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Protein Repair L-Isoaspartyl Methyltransferase in Plants[1]

Phylogenetic Distribution and the Accumulation of Substrate Proteins in Aged Barley Seeds

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Protein L-isoaspartate (D-aspartate) *O*-methyltransferases (MTs; EC 2.1.1.77) can initiate the conversion of detrimental L-isoaspartyl residues in spontaneously damaged proteins to normal L-aspartyl residues. We detected this enzyme in 45 species from 23 families representing most of the divisions of the plant kingdom. MT activity is often localized in seeds, suggesting that it has a role in their maturation, quiescence, and germination. The relationship among MT activity, the accumulation of abnormal protein L-isoaspartyl residues, and seed viability was explored in barley (*Hordeum vulgare* cultivar Himalaya) seeds, which contain high levels of MT. Natural aging of barley seeds for 17 years resulted in a significant reduction in MT activity and in seed viability, coupled with increased levels of "unrepaired" L-isoaspartyl residues. In seeds heated to accelerate aging, we found no reduction of MT activity, but we did observe decreased seed viability and the accumulation of isoaspartyl residues. Among populations of accelerated aged seed, those possessing the highest levels of L-isoaspartyl-containing proteins had the lowest germination percentages. These results suggest that the MT present in seeds cannot efficiently repair all spontaneously damaged proteins containing altered aspartyl residues, and their accumulation during aging may contribute to the loss of seed viability.

Biological macromolecules with functions essential to life are thermodynamically unstable and are subject to nonenzymatic breakdown reactions (Harding, 1985). In proteins aspartyl and asparaginyl residues are particularly susceptible to spontaneous covalent modification and are primary sites of age-related damage (Clarke et al., 1992). Deamidation, racemization, and isomerization of these residues can markedly affect protein structure and function. The major product of these degradative reactions is the L-isoaspartyl residue, in which the "kinked" polypeptide backbone proceeds through the side-chain carbonyl group. The accumulation of such aberrant residues can be detrimental to cellular metabolism. However, many prokaryotic and eukaryotic organisms express the protein L-isoaspartate (D-aspartate) O-MT (EC 2.1.1.77; O'Connor and Clarke, 1985; Johnson et al., 1991), which methylates L-isoaspartyl residues and initiates the "repair" pathway shown in Figure 1 (Johnson et al., 1987; Galletti et al., 1988; Brennan et al., 1994). The product of the methylation reaction, the L-isoaspartyl methyl ester, is itself unstable and is hydrolyzed by a two-step nonenzymatic pathway to form both isoaspartyl and normal aspartyl residues (Fig. 1). The continued action of the MT can thus ultimately convert abnormal isoaspartyl residues to normal aspartyl residues. This repair pathway has been demonstrated both in vitro (Johnson et al., 1987; Brennan et al., 1994) and in vivo in mouse tissues (Kim et al., 1997).

We were interested in determining whether plants maintain a similar pathway that could limit the accumulation of damaged proteins, and we recently found L-isoaspartyl MT activity in several types of plants (Mudgett and Clarke, 1993). As in mammalian systems, the plant MT is most active in tissues in which protein turnover is limited; the specific activity in mouse brain is 2- to 6-fold higher than in heart and liver, whereas in winter wheat (*Triticum aestivum*; Mudgett and Clarke, 1993, 1994) and the meadow weed *Arabidopsis thaliana* (Mudgett and Clarke, 1996), MT activity is localized primarily in seed tissues during the late stages of embryogenesis and maturation. Unlike the constitutively expressed mammalian enzyme, however, the MT in these plants is inducible and is expressed in vegetative tissue in response to exogenous treatment with ABA (Mudgett and Clarke, 1994, 1996).

Furthermore, the low level of enzyme activity present in wheat seedlings is significantly increased during periods of water deficit and salt stress (Mudgett and Clarke, 1994). It is likely that ABA serves as an endogenous signal in the cellular regulation of MT activity in seed-bearing plants, considering that endogenous ABA levels are elevated during late embryogenesis (Quatrano, 1986; Black, 1991) and in response to dehydration stress (Zeevaart and Creelman, 1988; Bray, 1991). The salt responsiveness of the L-isoaspartyl MT suggests that ABA-independent response pathway(s) may also contribute to regulation of the enzyme (Bostock and Quatrano, 1992). Together, these find-

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Abbreviation: MT, methyltransferase.

