

# Distinct Patterns of Expression But Similar Biochemical Properties of Protein L-Isoaspartyl Methyltransferase in Higher Plants<sup>1</sup>

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Protein L-isoaspartyl methyltransferase is a widely distributed repair enzyme that initiates the conversion of abnormal L-isoaspartyl residues to their normal L-aspartyl forms. Here we show that this activity is expressed in developing corn (*Zea mays*) and carrot (*Daucus carota* var. Danvers Half Long) plants in patterns distinct from those previously seen in winter wheat (*Triticum aestivum* cv Augusta) and thale cress (*Arabidopsis thaliana*), whereas the pattern of expression observed in rice (*Oryza sativa*) is similar to that of winter wheat. Although high levels of activity are found in the seeds of all of these plants, relatively high levels of activity in vegetative tissues are only found in corn and carrot. The activity in leaves was found to decrease with aging, an unexpected finding given the postulated role of this enzyme in repairing age-damaged proteins. In contrast with the situation in wheat and Arabidopsis, we found that osmotic or salt stress could increase the methyltransferase activity in newly germinated seeds (but not in seeds or seedlings), whereas abscisic acid had no effect. We found that the corn, rice, and carrot enzymes have comparable affinity for methyl-accepting substrates and similar optimal temperatures for activity of 45°C to 55°C as the wheat and Arabidopsis enzymes. These experiments suggest that this enzyme may have specific roles in different plant tissues despite a common catalytic function.

A widely distributed enzyme, the protein L-iso-Asp- (D-Asp) methyltransferase (EC 2.1.1.77) can specifically recognize proteins containing altered aspartyl residues. This enzyme catalyzes the methyl esterification of L-isoaspartyl (and with less affinity D-aspartyl) residues using S-adenosyl-L-methionine as the methyl group donor, and this reaction can initiate its conversion back into the normal L-aspartyl forms (Lowenson and Clarke, 1991, 1992; Brennan et al., 1994). The substrates for this enzyme are generally considered to arise from the deamidation, racemization, and isomerization of L-asparaginyl and L-aspartyl residues, which give rise to L-aspartyl, D-aspartyl, and D- and L-isoaspartyl residues (Geiger and Clarke, 1987; Patel and Borchardt, 1990; Tyler-Cross and Schirch, 1991; Clarke et al., 1992; Capasso et al., 1993). However, L-isoaspartyl residues can also possibly arise as the result of the incorporation of mischarged aspartyl residues during protein synthesis (Momand and Clarke, 1990). In either case, the accumulation of such altered residues in proteins can affect their function and lead to their inactivation (Manning et al., 1989; Stadtman, 1990; Liu, 1992; Paranandi and Aswad, 1995; Cacia et al., 1996; Capasso et al., 1996).

The L-isoaspartyl methyltransferase has been detected in gram-negative bacteria (Li and Clarke, 1992; Ichikawa and Clarke, 1998), plants (Trivedi et al., 1982; Johnson et al., 1991; Mudgett and Clarke, 1993, 1996; Kester et al., 1997; Mudgett et al., 1997; Kumar et al., 1999), nematodes (Kagan and Clarke, 1995), flies (O'Connor et al., 1997), and several mammals including humans (Clarke, 1985; O'Connor and Clarke, 1985). Mice lacking the methyltransferase show an accumulation of damaged proteins and die of seizures at an early age (Kim et al., 1997, 1999). *Caenorhabditis elegans* mutants having a disruption in the gene encoding this enzyme show poor dauer phase survival (Kagan et al., 1997), whereas *Escherichia coli* mutants lacking this enzyme are more sensitive to oxidative and other stresses in the stationary phase (Visick et al., 1998).

We are interested in the role of this enzyme in plants and in characterizing its expression and regulation. The methyltransferase activity is widely distributed both in dicots and monocots, and the activity has been found to be primarily localized in seeds, although vegetative tissues of certain plant species do display some activity (Mudgett and Clarke, 1993, 1994; Mudgett et al., 1997; Kumar et al., 1999). A role for this enzyme in seed aging was suggested from the findings that naturally aged barley seeds have reduced L-isoaspartyl methyltransferase levels and higher accumulation of "unrepaired" L-isoaspartyl residues coupled with lower germination rates (Mudgett et al., 1997). Seeds aged prematurely by heating do not have lower enzyme levels but still

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accumulate damage and have reduced viability indicating that the level of methyltransferase in seeds may not be sufficient to repair all of the damage (Mudgett et al., 1997). Furthermore, it has been shown that accelerated aging of tomato seeds at 45°C decreases germination as well as methyltransferase activity, whereas priming the aged seeds with KNO<sub>3</sub> restores the activity to the control level (Kester et al., 1997). On the other hand, Kumar et al. (1999) found that the methyltransferase activity increases in aged potato tubers, suggesting a response to a possible increase in damaged substrates. These results suggest that individual plant species may differentially control the expression of this enzyme with respect to aging.

Analysis of this enzyme in the monocot winter wheat (*Triticum aestivum* cv Augusta) indicated that the expression of the enzyme is developmentally regulated and is the highest in dry seeds, decreasing rapidly as the seeds germinated (Mudgett and Clarke, 1994). The decline in the activity correlated well with the decline in its mRNA. A relatively low and constant level of activity in leaves and whole seedlings is present after germination. Kester et al. (1997) also found that the methyltransferase activity is developmentally regulated in tomato where the activity is maximal in seeds, remains constant for 48 h postimbibition, and then declines. In wheat seedlings, the activity was found to be responsive to hormonal and stress applications. Abscisic acid (ABA) as well as dehydration and salt stress were found to induce both methyltransferase mRNA and activity (Mudgett and Clarke, 1994).

Analysis of mRNA levels and methyltransferase activity in the dicot *Arabidopsis* yielded a different profile. Although activity was also found in the seeds, it was undetectable in the vegetative tissues (Mudgett and Clarke, 1996). The mRNA levels of this enzyme did not correlate with the activity as in wheat. Here, methyltransferase mRNA was detected in the vegetative tissues but not in seeds. As in wheat, the mRNA was inducible by ABA but unlike wheat, drought and salt stress did not up-regulate mRNA expression. All of these results indicate that the regulation of this enzyme in different plants can be quite distinct and suggests it may be useful to examine the situation in other types of plants.

In the present study, we directly compared the L-isoaspartyl methyltransferase activity in the tissues of three plants: a dicot (carrot [*Daucus carota* var. Danvers Half Long]) and two economically important monocots (corn [*Zea mays*] and rice [*Oryza sativa*]). We have analyzed the activity levels in each tissue as a function of the development of the plant. Because the *Arabidopsis* and the wheat enzymes respond differently to stress conditions, we have also analyzed the effect of similar stresses on the corn enzyme activity. In addition, we have performed biochemical studies on the three plant enzymes to

determine their temperature optima and kinetic constants. These data provide evidence that the role of the L-isoaspartyl methyltransferase in plants may be more complex than originally thought and suggest that an evolutionary adaptation of the enzyme may have occurred to meet the individual needs of each plant species.

