with radicle protrusion through the covering layers (Bewley, 1997; Koornneef et al., 2002; Kucera et al., 2005) can be evaluated by following the two sequential steps of testa rupture and of endosperm rupture that is concomitant to root tip protrusion (Liu et al., 2005; Muller et al., 2006). In our experiments, germinated seeds were scored as soon as endosperm rupture was observed using a binocular microscope.

Tetrazolium Assay

Seeds were scarified and incubated in a 1% (w/v) aqueous solution of 2,3,5-triphenyltetrazolium chloride (Merck) at 30°C in darkness for 2 d according to the procedure described by Wharton (1955). Tetrazolium salts were metabolically reduced to highly colored end products called formazans by NADH-dependent reductases of the endoplasmic reticulum (Berridge et al., 1996).

Seed Storage Treatments

Seeds were harvested as dry mature seeds 45 d after flowering and then stored at 4°C. The storage treatment was conducted rapidly after harvest from ~200 mg of seed aliquots that were surface sterilized. Each step of the aging treatment was conducted in airtight tube carriers (Fisher) containing appropriate hydrated salts. Temperature and humidity were measured hourly with a Testo 175-H2 controller placed inside the tube carriers. The storage treatment was conducted at two regimes of seed water content. In the first, a high RH was used according to the described deterioration treatment by Tesnier et al. (2002). Seeds were equilibrated 3 d at 85% RH (15°C) achieved with appropriate NaCl quantities, and day 0 controls were immediately dried back for 3 d at 36% RH (20°C) in the presence of $CaCl_2$. The storage treatment at high RH was performed for various times (from 1 to 12 d) by storing the seeds at either 82 or 75% RH (40°C) (as obtained with appropriate NaCl amounts), which equilibrated the seeds to 15 to 20% water content. Then, seeds were dried back for 3 d at 32% RH (20°C) and stored at 4°C until germination tests. The storage treatment at low RH was performed for various times (2, 6, 9, and 12 weeks) by storing the seeds at 35% RH (40°C) [as achieved with Mg $(NO_3)_2$], which equilibrated the seeds to 8% water content. Seed moisture content was measured for each RH after seed equilibration by weighing the seeds before and after a drying treatment conducted for 24 h at 105°C. Measurements from four independently harvested seed lots of 200 mg were averaged.

Mutant Screening and Sequencing of T-DNA Insertion Sites

The collection of *Arabidopsis* T-DNA mutants screened for an insertion in *PIMT1* was generated in the Wassilewskija ecotype at INRA-Versailles (France) (Bechtold et al., 1993; Bouchez and Höfte, 1998). A total of 36,864 genomic DNA extracts from T-DNA–mutagenized lines, grouped in 48 hyperpools, were tested by PCR as described by Geelen et al. (2000). Combinations of two *PIMT1*-specific primers, *mt3in* (5′-ACCATCTGAGTTCTTATCCACCAC-3′) and *mt5b* (5′-CTACGGACCTTCAGTTTACAAGAC-3′), and of two T-DNA–specific primers, *Tag3.1* (5′-TTTCGGTATAAAGACTTCGCGCT-3′) and *Tag5* (5′-CTACAAATTGCCTTTTCTTATCGA-3′) were used to amplify DNA flanking the T-DNA at the *PIMT1*

locus. The PCR products were then sequenced using the BigDye terminator kit (PE-Applied Biosystems) and analyzed on an ABI Prism 310 sequencing machine (PE-Applied Biosystems).

RNA Isolation and RT-PCR

Total RNA was isolated from dry seeds and 5-d-old seedlings as described by Verwoerd et al. (1989). Total RNA (10 μg) was used to perform the purification of poly(A) RNAs with the Dynabeads oligo(dT)$_{25}$ system (Invitrogen) following the manufacturer's instructions.