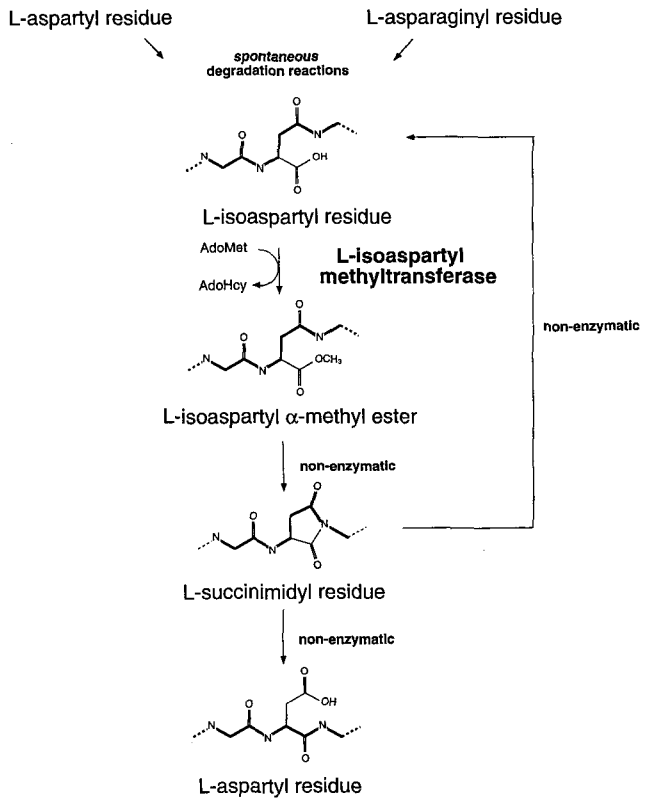

Figure 1. Role of the protein-repair MT in the conversion of damaged peptides containing L-isoaspartyl residues derived from the spontaneous isomerization of Asn and Asp residues to normal L-aspartyl residues. The peptide backbone is shown in bold to emphasize the kink in the polypeptide chain that occurs in the isoaspartyl bond and the linear nature of the chain in the normal aspartyl bond. AdoMet, *S*-Adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine.

ings demonstrate that MT activity increases when protein turnover is limited or when exogenous conditions promote protein denaturation, which can accelerate aspartyl/asparaginyl degradation.

If repair of damaged aspartyl residues does occur in seeds, the MT-linked pathway may be critical in maintaining seed viability during dormancy, in germination, and in early development of the seedling (Mudgett and Clarke, 1993). To investigate this possibility, we first assayed a broad variety of plants to determine whether the L-isoaspartyl MT has a widespread distribution in seed-bearing (and non-seed-bearing) plants. We then analyzed the effect of natural and artificial aging of barley seeds on MT activity, the accumulation of L-isoaspartyl residues, and seed viability. We found an inverse relationship between seed viability and the content of proteins containing altered aspartyl residues.

MATERIALS AND METHODS

Aged barley (*Hordeum vulgare* cv Himalaya) seeds (stored under ambient laboratory conditions) were obtained from Dr. Russell Jones (Department of Plant Biology, University of California, Berkeley). Broccoli, sporangia of an Australian tree fern, pollen of a lily, and seeds of Coulter pine, giant sequoia, yucca, big-leaf maple, cantaloupe, pumpkin, California black oak, and horse chestnut were either purchased locally in Los Angeles or collected in California or Oregon. *Colacium, Ectocarpus, Botrydium, Synedra, Amphidinium carterae, Bangia fuscopurpurea, Marchantia, Polytrichum, Lycopodium lucidulum, Selaginaella uncinata, Selaginella lepidophylla,* and *Equisetum hyemale* were purchased from Carolina Biological Supply (Burlington, NC). The remaining seeds used in this study were purchased from Chas. H. Lilly (Portland, OR), NK Lawn and Garden (Minneapolis, MN), or Ward's (Rochester, NY). Full Latin names are provided in the tables and text.

Preparation of Plant Homogenates

Crude cytosol was extracted from dry seeds and plant tissue by homogenization using a mortar and pestle. Frozen tissue (typically 0.1–2 g) was ground with a pestle in a liquid N_2-chilled mortar and then transferred to a tube containing extraction buffer (typically 0.5–5 mL; 100 mM sodium Hepes, pH 7.5, 1 mM DTT, 1 μM leupeptin, 1 mM phenylmethanesulfonyl fluoride, 10 mM sodium hydrosulfite, and 10 mM sodium metabisulfite at 4°C; Anderson and Rowan, 1967). The slurry was kept on ice, vortexed, and then centrifuged at 14,000*g* for 10 min to remove insoluble cell debris. The resulting supernatant, identified as the plant homogenate, was stored at −20°C and utilized as the source of MT.

Methylation Assays

L-Isoaspartyl MT activity was measured using a vapor-phase diffusion assay in which radiolabeled methyl groups transferred from *S*-adenosyl-L-[*methyl*-^{14}C]Met to a peptide substrate are released as [^{14}C]methanol by base hydrolysis and trapped in scintillation fluid. Generally, 12 μL of plant homogenate was incubated in a 40-μL reaction with 10 μM *S*-adenosyl-L-[*methyl*-^{14}C]Met (53 mCi/mmol, Amersham) and 0.33 M sodium Hepes, pH 7.5, in the presence or absence of the peptide substrate VYP-(isoAsp)-HA (500 μM), as described previously (Lowenson and Clarke, 1991).