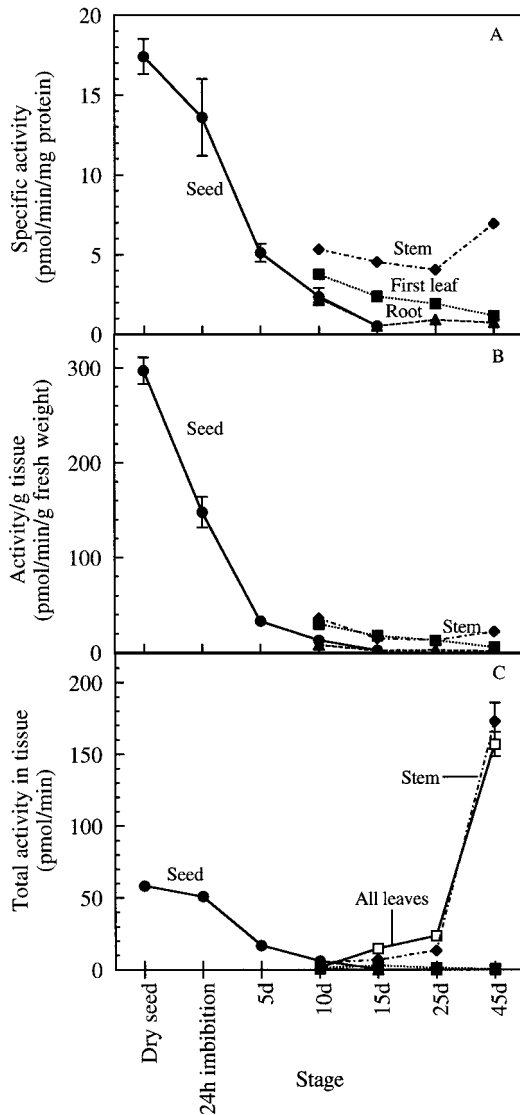
## RESULTS

### Pattern of Expression of L-Isoaspartyl Methyltransferase Activity in Developing Tissues of Corn, Rice, and Carrot

We measured methyltransferase activity in corn, rice, and carrot seeds, seedlings, and young plants. Activity levels were first measured in the dry seeds and imbibed seeds as described in "Materials and Methods." Seeds were then sown in soil and allowed to germinate. As the seedling emerged and the plant developed roots and leaves, activity levels were measured in the stem, first leaf, and roots and were correlated with the amount of protein and fresh tissue weight.

In corn plants, we found that the methyltransferase activity is abundant in the seeds on both a per gram protein and per gram tissue basis but decreases sharply as the seed undergoes hydration and germinates (Fig. 1, A and B). Germination of the corn seeds took approximately 4 to 5 d and was characterized by the emergence of the coleoptile. Developing tissues were then sampled at 5, 10, 15, 25, and 45 d post-germination. By the 15-d time point, the specific activity in the remnant seed was barely detectable. However from the 10-d time point, we found significant enzyme activity in the vegetative tissues with the highest specific activity in the stem. In the first leaf, the activity declined at longer times, whereas in stems and roots, the levels were fairly constant. Because the specific activity of the enzyme in the vegetative tissues was less than that seen in the original seed, we then wanted to ask whether the enzyme present in the vegetative tissues might arise from a redistribution of the seed protein. We thus compared the total activity in each tissue as the plant developed. As seen in Figure 1C, up to 25 d the total activity in all tissues was less than that originally present in the seed, but at 45 d the combined activity in the leaves and stem was over five times that of the seed. The data for leaves in Figure 1, A and B show the profile of the methyltransferase activity in the first leaf. In Figure 1C, we show data for both the first leaf and for the sum of all leaves. At the 45-d time point the plants had not yet developed ears and tassels.

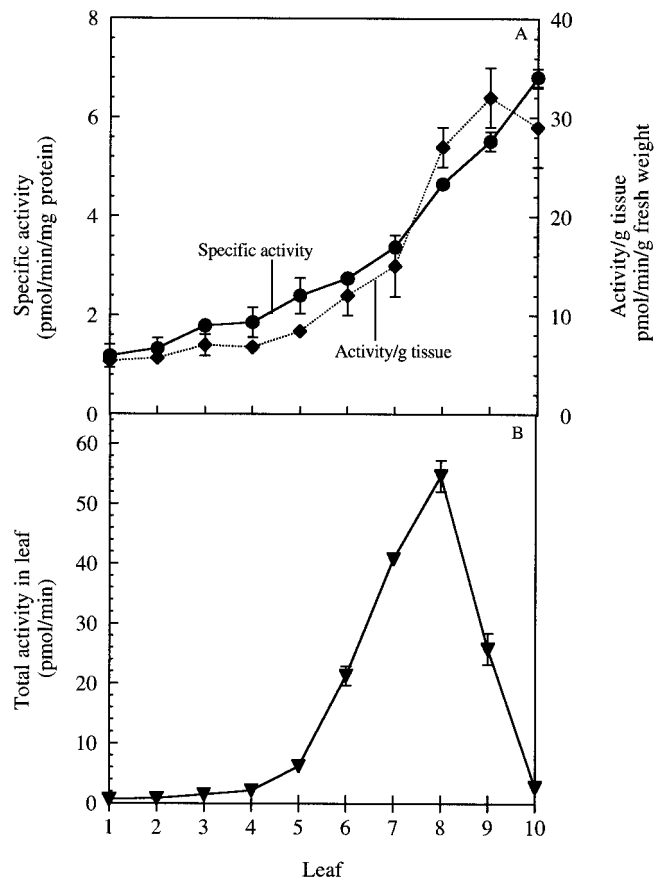
The unexpected presence of significant levels of methyltransferase activity in the corn leaves led us to examine it in the juvenile and adult leaves in greater detail. As the leaves are formed sequentially, the juvenile leaves are the oldest, whereas the younger



**Figure 1.** Activity of L-isoaspartyl methyltransferase in developing corn tissues. Days represent the time elapsed after germination of the seed. The leaf assayed was always the first leaf on the plant. The methyltransferase assay was performed as described in "Materials and Methods" with YYP-L-isoAsp-HA as a methyl-acceptor. Each reaction was done in duplicate and the values represent the mean  $\pm$  range. A, Specific activity of L-isoaspartyl methyltransferase in soluble extracts of seeds and vegetative tissues of corn. ●, Seeds; ◆, stem; ■, first leaf; ▲, root. B, Activity per unit tissue fresh weight. C, Total activity in each corn tissue; □, the sum total activity in all leaves. When no error bar is shown, the error is smaller than the width of the line.

leaves are found in the more adult population. At 45-d post-germination, leaves were excised for preparation of soluble extracts and assayed. As shown in Figure 2A, older leaves (leaves 1–5) have lower specific activity as compared with the younger leaves (leaves 6–10). However, in terms of total activity present in the leaf (Fig. 2B), the activity peaks for the 8th leaf and then declines due to the small size of the younger leaves. We next monitored the activity levels

in non-senescent individual leaves as they aged. Leaves of corn arising sequentially (numbered 1–10) were excised at different leaf stages, and their soluble extracts were assayed for methyltransferase activity. As shown in Table I, as the plant ages the activity in each individual leaf declines. For instance, the oldest 1st leaf shows more than a 3-fold decline in its activity at the 10-leaf stage versus the 1-leaf stage. A similar trend was observed for the other corn leaves. Reduction in the activity of the methyltransferase with age may reflect either developmental regulation of the enzyme or an age-dependent accumulation of methyltransferase inhibitors in the leaves. To assay the potential buildup of inhibitors, we performed a mixing experiment where the activity of the purified *Arabidopsis* recombinant methyltransferase (Thapar and Clarke, 2000) was monitored in the absence and presence of soluble extracts of corn leaves. No decrease in the activity of the recombinant enzyme activity was observed (data not shown). It is somewhat surprising that the total activity of the mixed



**Figure 2.** Activity of L-isoaspartyl methyltransferase in developing corn leaves. The numbering of the leaves refers to the sequence of their emergence from the plant; the first leaf is the oldest. Reactions were done as described in Figure 1, in duplicate, and the values represent the mean  $\pm$  range. A, Specific activity of methyltransferase (●) and activity per unit tissue fresh weight in each corn leaf (◆); B, total activity in each corn leaf (▼). When no error bar is shown, the error is smaller than the width of the line.

**Table 1.** Activity of *L*-isoaspartyl methyltransferase in corn leaves as a function of the seedling stage

Soluble extracts were prepared from the leaves of corn plants at different stages of growth and assayed for methyltransferase activity as described in Figure 1.