The poly(A) purified RNAs were reverse-transcribed (SuperScript II; Invitrogen) using an oligo(dT)$_{12-18}$ primer in a final volume of 60 μL. The first-strand cDNA mixture (1 μL) was used as a template for PCR analysis. The PCR (20 μL total volume) was done using 0.5 units of Taq polymerase (New England Biolabs) with *PIMT1* gene-specific primers named *FwP1* (5′-ATGAAGCAATTCTGGAGTC-3′) and *RevP1* (5′-GTCCCCTCTCAGCTGG-3′) and as a control, the constitutively expressed transcription factor *EF1α*-specific primers named *EF1αA4-up* (5′-ATGCCCCAGGACATCGTGATTTCAT-3′) and *EF1αA4-low* (5′-TTGGCGGCACCCTTAGCTGGATCA-3′). Each sample was submitted to 5, 10, 15, and 20 cycles of amplification and PCR products were analyzed, after electrophoresis and transfer on Nylon membrane, by hybridization against specific radiolabeled DNA probes and autoradiography. For each DNA molecule analyzed, the intensity of the amplified signal markedly increased between 5 and 20 cycles of reaction, and we used the 15th cycle PCR product that was in the logarithmic phase of the amplification for a quantitative analysis of the hybridization products. Results were quantified and normalized relative to the control *EF1α* using MacBAS software. Values were from three biological repetitions using 75 mg of seeds each (mean ± SD). Amplified bands were cloned and sequenced to check the specificity of RT-PCR products.

Description of Recombinant Protein Constructs

The constructs were made using Gateway cloning technology (Invitrogen). Briefly, cDNA of *PIMT1* was amplified by PCR using the primer *P1U* containing the attB1 recombination site and the start codon of the *PIMT1* gene (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGAAGCAATTCTGGAGTC-3′) and the primer *P1L* containing the attB2 recombination site and the end of the *PIMT1* gene without the stop codon (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCGTCCCCTCTCAGCTGG-3′). For *PIMT2*, cDNA was amplified by PCR using the primer *P2U* containing the attB1 recombination site and the start codon of the *PIMT2* gene (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGGAGAGTGGAACTGGCT-3′) and the primer *P2L* containing the attB2 recombination site and the end of the *PIMT2* gene without the stop codon (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCAAATCCTCCTAACTGTTCGT-3′). The two coding sequences were recombined into pDest17 (to obtain N-terminally fused 6x His-tagged proteins) expression vector (Invitrogen) to allow protein purification or recombined into the Gateway site of pGWB2 (http://bio2.ipc.shimane-u.ac.jp/PGWBS/INDEX.HTM) to produce the *35S:PIMT* constructs.

Wild-type seedlings from Wassilewskija ecotype were transformed by infiltration using *Agrobacterium tumefaciens* carrying the *35S:PIMT1* or *35S:PIMT2* constructs (Bechtold et al., 1993). T2 lines were analyzed for Mendelian segregation on hygromycin.

Recombinant Protein Purification

Recombinant protein synthesis was induced in the *Escherichia coli* strain BL21(DE3)RIL (Novagen), cultured at 28°C in 100 mL of Luria-Bertani medium containing 100 μg/mL of ampicillin until A_{600} reached 0.5. Upon induction with 0.3 mM isopropyl β-D-1-thiogalactopyranoside, the culture was grown for an additional 3 h. Bacterial cells were centrifuged at 3000*g* at 4°C for 10 min, resuspended in 1 mL of 100 mM HEPES, pH 7.5, frozen in liquid nitrogen, allowed to thaw at 4°C, and then sonicated 5 min. The lysate was centrifuged at 20,000*g* at 4°C for 10 min. Recombinant PIMT1 and PIMT2 proteins were purified using the MagneHis protein purification system according to the manufacturer's instructions (Promega). Recombinant protein purity was assessed by SDS-PAGE, stained with Coomassie Brilliant Blue, and sequenced by tandem mass spectrometry by Maya Belghazi at the Centre d'Analyze Protéomique (Marseilles, France; http://www.genopole.univ-mrs.fr/capmnord/) as described by Catusse et al. (2008). The peptide sequences were identical to expectation of the known *Arabidopsis PIMT1* and *PIMT2* coding sequences. Recombinant proteins were used in protein gel blot analyses as controls.