Incubations were performed at 25°C for 60 min and the reaction was quenched with 40 μL of 0.2 M NaOH and 1% (w/v) SDS. A 60-μL aliquot of this mixture was spotted onto a 1.5- × 8-cm pleated filter paper (Bio-Rad no. 165–090) and placed in the neck of a 20-mL scintillation vial containing 5 mL of counting fluor (Safety Solve, Research Products International, Mount Prospect, IL). The vials were capped and [^{14}C]methanol was allowed to diffuse through the vapor phase into the fluor while the nonvolatile [^{14}C]-methyl groups (e.g. unreacted *S*-adenosyl-L-[*methyl*-^{14}C]Met) remained on the paper. After 2 h at room temperature, the paper was removed from the necks of the vials and the vials were counted. Peptide-specific L-

isoaspartyl MT activity was determined by subtracting the generally low activity measured in incubations without peptide from that measured in parallel incubations containing peptide.

Plants that showed little or no MT activity were assayed for the presence of MT inhibitor(s). This was done by comparing the ability of wheat or cucumber MT (4 μL) to methylate L-isoaspartyl-containing peptide in the presence and absence of the inactive plant homogenate (8 μL) using the assay described above.

Recombinant human protein L-isoaspartate (D-aspartate) *O*-MT (MacLaren and Clarke, 1995) was used to quantify L-isoaspartyl residues in proteins isolated from aged (natural and accelerated) barley seeds. In a total reaction volume of 40 μL, 1.1 μg of human recombinant MT (specific activity 10,000 pmol methyl esters min^{-1} mg^{-1} protein) was incubated with 5 μL of barley seed homogenate and 10 μM S-adenosyl-L-[*methyl*-^{14}C]Met in 0.2 M bis-Tris, pH 6.0. Incubations were performed at 37°C for 90 min, which is sufficient for the highly active human enzyme to fully methylate L-isoaspartyl residues in a variety of sequences (Kim et al., 1997). The resulting protein methyl esters were then quantified as described above.

Protein Determination

A modification of the Lowry procedure (Bailey, 1967) was used to determine the concentration of protein following precipitation with an equal volume of 10% (w/v) TCA. BSA was used for the standard curve. Typical extract protein concentrations ranged from 0.5 to 10 mg/mL.

Germination of Seeds

For developmental analysis seeds were surface-sterilized and then sown on a piece of Whatman filter paper (type 1) placed on a 150-mm Petri dish containing approximately 150 mL of 0.7% agar. Plates were uncovered and the seedlings were watered daily following germination under continuous light (approximately 200 μE m^{-2} s^{-1}) at room temperature.

Accelerated Aging of Barley Seeds

Accelerated aging of barley seeds collected in 1991 was performed in 1996 by a modification of the method of Parrish and Leopold (1978). Seeds were placed in a single layer on a dish and incubated in a covered water bath at 40°C and approximately 100% RH. After 0 to 18 d, seeds were removed and allowed to return to normal room humidity and temperature overnight.

RESULTS

Survey of Plant Kingdom for L-Isoaspartyl MT Activity

L-Isoaspartyl MT has been found to limit the accumulation of damaged aspartyl residues in mice (Kim et al., 1997), and we hypothesized that it fulfills a similar role in plant tissues. Preliminary results showed that winter wheat and *A. thaliana* have much higher levels of L-isoaspartyl MT activity in their seeds than in their other tissues, suggesting that seeds may be particularly sensitive to damaged proteins (Mudgett and Clarke, 1994, 1996).

To determine whether the protein-repair MT is of widespread occurrence or of limited distribution, we surveyed the plant kingdom by assaying tissue homogenates for the ability to specifically methylate an L-isoaspartyl-containing peptide, VYP-isoD-HA. We found peptide-specific MT activity in 39 of 42 seed homogenates assayed (Table I), as well as in homogenates of 6 of 13 non-seed plants (Table II). Although the specific activity in seeds from different plants varied considerably, the values were mostly between 0.5 and 8.0 pmol methyl groups transferred min^{-1} mg^{-1} protein, which is comparable to the activity levels found in mammalian tissues and bacteria (Johnson et al., 1991; Li and Clarke, 1992).

MT activity was undetectable only in seeds from the giant sequoia (*Sequoiadendron giganteum*), big-leaf maple (*Acer macrophyllum*), and California black oak (*Quercus kelloggii*). Subsequent studies, however, revealed that these homogenates contain substances that inhibit the activity of the L-isoaspartyl MT in cucumber and wheat homogenates (Table III). Furthermore, the activity levels in some plants (e.g. *Gingko biloba* and *A. thaliana*) might be underestimated because these seeds contain intermediate levels of the inhibitor(s). The nature of these inhibitory components was not determined. However, we suspect that tannin or tannin-like substances may inactivate or otherwise interfere with the activity of this MT. These substances are most abundant in the plant species possessing the highest levels of MT inhibitor (Gibbs, 1974). Therefore, it is possible that seeds in which MT was not detected do in fact express this enzyme, but the release of an inhibitor during homogenization masks the activity.