	Activity (pmol min <sup>-1</sup> mg <sup>-1</sup> protein) <sup>a</sup>			
	Seedling Stage			
	1 Leaf	3 Leaf	4 Leaf	10 Leaf
Leaf 1 <sup>b</sup>	3.76 ± 0.51	2.39 ± 0.41	1.93 ± 0.16	1.18 ± 0.23
Leaf 2	–	2.68 ± 0.53	2.2 ± 0.29	1.33 ± 0.21
Leaf 3	–	3.9 ± 0.33	2.99 ± 0.46	1.79 ± 0.11
Leaf 4	–	–	3.42 ± 0.34	1.86 ± 0.3
Leaf 5	–	–	–	2.40 ± 0.36
Leaf 6	–	–	–	2.75 ± 0.11
Leaf 7	–	–	–	3.38 ± 0.12
Leaf 8	–	–	–	4.66 ± 0.12
Leaf 9	–	–	–	5.52 ± 0.19
Leaf 10	–	–	–	6.80 ± 0.18

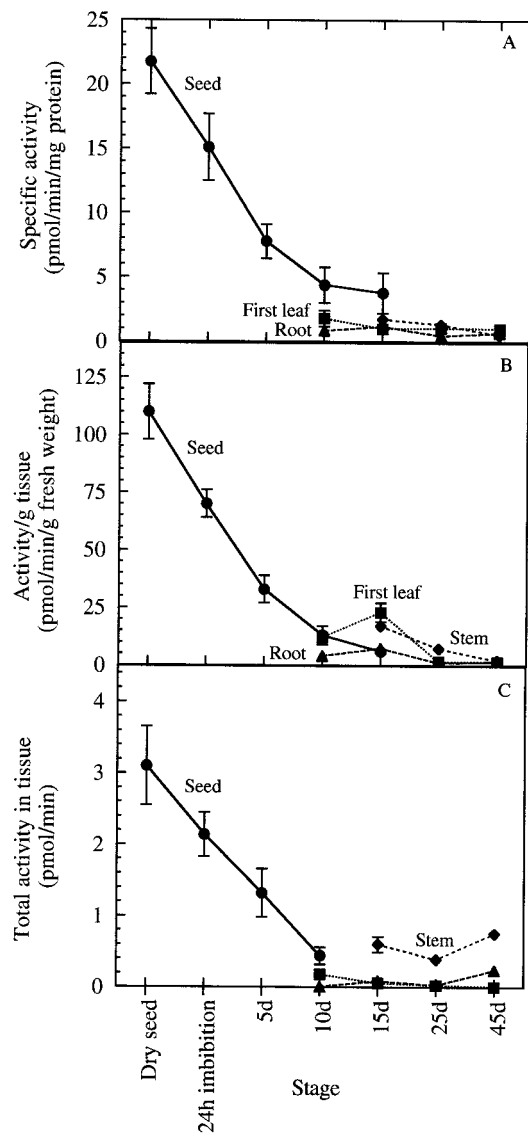
<sup>a</sup> Methylation reactions were performed in duplicate. Values represent the mean ± range. <sup>b</sup> Leaf 1 represents the oldest leaf and leaf 10 represents the youngest leaf.

samples was slightly larger than expected from the contributions of the *Arabidopsis* enzyme and the corn leaf extracts. From the above results, it is clear that the decrease in the methyltransferase activity in the leaves with age is not due to the accumulation of endogenous inhibitors but rather reflects a developmental cue. The fact that the *L*-isoaspartyl methyltransferase activity was either present at very low levels or not detected in wheat and *Arabidopsis* leaves, respectively (Mudgett and Clarke, 1994; Mudgett and Clarke, 1996), suggests that in corn this enzyme may play a specific role.

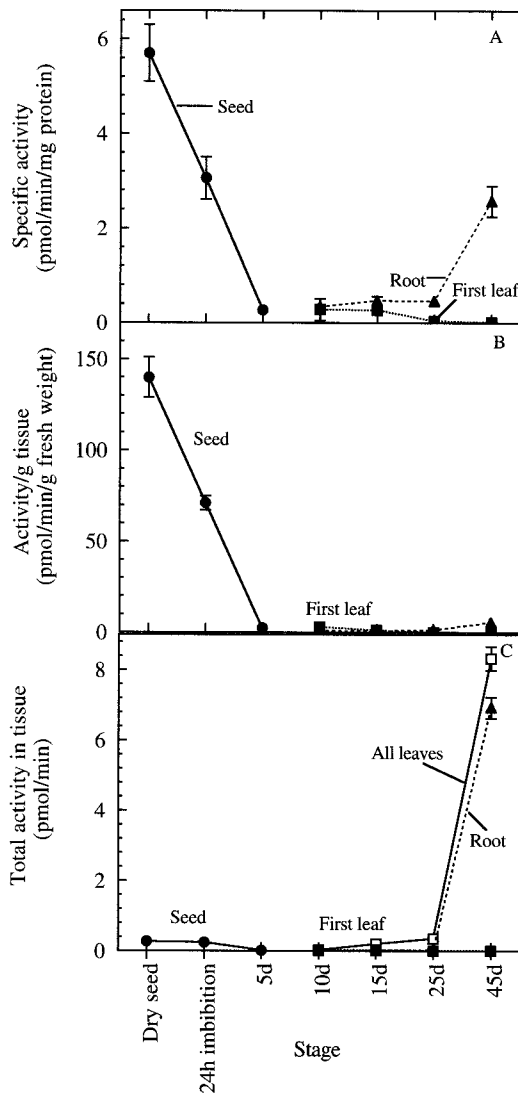
To ask how general the situation with corn might be, we then monitored the methyltransferase activity in developing rice plants. Figure 3, A and B show the distribution of the enzyme activity in different tissues as a function of growth stage. As seen with corn in Figure 1, high levels of activity were found in dry seeds, but these levels declined as the plant developed. We also found that the vegetative tissues had methyltransferase activity that remained fairly constant with age. In terms of the total activity, the stem had higher levels than leaves and roots and showed a marginal increase at d 45 post-germination (Fig. 3C). The decline in methyltransferase activity in germinating seeds and the relatively low level of activity in leaves and roots is similar to the situation previously observed in developing wheat (Mudgett and Clarke, 1994).

We then decided to analyze the methyltransferase activity in the dicot carrot to compare the situation with the dicot *Arabidopsis* where activity was only detected in seeds (Mudgett and Clarke, 1996). In carrot, the maximum specific activity was seen in the dry seeds but this decreased to barely detectable levels at d 5 post-germination (Fig. 4A). However, activity was also detected in the developing vegeta-

tive tissues (Fig. 4, A–C). The roots had significantly higher methyltransferase levels especially at d 45 post-germination when the root comprises the major portion of the plant. Relatively high methyltransferase activity in carrot roots was described previously (Mudgett and Clarke, 1993). We also measured activity in the individual leaves at different ages (Fig. 5, A and B). We found, as with corn leaves, that the older leaves had the lowest activity (Fig. 5A). Leaves from carrot were also sampled at different leaf stages of the seedling/plant (Table II) and found to exhibit



**Figure 3.** Activity of *L*-isoaspartyl methyltransferase in developing rice. Days represent the time elapsed after germination of the seed. Assays were performed as indicated in Figure 1, in duplicate, and the values represent the mean ± range. A, Specific activity of methyltransferase in soluble extracts of seeds and vegetative tissues of rice. ●, Seeds; ◆, stem; ■, first leaf; ▲, root. B, Activity per unit tissue fresh weight; C, total activity in each rice tissue. When no error bar is shown, the error is smaller than the width of the line.



**Figure 4.** Activity of L-isoaspartyl methyltransferase in developing carrot. Days represent the time elapsed after germination of the seed. Assays were performed as indicated in Figure 1, in duplicate, and the values represent the mean  $\pm$  range. A, Specific activity of methyltransferase in soluble extracts of seeds and vegetative tissues of carrot. ●, Seeds; ■, first leaf; ▲, root. B, Activity per unit tissue fresh weight. C, Total activity in each carrot tissue; □, the sum total activity in all leaves. When no error bar is shown, the error is smaller than the width of the line.