Preparation of Plant Homogenates for Protein Blot Analyses and Quantitation of Damaged Aspartyl and Asparaginyl Residues

Soluble proteins were extracted from freshly harvested dry mature seeds and green siliques. Frozen tissue was ground with a pestle in a liquid-nitrogen chilled mortar. The powder was transferred in a mortar containing an extraction buffer (typically 300 μL) containing 100 mM HEPES, pH 7.5, 500 μM EDTA, 100 μM DTT, 10% (w/v) sucrose, and 1% protease inhibitor cocktail (Sigma-Aldrich) and ground further. The slurry was centrifuged at 20,000*g* at 4°C for 10 min. The resulting supernatant was stored at −20°C. Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as standard. For quantitation of damaged aspartyl and asparaginyl residues, an aliquot of the supernatant was precipitated by 65% ammonium sulfate. Typically, an amount of 410 mg of ammonium sulfate was dissolved in 1 mL of protein extracts. Following incubation for 1 h at 4°C, precipitated proteins were collected by centrifugation at 20,000*g* at 4°C for 15 min. The pellets were resuspended in 200 μL of 100 mM HEPES, pH 7.5.

Preparation of Plant Extracts for Measuring Seed PIMT Methyltransferase Activity

Crude cytosol was prepared from *Arabidopsis* seed samples as described by Villa et al. (2006) with minor changes. Dry mature and 1 d imbibed seeds were ground in liquid nitrogen with a chilled mortar and pestle. The seed powder was homogenized in 1 mL of extraction buffer composed of 100 mM HEPES, pH 7.5, 10 mM β-mercaptoethanol, 10 mM sodium hydrosulfite, 10 mM sodium metabisulfite, 10% glycerol (v/v), 1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The suspensions were spun at 14,000*g* for 10 min at 4°C, and the supernatants were collected. These soluble extracts were used as the source of the PIMT methyltransferase enzyme and stored at −80°C until use.

Measurement of PIMT Methyltransferase Activity

A vapor diffusion assay was performed as described by Mudgett and Clarke (1993) with minor changes. It involves the transfer of a radiolabeled methyl group from *S*-adenosyl-L-[methyl-^{14}C]methionine ([^{14}C]AdoMet) to the methyl acceptor L-isoaspartyl-containing protein ovalbumin. The reaction mixture (total of 40 μL) consisted of 12 μL of soluble protein extract described above, 10 μM [^{14}C]AdoMet (52 mCi mmol^{-1}; Perkin-Elmer), and 600 μg of isoAsp-containing ovalbumin (Grade V; Sigma-Aldrich) in 0.2 M sodium citrate buffer, pH 6.0. Control reactions were performed by incubating the enzyme extract with buffer alone instead of the ovalbumin protein substrate. The reaction was allowed to proceed at 37°C for 1 h and stopped by quenching with 40 μL of 0.2 M NaOH/1% (w/v) SDS, which resulted in the hydrolysis of methyl esters to methanol. A total of 60 μL of this mixture was immediately spotted on a 1 × 8.5-cm piece of thick filter paper (Bio-Rad) prefolded in an accordion pleat and placed in

the neck of a 20-mL plastic scintillation vial containing 10 mL of ACS II counting fluor (Amersham) and capped. Vials were left at room temperature for 2 h. During this time, volatile [^{14}C]methanol diffused into the fluor and the unreacted [^{14}C]AdoMet remained on the filter paper. After removal of the filter paper, radioactivity in the vials was counted by liquid scintillation. Peptide-specific activity was calculated by subtracting the radioactivity from the control.