The L-isoaspartyl MT activity detected in non-seed plants, including species from the divisions Bryophyta, Microphyllophyta, Arthrophyta, and Pteridophyta, is present at levels much lower than those found in most seed plants (Tables I and II). Previous work had shown that the enzyme is also present in the division Chlorophyta (Mudgett and Clarke, 1993). Representatives from the other divisions of the non-seed plants had no detectable MT activity under the specifications of the assay used herein. All of the plant homogenates surveyed did possess malate dehydrogenase activity (data not shown), demonstrating that the lack of MT activity was not due to problems in the homogenate preparation. When some of the homogenates were assayed for the presence of MT inhibitor(s), it was found that inhibition was not responsible for the low MT activities in these plants (Table III). *Alisophila* sporangia and leaves from *S. lepidophylla* had low MT activity but only about 10% of the inhibitor present in *A. macrophyllum*, whereas even less inhibitor was detected in *Polytrichum* spores, in which MT was undetectable. It remains unclear whether the liverwort (*Marchantia* sp.) has low MT activity, because it also contains about 30% of the relative MT inhibitor found in *A. macrophyllum*.

Table I. *Occurrence of peptide-specific L-isoaspartyl MT activity in homogenates of seed plants (division Spermatophyta; Bold et al., 1987)*

Class (Subclass)	Family	Genus and Species	Common Name	MT Activity[a]	Tissue
				pmol min^{-1} mg^{-1} protein	
Conopsida	Ginkgoaceae	*Ginkgo biloba*	Ginkgo	0.31	Seeds
	Pinaceae	*Pinus coulteri*	Coulter pine	2.61	Seeds
		Pinus taeda	Loblolly pine	4.38	Seeds
	Taxodiaceae	*Sequoiadendron giganteum*	Giant sequoia	ND[b]	Seeds
Angiospermae					
Monocotyledoneae					
	Gramineae	*Hordeum vulgare*	Barley	2.80	Seeds
		Triticum aestivum	Wheat	3.97	Seeds
		Zea mays	Corn	6.10	Seeds
	Liliaceae	*Allium schoenoprasum*	Onion	2.52	Seeds
		Lilium auratum	Lily	0.75	Pollen
		Yucca sp.	Yucca	3.22	Seeds
Dicotyledoneae					
	Acanthaceae	*Rudbeckia hirta*	Black-eyed Susan	1.27	Seeds
		Rudbeckia sp.	Purple coneflower	1.08	Seeds
	Aceraceae	*Acer macrophyllum*	Big leaf maple	ND	Seeds
	Asclepiadaceae	*Asclepias tuberosa*	Butterfly weed	0.99	Seeds
	Chenopodiaceae	*Spinacia* sp.	Spinach	0.44	Seeds
	Compositae	*Achillea millefolium*	Yarrow	1.95	Seeds
		Coreopsis sp.	Dwarf-red coreopsis	2.66	Seeds
		Helianthus annuus	Sunflower	2.19	Seeds
		Gaillardia sp.	Indian blanket	1.06	Seeds
		Lactuca sativa	Lettuce	0.52	Seeds
		Zinnia sp.	Dwarf zinnia	1.05	Seeds
	Cruciferae	*Arabidopsis thaliana*	Thale cress	1.00	Seeds
		Brassica oleracea	Broccoli	0.98	Meristems
		Raphanus sativus	Radish	2.06	Seeds
	Cucurbitaceae	*Cucumis melo*	Cantaloupe	3.61	Seeds
		Cucumis sativus	Cucumber	7.74	Seeds
		Citrullus lanatus	Watermelon	5.14	Seeds
		Cucurbita pepo	Pumpkin	3.24	Seeds
		Cucurbita pepo	Zucchini	3.35	Seeds
	Fagaceae	*Quercus kelloggii*	California black oak	ND	Seeds
	Hippocastanaceae	*Aesculus hippocastanum*	Horse chestnut	4.64	Seeds
		Lupinus albus	Lupine	0.93	Seeds
		Medicago sativa	Alfalfa	3.08	Seeds
		Phaseolus vulgaris	Bush bean	0.53	Seeds
	Labiatae	*Ocimum basilicum*	Sweet basil	4.35	Seeds
	Loasaceae	*Chamaelirium luteum*	Blazing star	0.54	Seeds
	Onagraceae	*Oenothera* sp.	Evening primrose	0.05	Seeds
	Papaveraceae	*Eschscholzia californica*	California poppy	1.75	Seeds
	Polemoniaceae	*Phlox drummondii*	Phlox	0.85	Seeds
	Solanaceae	*Lycopersicon esculentum*	Tomato	6.90	Seeds
		Nicotiana tabacum	Tobacco	1.27	Seeds
	Umbelliferae	*Daucus carota*	Carrot	0.93	Seeds

[a] Methylation assays were performed in duplicate. [b] ND, No detectable MT activity.