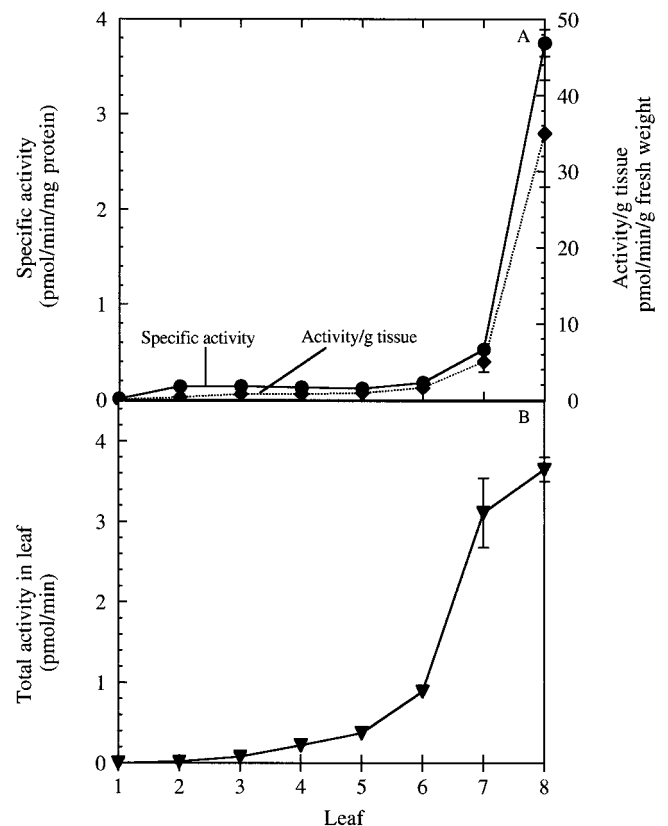
a profile similar to that observed in corn, although the decline was much greater.

A comparison of methyltransferase activity levels in developing corn, rice, and carrots is summarized in Figure 6, A and B. In each of these plants, the overall methyltransferase specific activity was found to be greatest in the seed with carrot seeds having a significantly lower activity ( $5.7 \text{ pmol min}^{-1} \text{ mg}^{-1}$  protein) than corn ( $17.5 \text{ pmol min}^{-1} \text{ mg}^{-1}$  protein) or rice ( $21.5 \text{ pmol min}^{-1} \text{ mg}^{-1}$  protein). In all cases, the activity declines after germination but lower levels are found in the developing vegetative tissues (Fig. 6, A and B).

### Distinct Stress Regulation of L-Isoaspartyl Methyltransferase Activity

Plants are exposed to stress when grown in a natural environment and their proteins are subject to accelerated damage including the accumulation of L-isoaspartyl residues (Mudgett et al., 1997). To counteract these stresses, increased expression of a number of genes can provide protective functions. We wanted to ask whether the L-isoaspartyl methyltransferase might have such a function in corn. We chose corn to study because it germinates rapidly and provides abundant tissue for analysis. Up-regulation of the methyltransferase activity by ABA treatment as well as salt and dehydration stress has been shown in wheat seedlings (Mudgett and Clarke, 1994). The phytohormone ABA is involved in interactions that control water balance and is known to induce several genes in response to water stress (Chandler and Robertson, 1994).

We analyzed the effect of short-term application of stress on the activity of the enzyme in seeds and seedlings at different developmental stages. We exposed (a) dry seeds, (b) seeds that had just germi-



**Figure 5.** Activity of L-isoaspartyl methyltransferase in developing carrot leaves. Assays were performed as indicated in Figure 1, in duplicate, and the values represent the mean  $\pm$  range. A, Specific activity of methyltransferase (●) and activity per unit tissue fresh weight in each carrot leaf (◆); B, total activity in each carrot leaf (▼). When no error bar is shown, the error is smaller than the width of the line.

**Table II.** Activity of *L*-isoaspartyl methyltransferase in carrot leaves as a function of the seedling stage

Soluble extracts were prepared from the leaves of carrot plants at different stages of growth and assayed for methyltransferase activity as described in Figure 1.

	Activity at Seedling Stage <sup>a</sup>			
	1-Leaf	2-Leaf	3-Leaf	4-Leaf
	<i>pmol min<sup>-1</sup> mg<sup>-1</sup> protein</i>			
Leaf 1 <sup>b</sup>	0.28 ± 0.04	0.27 ± 0.02	0.04 ± 0.01	0.01 ± 0.001
Leaf 2	–	1.76 ± 0.13	0.45 ± 0.02	0.14 ± 0.01
Leaf 3	–	–	0.9 ± 0.15	0.14 ± 0.002
Leaf 4	–	–	–	0.13 ± 0.02
Leaf 5	–	–	–	0.12 ± 0.01
Leaf 6	–	–	–	0.18 ± 0.01
Leaf 7	–	–	–	0.53 ± 0.02
Leaf 8	–	–	–	3.75 ± 0.14

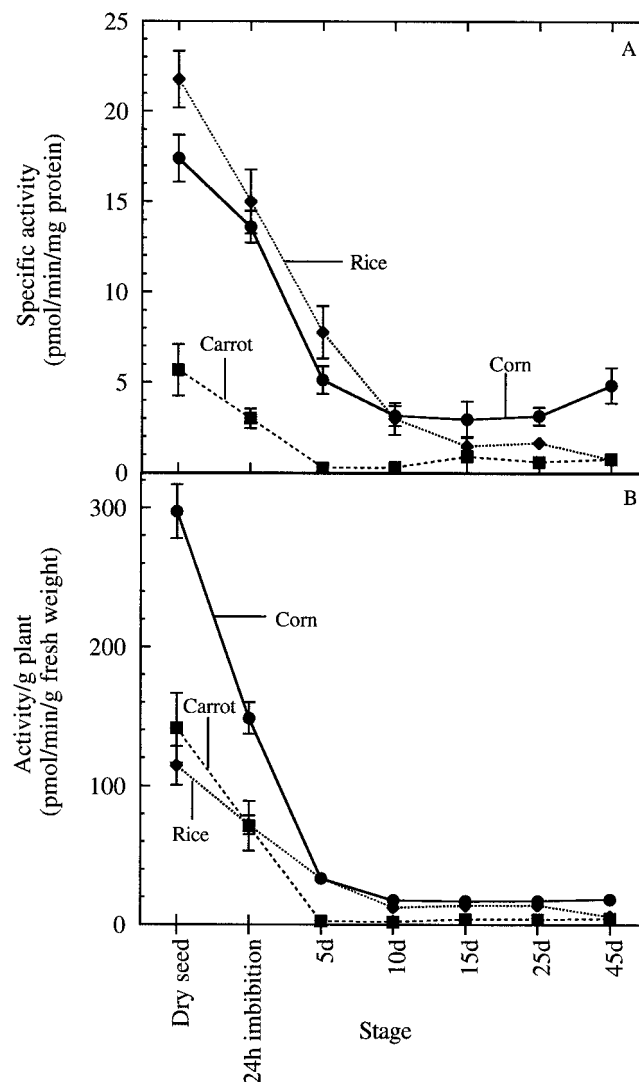
<sup>a</sup> Methylation reactions were performed in duplicate. Values represent the mean ± range. <sup>b</sup> Leaf 1 represents the oldest leaf and leaf 10 represents the youngest leaf.

nated, and (c) 4- to 5-d-old seedlings to various conditions for 10 h including ABA, high levels of sorbitol and NaCl, cold and heat, and darkness. Dry seeds as well as seedlings did not show any major changes in the activity under these conditions, and levels were maintained close to those observed in control samples (Fig. 7). However, significant effects were observed when germinated seeds were stressed. Here, methyltransferase activity was found to increase in response to salt and osmotic stress mediated via NaCl and sorbitol in a concentration-dependent manner, but ABA treatment did not affect the activity. At the 1 M NaCl and 0.5 M sorbitol concentrations, there was a 2- to 2.4-fold increase in the activity ( $P < 2 \times 10^{-4}$  for 1 M NaCl and  $P < 10^{-6}$  for 0.5 M sorbitol). The only other significant change found was that the activity was reduced to approximately 50% when seedlings were placed in the dark (Fig. 7,  $P < 2 \times 10^{-6}$ ). It is interesting that samples that were incubated under total dehydration conditions did not show any significant change in the activity (Fig. 7). From these results it appears that seeds are most susceptible to *L*-isoaspartyl residue-associated damage when they are in a state of high metabolic activity such as germination.