Quantitation of Damaged Aspartyl and Asparaginyl Residues

The ISOQUANT isoaspartate detection kit (Promega) was used to quantify L-isoaspartyl residues in seeds. Methylation reactions were performed in 25-μL final volumes. Typically, 10 μL of total proteins (25 μg) were incubated 30 min at 30°C with 5 μL 5× reaction buffer (0.5 M sodium phosphate, pH 6.8, 5 mM EGTA, 0.02% sodium azide, and 0.8% Triton X-100), 5 μL 100 μM AdoMet, and 5 μL PIMT enzyme furnished with the kit. Reactions were stopped by adding 5 μL stop solution (0.3 M phosphoric acid), and then the proteins were precipitated by centrifugation at 20,000*g* at 4°C for 10 min and the supernatants (30 μL) were taken for HPLC analyses of *S*-adenosyl-L-homocystein (AdoHcy) production. The amount of AdoHcy initially present in the extract (without incubation) was subtracted from the values obtained with incubation.

HPLC and UV Detection of AdoHcy

Methylation reactions and AdoHcy standards were analyzed on a Synergi Hydro-RP column (Phenomenex). Solvent A was 50 mM potassium phosphate, pH 6.2, and solvent B was 100% methanol. After equilibrating the column with 8% B, the sample (25 μL) was injected and a gradient from 8 to 30% B was started immediately using a flow rate of 0.25 mL min^{-1}. UV absorbance was monitored online at 260 nm with a Gilson 117 detector. Quantitation of AdoHcy eluted from the column was determined by peak area with reference to a set of AdoHcy standards subjected to the same HPLC analysis.

Protein Gel Blot Analyses

Specific peptides containing 18 amino acids (C-LQVVDKNSDGSVIKDET) of PIMT1 sequence and 11 amino acids (SGTGSSGKRG-C) of PIMT2 sequence were synthesized by BioGenes and used to immunize rabbits. The antisera were immunoaffinity purified by BioGenes against their corresponding peptides bound to Sepharose matrix. The affinity-purified antipeptide antibodies were diluted 1:10,000 for protein gel blot analyses.

Total protein extracts (30 μg) were suspended in Laemmli buffer (Laemmli, 1970) and resolved by SDS-PAGE in 12% polyacrylamide gels. After electrophoresis, proteins were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes (Hybond-C Extra; Amersham) that were then air-dried for 30 min and blocked overnight at 4°C in a saline solution supplemented with Tween 20 (PBS-T; 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 15 mM KH_2PO_4, and 0.003% [v/v] Tween 20, pH 7.6) containing 10% (w/v) defatted powder milk. The membranes were probed for 5 h at 25°C with 1:10,000 rabbit affinity-purified primary antibodies in 20 mL PBS-T containing 10% (w/v) defatted powder milk. The blots were washed with PBS-T (3 × 10 min) and probed for 2 h at 25°C with 1:8000 goat anti-rabbit peroxidase-conjugated antibodies (Sigma-Aldrich) in 20 mL of PBS-T. After the blot was washed two times with PBS-T for 15 min each, chemiluminescence of the signal was developed using an ECL kit (Pierce) and the blot was exposed to radiographic film (Amersham).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *PIMT1* (AT3G48330) and *PIMT2* (AT5G50240).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. DNA Gel Blot Analysis of Wild-Type and *pimt1-1* Mutant Genomic DNA Hybridized with Kanamycin-Specific Labeled Probe.

Supplemental Figure 2. ABA Dose Response for Germination Inhibition of Wild-Type and *pimt1-1* Seeds.

Supplemental Figure 3. Specificity of Polyclonal Antibodies Raised against *Arabidopsis* PIMT1 and PIMT2.

Supplemental Figure 4. Comparison of PIMT1 Accumulation Patterns in *Arabidopsis* Rosette Leaves of Wild-Type PIMT1 Over- and Underaccumulating Lines.

Supplemental Figure 5. Phenotypical Comparison of Postgerminative Development in *Arabidopsis* Wild-Type, *pimt1-1* Mutant, PIMT1 Overaccumulating Line O1 and PIMT1 Underaccumulating Line U1.

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Protein Repair l-Isoaspartyl Methyltransferase1 Is Involved in Both Seed Longevity and Germination Vigor in *Arabidopsis*

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