L-Isoaspartyl MT in Aged Barley Seeds

The occurrence of the L-isoaspartyl MT in the diversity of species surveyed here supports a fundamental role for this enzyme in plants. Furthermore, the predominance of this activity in seed-bearing plants suggests that it is most important during seed maturation, quiescence, and germination. We hypothesize that the MT in seeds may repair damaged aspartyl residues generated during the natural course of seed desiccation and aging, thus reducing the extent of this type of protein deterioration and assisting in the preservation of a viable embryo. To test the feasibility of this hypothesis, we obtained aged barley seeds that had been collected and stored during the past 17 years and examined the effect of seed age on MT activity and the ability to germinate. For each of four age groups, 10 seeds were individually assayed in duplicate and the results were averaged. We found that the L-isoaspartyl MT-specific activity was significantly reduced in the seeds from 1979 and 1985 compared with the seeds from 1991 (Table IV). Although the total MT activity in the seeds decreased slightly between 5 and 11 years of storage, it was significantly reduced after 17 years (Table IV).

Table II. *Occurrence of peptide-specific L-isoaspartyl MT activity in homogenates of non-seed plants (divisions as classified by Bold et al., 1987)*

Division	Genus and Species	Common Name	MT Activity[a]	Tissue
			pmol min^{-1} mg^{-1} protein	
Euglenophyta	*Colacium* sp.	Euglenoids	ND[b]	Vegetative
Phaeophyta	*Ectocarpus* sp.	Brown algae	ND	Vegetative
Chrysophyta	*Botrydium* sp.	Yellow-green algae	ND	Vegetative
	Synedra sp.	Diatom	ND	Vegetative
Pyrrhophyta	*Amphidinium carterae*	Dinoflagellate	ND	Vegetative
Rhodophyta	*Bangia fuscopurpurea*	Red algae	ND	Vegetative
Hepatophyta	*Marchantia* sp.	Liverwort	ND	Thallus
Bryophyta	*Polytrichum* sp.	Hair-cap moss	0.21	Capsules
			ND	Spores
			0.08	Leaves/stems
Microphyllophyta	*Lycopodium lucidulum*	Shining club moss	0.55	Leaves
	Selaginella uncinata	Blue spike moss	0.69	Leaves
	Selaginella lepidophylla	Resurrection plant	0.40	Dry leaves
			0.05	Wet leaves
Arthrophyta	*Equisetum hyemale*	Horsetail	0.35	Strobilus
			0.37	Stems
Pteridophyta	*Alsophila* sp.	Australian tree fern	0.07	Sporangia

[a] Methylation assays were performed in duplicate. [b] ND, No detectable MT activity.

Given that the endogenous barley MT was still active in the aged seeds, we next wished to quantify the number of L-isoaspartyl residues in the different seed populations. No increase in methylation sites was detected in the proteins of the older seeds when assayed with endogenous barley MT (data not shown). This suggests that either aspartyl and asparaginyl residues are not damaged during seed storage or the damage does occur but the seed MT is capable of repairing it. We were, however, able to detect a 2.7-fold increase in endogenous L-isoaspartyl-containing polypeptides in extracts of 1979 barley seeds compared with extracts from 1991 seeds using human MT (MacLaren and Clarke, 1995; Table IV). This enzyme is useful for this assay because it is much more concentrated and possesses a higher affinity for L-isoaspartyl residues in peptide and protein substrates than the endogenous MT (Mudgett and Clarke, 1993; Kagan and Clarke, 1995). This increase in L-isoaspartyl residues in the aged seeds implies that aspartyl damage may in fact occur during seed storage but that the barley MT recognizes and repairs only a subpopulation of the damaged residues, leaving the rest to accumulate.

The accumulation of L-isoaspartyl-containing proteins observed in the aged population of barley seeds coincides with a severe reduction of seed viability (Fig. 2). Whereas all 20 seeds examined from the 5-year-old population germinated within 1 d of imbibition, only 50% of the 7- and 11-year-old seeds germinated within 6 d of imbibition. None of the 17-year-old seeds were viable (Fig. 2). The 50% loss in viability between the 5-year-old population and the 7- and 11-year-old population correlates with the largest

Table III. *Inhibition of peptide-specific L-isoaspartyl MT activity by plant extracts*

Plant Extract	Source of MT	MT Activity[a]	Inhibition
		pmol min^{-1} mg^{-1} protein	%
No addition	Wheat	2.69	0
No addition	Cucumber	8.13	0
Polytrichum spores	Cucumber	7.86	3.3
Evening primrose seeds	Cucumber	7.58	6.8
Resurrection plant dry leaves	Cucumber	7.50	7.7
Alsophila sporangia	Cucumber	6.96	14.4
Polytrichum leaves/stems	Cucumber	6.75	17.0
A. thaliana seeds	Cucumber	6.41	21.2
Liverwort thallus	Cucumber	5.33	34.4
California black oak seeds	Cucumber	2.16	73.4
Gingko seeds	Cucumber	1.32	83.8
Giant sequoia seeds	Wheat	0.43	83.9
Big leaf maple seeds	Cucumber	0.08	99.0
Big leaf maple seeds	Wheat	0.00	100

[a] Methylation assays were performed in duplicate.