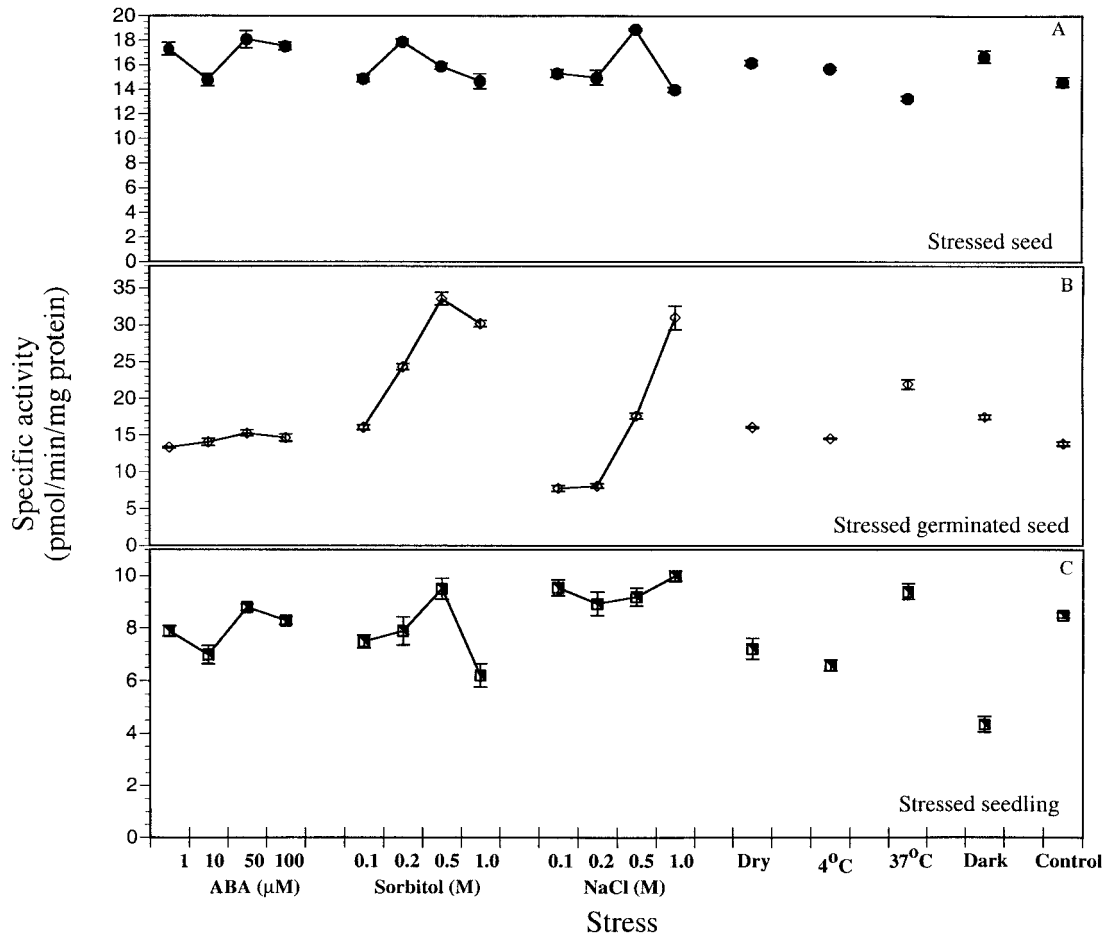
#### Comparison of the Enzymatic Properties of Plant *L*-Isoaspartyl Methyltransferases

We now wanted to ask if the differences seen here in the developmental regulation of the corn, rice, and carrot enzymes from that observed previously in wheat and *Arabidopsis* might be reflected in differences in the catalytic nature of these enzymes. We first examined the relative efficiency of each of the plant methyltransferases for recognizing damaged peptides. Soluble extracts of corn, rice, wheat, and carrot seeds as well as corn leaves were assayed for initial velocity toward various concentrations of a

peptide substrate (VYP-*L*-iso-Asp-HA) as well as a protein substrate (ovalbumin) to determine the  $K_m$  and  $V_{max}$  values. The results shown in Table III demonstrate that these values are very similar to each other. For the seed extracts, the  $K_m$  values for the peptide substrate varied by a maximum of 1.6-fold and for ovalbumin by a maximum of 2.8-fold. It is of interest that the relative  $V_{max}$  values for peptide and protein substrates were also similar with the  $V_{max}$  for peptide being 2- to 3-fold higher than for ovalbumin with the exception of the wheat enzyme where these values were found to be similar. The  $K_m$  of the corn leaf enzyme for the peptide substrate was lower than that for the seed enzyme; ovalbumin proved to be a poorer substrate. These results suggest that despite the developmental and hormonal regulatory differ-



**Figure 6.** Comparison of *L*-isoaspartyl methyltransferase activity levels in corn (●), rice (◆), and carrot (■) as a function of the development of the plant. A, Specific methyltransferase activity in the whole plant; B, activity per unit plant weight. When no error bar is shown, the error is smaller than the width of the line.



**Figure 7.** L-Isoaspartyl methyltransferase activity in soluble extracts prepared from corn seeds, germinated seeds, and seedlings treated under various stress conditions. Activity of methyltransferase in soluble extracts of washed seeds (A, ●), germinated seeds (B, ◇), and 4-d-old seedlings (C, ▣) exposed to various treatments for 10 h at 25°C under 24 h of continuous light unless otherwise specified. For the application of salt and osmotic and hormonal stress, samples were incubated in sodium chloride, sorbitol, and ABA solutions, respectively. For temperature stress, samples were incubated in water at 4°C and 37°C under a continuous dark period. For dark stress, samples were incubated in water in the dark at 25°C. "Dry" indicates samples incubated in tubes without any solution under continuous light conditions. After 10 h of stress, the seeds/seedlings were harvested, and soluble extracts of whole seedlings were prepared as mentioned in "Materials and Methods." Assays were performed in triplicate as described in Figure 1, and the values represent the mean  $\pm$  SE. The control represents seeds/seedlings incubated in water alone at 25°C under 24 h of continuous light.

ences, the seed enzymes are catalytically similar. However, the corn leaf enzyme does appear to have distinct kinetic parameters, recognizing the peptide substrate with more affinity and ovalbumin with less affinity (Table III). It is important to point out that these rate constants are determined for the enzyme present in soluble extracts and may be influenced by different factors in these extracts.