Table IV. *Peptide-specific L-isoaspartyl MT activity and L-isoaspartyl-containing methyl-accepting substrates in individual aged barley seeds*

Year Seed Produced	Barley MT Activity[a]		L-Isoaspartyl-Containing Methyl-Acceptor Proteins[b]	
	Specific activity[c]	Total activity	Total level	Increase over 1991 level
	pmol min^{-1} mg^{-1} protein	*pmol min^{-1}*	*pmol mg^{-1} protein*	*-fold*
1991	2.41 ± 0.44	1.86 ± 0.43[d]	251 ± 35	1.0
1989	2.35 ± 0.25	1.70 ± 0.23	552 ± 48	2.2
1985	2.11 ± 0.29	1.74 ± 0.31	482 ± 75	1.9
1979	1.86 ± 0.28	1.50 ± 0.23[d]	670 ± 82	2.7

[a] Values from 10 seeds each assayed in duplicate (mean ± SD). [b] Values from 10 seeds (mean ± SD) determined using human recombinant MT. [c] Using the LSD procedure for multiple comparisons among means (Steel and Torrie, 1980), the MT-specific activity of the 1989 seed is not significantly different from that of the 1991 seed. However, the specific activities of the 1985 and 1979 seeds are significantly different from that of the 1991 seed ($P = 0.05$ and 0.001, respectively). [d] MT total activities for seeds produced in 1991 and 1979 are significantly different ($P = 0.003$).

increase (2.2- and 1.9-fold, respectively), in endogenous L-isoaspartyl residues, but the 17-year-old seeds had only 1.2-fold more L-isoaspartyl residues than the 7-year-old seeds (Table IV). Clearly, types of deterioration other than spontaneous aspartyl damage accompany seed aging and are likely to affect the viability of the embryo.

Because the barley seeds analyzed as described above were from lots grown in different years and under different environmental conditions, we were interested in performing a more controlled analysis of seed aging on a single lot of seeds. To accelerate seed deterioration, barley seeds from a lot collected in 1991 were exposed to 40°C and approximately 100% RH for up to 18 d. This accelerated aging of the barley seeds did not reduce MT activity. Rather, MT-specific activity and total activity increased (Table V; 67 and 154%, respectively), after 4 d of accelerated aging. MT levels decreased after 7 d of accelerated aging and yet remained elevated compared with the unaged seeds (Table V). The germination percentage of the aged barley seeds was greatly reduced after 7 d of accelerated aging (Fig. 3), and less than 25% of the seeds were viable after 18 d of treatment. We also observed increased levels of protein aspartyl damage in seeds exposed to high temperature and humidity (Table V); after 4 d, the increase was about 1.5-fold, whereas after 18 d, it was 2.9-fold, which is similar to that seen in the 17-year-old naturally aged seeds. We found that there was a generally very good correlation between the content of methylatable substrates (damaged proteins) in seeds and the loss of viability (Fig. 4).

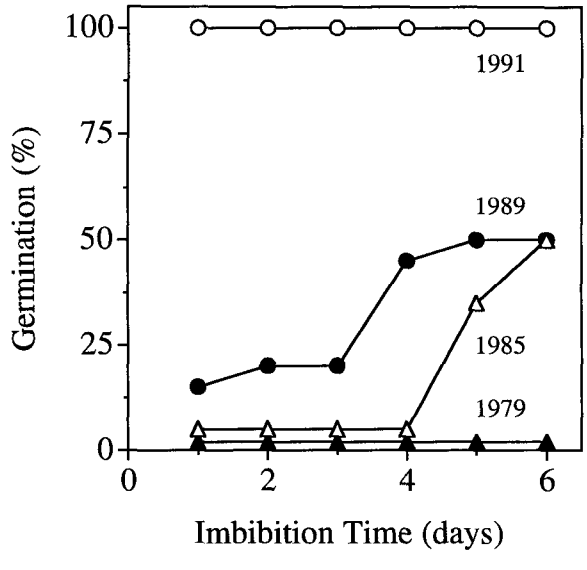

Figure 2. Germination of naturally aged barley seeds. The average percentage of germination of groups of 20 barley seeds collected in 1979 (▲), 1985 (△), 1989 (●), and 1991 (○) are shown.

To ascertain where the damaged proteins were accumulating in the aged seeds, we assayed embryo and endosperm seed sections from unaged and 18-d-accelerated aged barley seeds for both MT activity and substrates for the human MT. We found the specific activity of the barley MT to be approximately 3- to 4-fold higher in the embryo than in the endosperm for both the unaged and 18-d-aged seeds (Table VI). The level of aspartyl-damaged proteins increased 2.2-fold in the endosperm extracts and 5.3-fold in the embryo extracts (Table VI). Accelerated aging for 18 d therefore resulted primarily in the spontaneous deterioration of embryo proteins and the loss of seed viability. The high level of methylatable substrates in the 14- and 18-d artificially aged seeds (Table V), which was comparable to the level in seeds naturally aged for 7 to 17 years (Table IV), suggests that the rate of spontaneous damage was significantly increased under these accelerated aging conditions. Although the barley MT remained active within the seeds, it appeared to be unable to recognize and repair the L-isoaspartyl residues at the same rate that these residues are being generated.

DISCUSSION

Ultimately, the viability of an aging plant will depend on how well it protects its enzymes and structural proteins from developmentally and environmentally imposed stresses. Although some types of damage (e.g. proteolysis) require replacement of the protein, other types of damage

Table V. *Peptide-specific L-isoaspartyl MT activity and L-isoaspartyl-containing methyl-accepting substrates in accelerated aged barley seeds*

Days Aged	Barley MT Activity[a]		L-Isoaspartyl-Containing Methyl-Acceptor Proteins[b]	
	Specific activity	Total activity	Total level	Increase over 0-d-aged seeds
	pmol min^{-1} mg^{-1} protein	*pmol min^{-1}*	*pmol min^{-1} mg^{-1} protein*	*-fold*
0	1.66	11.5	180	1.0
4	2.78	29.2	266	1.5
7	2.31	19.8	280	1.6
10	2.36	18.2	350	2.0
14	2.30	19.1	383	2.1
18	2.46	20.2	518	2.9

[a] Values from a homogenate of 10 seeds assayed in duplicate. [b] Values from a homogenate of 10 seeds using human recombinant MT.

can be corrected with much less expenditure of energy. The L-isoaspartyl MT has recently been shown to function in mammals by repairing L-isoaspartyl residues, a particularly common form of protein damage (Kim et al., 1997). The widespread presence of this enzyme in the plant species surveyed here suggests that these plants may be equipped with a similar methylation-dependent protein repair pathway.

Changes in MT regulation in response to varying levels of exogenous (Mudgett and Clarke, 1994, 1996) and endogenous ABA, as well as to water-deficit and high-salt conditions (Mudgett and Clarke, 1994), indicate that seed plants are able to modify the levels of enzyme activity in both seed tissue and vegetative tissue in response to environmental fluxes that may lead to spontaneous protein degradation. The apparent absence of MT in some non-seed-bearing plants is similar to what has been observed in a survey of bacterial species: a number of Gram-negative bacteria possess L-isoaspartyl MT activity, whereas other Gram-negative and all Gram-positive bacteria tested to date do not; presumably, the latter have developed other methods for avoiding isoaspartyl damage (Li and Clarke, 1992).

The pervasiveness of MT in seeds supports the idea that this enzyme may be most important in cells in which protein turnover is limited; checking at least a fraction of the damaged aspartyl residues that are slowly generated in the seed proteins over time may assist in the preservation of a viable embryo. Nonetheless, it is clear from our results that, during the natural aging of barley seeds, a population of L-isoaspartyl residues is arising that cannot be efficiently repaired by endogenous MT.

We suspect that the problem of spontaneous protein degradation is exacerbated in seed proteins undergoing extreme changes in solvation during the desiccation period of seed maturation and the solvation period of seed germination. For example, it has been shown that the rate of

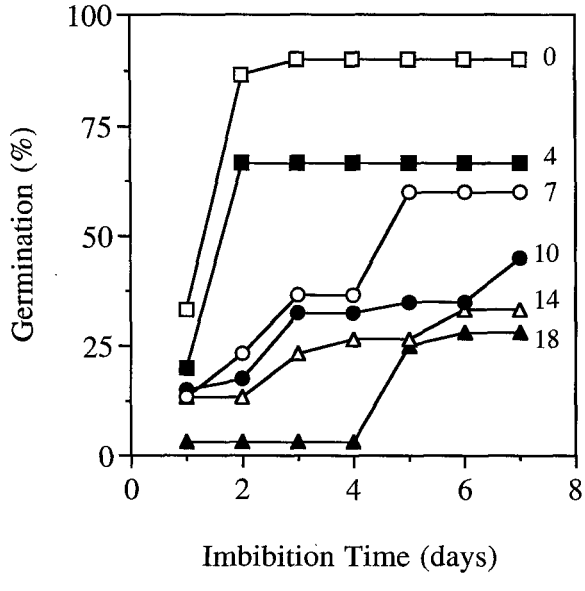

Figure 3. Germination of barley seeds after accelerated aging. The average percentage of germination is shown for groups of 30 barley seeds aged at 40°C and 100% RH for 0 d (□), 4 d (■), 7 d (○), 10 d (●), 14 d (△), and 18 d (▲).