Finally, soluble extracts were prepared from corn, rice, wheat, and carrot dry seeds and assayed for methyltransferase activity at 10°C intervals over a range of 25°C to 65°C. As shown in Figure 8A, activity was maximal at 45°C to 55°C for each of the enzymes except for the corn leaf enzyme where it rapidly fell at 55°C. It was also observed that the corn enzyme both from seeds and leaves retained a detectable activity level at 65°C. What is most interest-

ing, however, is that significant activity was found over the entire range tested for all of the plant enzymes. We then asked whether this also would be the case for the enzymes from the bacterium *E. coli*, the nematode *C. elegans*, and recombinant human enzyme under similar conditions. We found that the nematode enzyme activity was relatively constant over the range of 25°C to 65°C (Fig. 8B). The bacterial enzyme had an optimal activity at 45°C but also had significant activity over the entire range. It is surprising that the human recombinant enzyme was most active at 55°C with approximately 4-fold more activity than at the physiological temperature of 37°C. The ability of all these enzymes to be catalytically active over this range of temperatures suggests that repair can occur even under "heat shock" conditions where the spontaneous damage to proteins could be ex-

**Table III.** Kinetic constants of L-isoaspartyl methyltransferase activity in soluble extracts of various plant seeds as well as corn leaves for an L-isoaspartyl containing peptide and ovalbumin

The methylation reaction consists of either the peptide substrate (final concentration ranging from 0.01–2 mM) or ovalbumin (final concentration ranging from 0.1–3 mM) incubated with 10  $\mu\text{M}$  [ $^{14}\text{C}$ ]AdoMet and 12  $\mu\text{L}$  of the enzyme extract to a final volume of 40  $\mu\text{L}$  in 0.2 M sodium citrate buffer (pH 6.0). The reaction was performed at 45°C for 1 h. Reactions for each substrate concentration were performed in duplicate and the values represent the mean  $\pm$  range.  $K_m$  and  $V_{\text{max}}$  values were calculated by fitting the data to the Michaelis-Menten equation using the DeltaGraph program (Version 4.0).

	VYP-L-isoAsp-HA		Ovalbumin	
	$K_m$ mM	$V_{\text{max}}$ $\text{pmol min}^{-1} \text{mg}^{-1} \text{protein}$	$K_m$ mM	$V_{\text{max}}$ $\text{pmol min}^{-1} \text{mg}^{-1} \text{protein}$
Corn seed	0.29 $\pm$ 0.04	31.5 $\pm$ 1.4	2.77 $\pm$ 0.32	10.2 $\pm$ 1.0
Rice seed	0.33 $\pm$ 0.05	29.5 $\pm$ 1.4	1.03 $\pm$ 0.06	14.9 $\pm$ 1.3
Carrot seed	0.24 $\pm$ 0.01	9.9 $\pm$ 0.2	2.53 $\pm$ 0.35	3.4 $\pm$ 0.1
Winter wheat seed	0.38 $\pm$ 0.01	11.2 $\pm$ 0.1	2.32 $\pm$ 0.42	11.9 $\pm$ 0.9
Corn leaf	0.13 $\pm$ 0.02	1.16 $\pm$ 0.12	12.7 $\pm$ 2.0	2.2 $\pm$ 0.3

pected to be enhanced. To determine the individual heat sensitivity of the corn enzyme from the seeds versus the leaves, crude extracts were pre-incubated at temperatures ranging from 37°C to 65°C for 30 min and then tested for activity toward the peptide substrate at the optimal 45°C temperature (Fig. 8A). As observed in Figure 9, the corn seed enzyme activity remained fairly constant until a temperature of 50°C and then fell rapidly. On the other hand, the leaf enzyme showed a rapid decline at 50°C and fell to undetectable levels at and above 55°C. It is interesting that the corn seed enzyme still retained activity at these high temperatures reflecting its ability to tolerate heat stress to a greater extent than the leaf enzyme.

## DISCUSSION

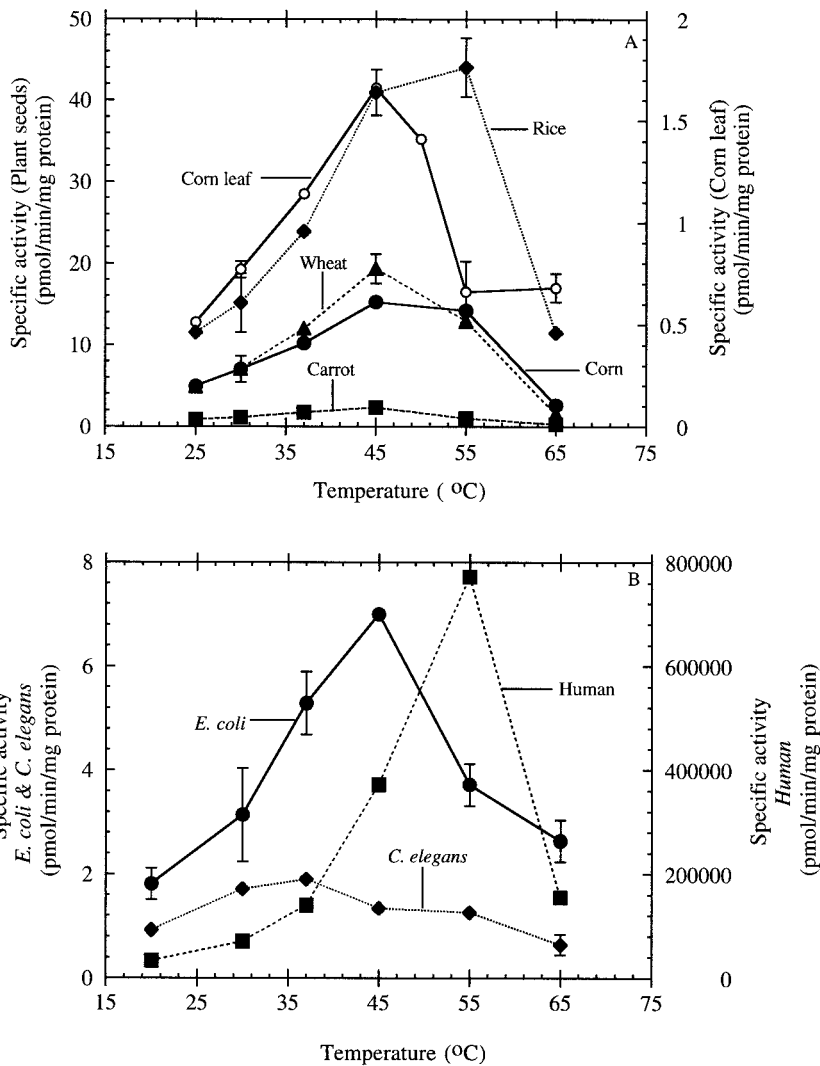
Plant viability and vigor are dependent on the integrity of its proteins (Priestley, 1986). These molecules are susceptible to spontaneous degradation reactions that progress irreversibly. How can cells avoid the accumulation of these age-damaged species? One mechanism involves protein L-isoaspartyl methyltransferase that can play a role in repairing at least one portion of such age-accumulated damage in proteins due to spontaneous deamidation, racemization, and isomerization reactions (Lowenson and Clarke, 1991, 1992; Brennan et al., 1994).

In this work, we analyzed L-isoaspartyl methyltransferase activity in corn, rice, and carrot as a function of the development of the plant. In all three plants, we found that enzyme activity is high in seeds, rapidly decreases in the seedling, and then re-emerges to different extents in the vegetative tissues. Significant levels of activity are found in the leaves and stems of corn and rice and in the leaves and roots of carrots. A similar loss in enzyme activity in germinating seeds has been found to occur in wheat followed by a re-emergence in the leaves to levels somewhat lower to those seen here in rice (Mudgett and Clarke, 1994). A slightly more moderate decline in enzyme activity in germinating tomato

seeds has also been described (Kester et al., 1997). The developmental patterns seen in corn, rice, wheat, and carrots stand in contrast to that observed in Arabidopsis where the activity has been detected only in the seeds and not in any of the vegetative tissues (Mudgett and Clarke, 1996).

These results suggest that plant L-isoaspartyl methyltransferase activities can be developmentally regulated in distinct programs, presumably to meet the physiological needs of each species. In dry seeds, a large population of proteins is present, which needs to be maintained in the normal form in the absence of active protein synthesis. Thus, it is not surprising that high levels of methyltransferase activity are found here, as shown in this study and earlier work (Mudgett and Clarke, 1993, 1994, 1996; Kester et al., 1997; Mudgett et al., 1997). However, the reasons for the increase in activity in vegetative tissues are less clear. High levels of enzyme activity have been previously shown to be present in carrot roots (Mudgett and Clarke, 1993) and in potato tubers (Kumar et al., 1999). In these tissues, the situation may be comparable with that of seeds where proteins need to be maintained for relatively long periods of time. However, this explanation does not seem to suffice for leaf tissues, especially in light of our observations that methyltransferase activity declines in aging leaves of corn and carrots.

It is at first difficult to understand the increased activity of the repair methyltransferase in younger leaves because the proteins in these leaves would have been present for less time than those in the older leaves and might be expected to contain fewer damaged aspartyl residues to be metabolized by the methyltransferase. However, two possibilities may complicate this simple type of picture. In the first place, damaged isoaspartyl residues can be generated during protein synthesis by the misincorporation of isoaspartyl-tRNAs where the RNA linkage is to the side chain rather than the main chain carboxylic acid group (Momand and Clarke, 1990). Here, the faster rate of protein synthesis in the newly forming leaf may result in a larger concentration of



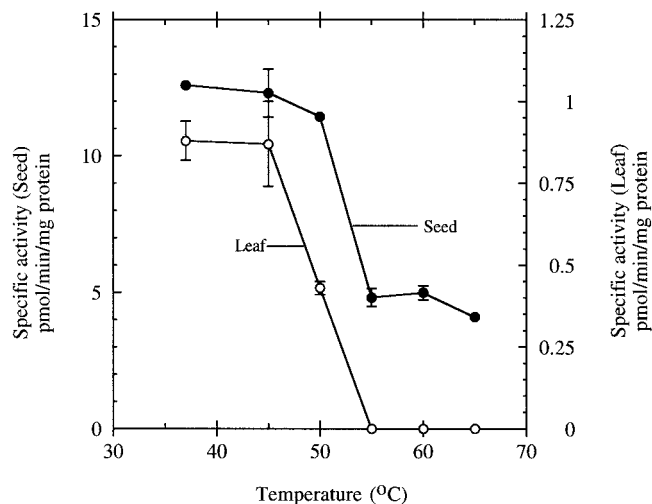
**Figure 8.** L-Isoaspartyl methyltransferase activity from various organisms assayed at different temperatures. A, Methyltransferase activity in soluble seed extracts of corn (●), rice (◆), wheat (▲), carrot seeds (■), and corn leaves (○) at different reaction temperatures. The methylation reaction composition was the same as described in Figure 1. B, Methyltransferase activity in crude extracts of *E. coli* (●), *C. elegans* (◆), and partially purified human recombinant enzyme (■). The reactions were performed as described in "Materials and Methods," in duplicate, and the values represent the mean ± range. When no error bar is shown, the error is smaller than the width of the line.

isoaspartyl-containing polypeptides. Second, it is known that reactive oxygen intermediates can increase the rate of formation of isoaspartyl residues (O'Connor and Yutzey, 1988; Ingrosso et al., 2000). In plants, the youngest leaves are often the ones that are most exposed to full sunlight, which can result in the generation of reactive oxygen species (Niyogi, 1999). In both cases, the fastest rate of damaged aspartyl residue formation might be expected to be found in the youngest leaves and this is exactly where we find the highest concentrations of the protein repair L-isoaspartyl methyltransferase activity. We are now interested in measuring the levels of damaged proteins themselves in leaves of different ages to test these hypotheses.

The differences in the methyltransferase activity of older and younger leaves might be linked to the "phase shift" phenomenon seen in leaf development (Kerstetter and Poethig, 1998) where juvenile and adult leaves have distinct anatomies (Orkwiszewski and Poethig, 2000). Although the molecular basis of the phase shift has not been established, it is possible

that methyltransferase expression is turned down in juvenile leaves and up in adult leaves. However, we do observe a decrease in methyltransferase activity even in the same numbered leaf when assayed over time at different seedling stages (Tables I and II) so that our results cannot be explained solely by phase shift changes. It would be very interesting to monitor the phase-specific expression of the methyltransferase mRNA in juvenile and adult leaves.

Plants can respond to various stresses by either up-regulation or down-regulation of specific genes. Here, we analyzed the effect of various stress treatments on the L-isoaspartyl methyltransferase activity. We found that the activity in corn seeds that have just germinated is up-regulated in response to salt and osmotic stress. It is surprising that no effect was observed when seeds or seedlings were exposed to similar stress conditions. This result is different from that observed in wheat seedlings where the activity was found to be induced by salt and dehydration stresses (Mudgett and Clarke, 1994). Our results in corn suggest that the stage at which the seed is



**Figure 9.** Temperature stability of corn methyltransferase from seeds and leaves. Crude soluble extracts prepared from corn seeds and leaves as described in "Materials and Methods" were pre-incubated at the indicated temperatures (37°C–65°C) for 30 min and then assayed for activity at 45°C as mentioned in Figure 1. All reactions were done in triplicates, and the values represent the mean  $\pm$  SD. When no error bar is shown, the error is smaller than the width of the line.

exposed to stress is important in determining the response of the activity. ABA did not appear to affect the methyltransferase activity in our experiments. In wheat seedlings, both ABA and salt application cause an increase in methyltransferase mRNA as well as activity (Mudgett and Clarke, 1994), whereas in *Arabidopsis*, salt stress does not up-regulate the mRNA but ABA treatment does induce the message (Mudgett and Clarke, 1996). ABA plays a major role in the regulation of embryo maturation as well as responses to osmotic stress, salt, and cold treatments where endogenous ABA levels increase (Henson, 1984; Quatrano, 1986; Mohapatra et al., 1988). Genes that are regulated by natural environmental stresses can be turned on/off by exogenous ABA application (Chandler and Robertson, 1994).

The comparison of the methyltransferase activity from different plants suggests that although the enzyme is developmentally regulated, its patterns of expression as well as its responses to various stress treatments can be quite distinct. Thus, we were interested in determining whether these enzymes differ in their biochemical characteristics. We found, however, that the kinetic properties of the corn, wheat, rice, and carrot enzymes were very similar. These results indicate that the structure of plant L-isoaspartyl methyltransferases may be much more similar than the regulation of their gene expression in each organism. It will be very interesting to analyze the physiological rationale for the differences in the expression seen in this work for each of these species.

## MATERIALS AND METHODS

### Plant Material

Seeds of corn (*Zea mays* hybrids White Knight, Kandy Korn, and Silver Queen) and carrot (*Daucus carota* var. Danvers Half Long) were from Lilly Miller (Portland, OR) and purchased locally. Seeds of rice (*Oryza sativa*, M-101 and M-202) were provided by Dr. Charles West (UCLA, Los Angeles). Yellow cornmeal (enriched and degermed, Albers, Nestle USA, OH) was purchased locally. Winter wheat (*Triticum aestivum* cv Augusta) seeds were provided by Dr. Robert Forsberg (University of Wisconsin, Madison, WI).

### Substrates for L-Isoaspartyl Methyltransferase

The peptides VYP-L-isoAsp-HA and KASA-L-isoAsp-LAKY were synthesized and HPLC-purified by California Peptide Research, Napa, CA. Ovalbumin (chicken egg, Grade VII) was from Sigma (St. Louis).

### Preparation of Seed Extracts

Rice seeds were ground to a fine powder in a household coffee grinder, whereas corn, wheat, and carrot seeds were ground to a fine powder in liquid nitrogen in a mortar with a pestle. For corn, a commercial preparation of cornmeal powder was also used as the source of enzyme for kinetic assays. The powders of the various seed extracts were then suspended in approximately 5 mL of chilled extraction buffer (100 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, pH 7.5, 10 mM  $\beta$ -mercaptoethanol, 1  $\mu$ M leupeptin, 1 mM phenylmethanesulfonyl fluoride, 10 mM sodium hydrosulfite, 10 mM sodium metabisulfite) per gram of seeds and the contents stirred at 4°C for 10 min. The suspensions were then spun at 1,240g for 30 min at 4°C. The supernatants were collected and centrifuged at 186,000g for 1 h at 4°C. The supernatants of this spin were then filtered through one layer of Miracloth (Calbiochem, La Jolla, CA) and stored at  $-20^{\circ}\text{C}$ . These soluble extracts were used as the source of the methyltransferase enzyme.