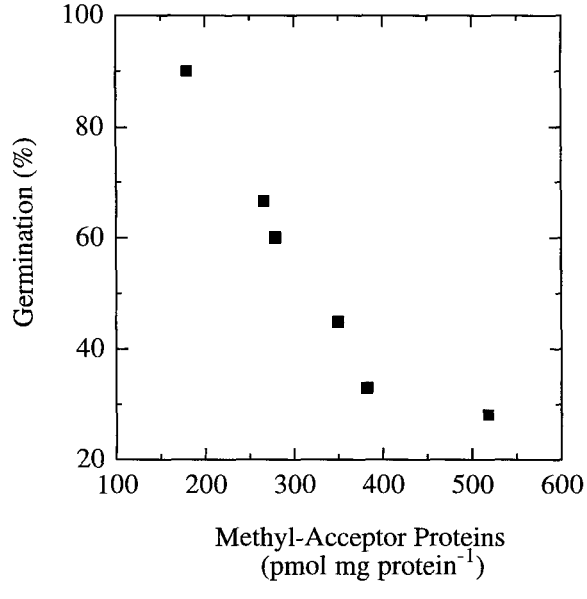

Figure 4. Correlation of barley seed germination and the accumulation of damaged protein substrates for the L-isoaspartyl MT in seeds undergoing accelerated aging. Data were taken from Figure 3 and Table V.

Table VI. *Peptide-specific L-isoaspartyl MT activity and L-isoaspartyl-containing methyl-accepting substrates in embryo and endosperm tissue isolated from natural and accelerated aged barley seeds*

Tissue	Days Aged	Barley MT-Specific Activity[a]	L-Isoaspartyl-Containing Methyl-Acceptor Proteins[a]	
			Total level	Increase over 0-d-aged seeds
		pmol min^{-1} mg^{-1} protein	*pmol mg^{-1} protein*	*-fold*
Endosperm	0	2.49	277	1.0
Embryo	0	6.89	236	1.0
Endosperm	18	1.92	602	2.2
Embryo	18	7.36	1258	5.3

[a] Values from homogenates of endosperm and embryo tissue isolated from 10 seeds, each assayed in duplicate.

succinimide formation from L-asparaginyl residues in peptides, the reaction that leads directly to L-isoaspartyl formation, increases with the dielectric strength of the medium (Brennan and Clarke, 1993). Thus, the increase in the intracellular salt concentration brought on by desiccation or salt stress may result in an acceleration of L-isoaspartyl residue formation. In addition, denaturation of proteins is known to promote the accumulation of these residues (Clarke et al., 1992). Aggregation of damaged proteins can further limit accessibility of MT to L-isoaspartyl residues. Although it is also possible that lack of water or *S*-adenosylmethionine would inhibit the repair reactions in seeds, this seems less likely because L-isoaspartyl residues that can be recognized by the plant MT do not increase with seed age.

Results similar to those presented here have recently been observed in mammalian tissues. The specific activity of the L-isoaspartyl (D-aspartyl) *O*-MT in mice is about 6-fold higher in brain tissue than in liver tissue, possibly because cell turnover is slower in the brain (Kim et al., 1997). However, the number of L-isoaspartyl residues in endogenous proteins in these mice is 4- to 6-fold higher in the brain than in the liver, presumably for the same reason. Like the seed MT, the brain enzyme is not perfectly efficient. When mice lacking MT are generated by gene replacement, the number of L-isoaspartyl residues in brain cells increases 6-fold above the level seen in wild-type siblings, and the mice die from brain dysfunction at an average of 42 d after birth (Kim et al., 1997). Therefore, cells with the highest MT activity appear to be those most susceptible to the accumulation of isoaspartyl residues in both plants and mammals.

Does the accumulation of L-isoaspartyl-containing proteins in naturally aged barley seeds contribute to the observed loss of viability? Barley seeds accumulated L-isoaspartyl-containing proteins as viability decreased during exposures to high temperature and humidity (Fig. 4). It remains possible, however, that the altered aspartyl residues simply arise as a side product of some other degradation reaction that leads to the loss of viability. Work is currently being done to answer this question by generating mutant or transgenic *A. thaliana* that lack the L-isoaspartyl MT. The effect of L-isoaspartyl residues in plant proteins and the physiological role of the MT will be determined by observing whether these plants accumulate the products of spontaneous aspartyl damage and whether the presence of such aberrant proteins limits their lifespan.

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