### Protein Determination

Protein content was determined by precipitating the protein with 1 mL of 10% (w/v) trichloroacetic acid and then using a modification of the Lowry method (Bailey, 1967). Bovine serum albumin (Sigma) was used for creating a standard curve.

### Assay of Methyltransferase Activity

A vapor-diffusion assay (Gilbert et al., 1988) was used to determine the methyltransferase activity. The method involves the transfer of radiolabeled methyl groups by the enzyme from S-adenosyl-L-[methyl- $^{14}\text{C}$ ]Met to a peptide substrate such as VYP-L-isoAsp-HA or KASA-L-isoAsp-LAKY, or to the protein substrate ovalbumin. The methyl esters subsequently are hydrolyzed with a base and the resulting  $^{14}\text{C}$ -methanol is quantified. Unless otherwise de-

scribed, the reaction mixture (total of 40  $\mu\text{L}$ ) consists of 500  $\mu\text{M}$  of VYP-*L-iso-Asp*-HA, 10  $\mu\text{M}$  of [ $^{14}\text{C}$ ]AdoMet (57 mCi  $\text{mmol}^{-1}$ , Amersham Pharmacia Biotech, Piscataway, NJ), 0.2 M sodium citrate buffer (pH 6.0), and 12  $\mu\text{L}$  of the crude enzyme preparation. As a control, the endogenous activity was measured by incubating the enzyme with buffer alone instead of the peptide. The reaction was allowed to proceed at 45°C for 1 h and stopped by quenching with 40  $\mu\text{L}$  of 0.2 N NaOH/1% (w/v) SDS. The contents were vortexed and 60  $\mu\text{L}$  of this mixture was then spotted onto a 1.5  $\times$  8-cm pleated filter paper (no. 1650962, Bio-Rad Laboratories, Hercules, CA), which was placed in the neck of a 20-mL scintillation vial containing 5 mL of counting fluor (Safety Solve High Flashpoint Cocktail, Research Products International, Mt. Prospect, IL). The vials were capped and incubated for 2 h at room temperature. During this period  $^{14}\text{C}$ -methanol diffuses into the fluor, and the un-reacted [ $^{14}\text{C}$ ]AdoMet stays on the filter paper. Quantification was done by removal of the paper and counting the vials in a scintillation counter. Peptide-specific activity was calculated by subtracting the endogenous activity from the activity in the presence of the peptide. We found that this "endogenous" activity was always very low, generally similar to that seen when [ $^{14}\text{C}$ ]AdoMet was incubated with peptide and buffer alone in the absence of extract, and thus did not appear to represent the methylation of endogenous damaged substrates by the L-isoaspartyl methyltransferase.

### Growth of Plants for Developmental Analysis and Preparation of Tissue Extracts

After weighing, seeds of corn (White Knight hybrid) and carrot were soaked in sterile distilled water for 24 h at 25°C under continuous illumination while seeds of rice were soaked in water for 5 d at 15°C in the dark followed by 3 d at 25°C under continuous light. After this imbibition period, seeds were sown directly on germination mix (Gardener's Supply Company, Burlington, VT) in pots. The pots were kept in a greenhouse maintained at 30°C and 70% relative humidity under natural sunlight conditions.

For developmental analysis, tissues of the plant were collected at various time points, starting from 5 d post-germination to 45 d post-germination. The leaves, stems, and roots typically were collected every 5 d after germination and weighed. Tissue samples (0.05–8.00 g) were frozen in liquid nitrogen in a mortar and then ground with a pestle to a fine powder. Immediately after the liquid nitrogen evaporated, chilled extraction buffer (7–10 mL/g tissue) was added and the suspension was ground further. The contents were spun at 16,000g for 15 min at 4°C, and the resulting supernatant was used as the source of the methyltransferase enzyme. These extracts were stored at  $-20^\circ\text{C}$ .

### Assay of Corn Seeds, Germinated Seeds, and Seedlings under Stress Conditions

Seeds of corn (Kandy Korn hybrid) were first surface sterilized by rinsing with 50% (v/v) ethanol for 30 s and were then washed with sterile de-ionized water twice.

They were then soaked in a 2.5% (w/v) sodium hypochlorite solution for 10 min at 25°C on a rotary shaker. The seeds were washed extensively with 4 volumes of sterile de-ionized water. For short-term exposure to stress, washed seeds were placed in 10-mL sterile glass tubes containing 3 mL of either NaCl solution (0.1–1.0 M), sorbitol solution (0.1–1.0 M), or  $\pm$  cis,trans-abscisic acid (Sigma) solution (1–100  $\mu\text{M}$ ) all prepared in water. Control seeds were placed in water alone. Seeds exposed to light stress were placed in water alone and incubated at 25°C in the dark, whereas seeds exposed to temperature stress were placed in water alone and incubated at 4°C and 37°C in the dark. For stress by dehydration, seeds were placed in an empty tube. Otherwise, all seeds were exposed under continuous light conditions at 25°C. After a period of 10 h of exposure, seeds were harvested and soluble extracts prepared for methyltransferase activity as described above. For exposing germinated seeds to similar stresses, seeds were surface sterilized as before and sown onto two sets of 150-mm petriplates containing a Whatman 3MM filter paper soaked in water. The plates were then incubated at 25°C under continuous light until germination occurred. Immediately upon germination, the germinated seeds from the first set were exposed to 10-h stress treatments as described above and their soluble extracts prepared. For exposing seedlings to stress, the seeds, which had germinated on the plate of the second set, were allowed to grow into seedlings that took approximately 4 to 5 d under those conditions. These seedlings were then exposed to 10 h of stress as before. Student's *t*-test was used to calculate the *P* values.

### Temperature Dependence of Methyltransferase Activity

Soluble extracts of *Escherichia coli* (MC1000 strain), *Caenorhabditis elegans* (N2 strain), and plant seeds as well as a partially purified fraction of human recombinant L-isoaspartyl methyltransferase (MacLaren and Clarke, 1995) were assayed for methyltransferase activity at various temperatures ranging from 25°C to 65°C. The methylation reaction for the bacterial and the nematode enzyme consisted of 0.2 M sodium citrate buffer (pH 6.0), 0.2 mM peptide substrate (KASA-*L-isoAsp*-LAKY), 10  $\mu\text{M}$  [ $^{14}\text{C}$ ]AdoMet, and 10  $\mu\text{L}$  of enzyme extract to a final volume of 40  $\mu\text{L}$ . Incubation was performed for 45 min at each temperature. The methylation reaction for the human enzyme consisted of 0.2 M sodium citrate buffer (pH 6.0), 0.2 mM peptide substrate (KASA-*L-isoAsp*-LAKY), 10  $\mu\text{M}$  [ $^{14}\text{C}$ ]AdoMet, and 2  $\mu\text{L}$  of enzyme extract to a final volume of 40  $\mu\text{L}$ . Incubation was performed for 15 min at each temperature. The methylation reactions for the plant extracts were performed as described above with an incubation period of 1 h at each temperature.

### Methyltransferase Kinetics

For kinetic studies, a peptide substrate (VYP-*L-isoAsp*-HA) and a protein substrate (ovalbumin) were tested. The source of methyltransferase enzyme for rice, wheat, and carrot was the soluble extract prepared from seeds as de-

scribed earlier. For the corn enzyme, 20 g of cornmeal was suspended in 75 mL of chilled extraction buffer and stirred at 4°C for 30 min. This suspension was then spun at 1,240g for 30 min at 4°C and the resulting supernatant was further spun at 100,000g for 1 h at 4°C. This supernatant was used for assays. The source of the corn leaf enzyme was the extract prepared from the leaves of the plant arising from the untreated control seeds in the stress experiment described above. This extract was prepared as described for the developmental analysis with the exception that the soluble fraction was centrifuged at 100,000g for 2 h at 4°C to remove any insoluble material. Methylation reactions were performed as described above.

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