Maintaining Methylation Activities during Salt Stress. The Involvement of Adenosine Kinase[1]

Elizabeth A. Weretilnyk[2]*, Kristin J. Alexander, Martina Drebenstedt, Jamie D. Snider, Peter S. Summers, and Barbara A. Moffatt[2]

Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1 (E.A.W., K.J.A., M.D., P.S.S.); and Biology Department, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1 (J.D.S., B.A.M.)

Synthesis of the compatible osmolyte Gly betaine is increased in salt-stressed spinach (*Spinacia oleracea*). Gly betaine arises by oxidation of choline from phosphocholine. Phosphocholine is synthesized in the cytosol by three successive *S*-adenosyl-Met-dependent *N*-methylations of phosphoethanolamine. With each transmethylation, a molecule of *S*-adenosylhomo-Cys (SAH) is produced, a potent inhibitor of *S*-adenosyl-Met-dependent methyltransferases. We examined two enzymes involved in SAH metabolism: SAH hydrolase (SAHH) catabolizes SAH to adenosine plus homo-Cys and adenosine kinase (ADK) converts adenosine to adenosine monophosphate. In vitro SAHH and ADK activities increased incrementally in extracts from leaves of spinach plants subjected to successively higher levels of salt stress and these changes reflected increased levels of SAHH and ADK protein and transcripts. Another Gly betaine accumulator, sugar beet (*Beta vulgaris*), also showed salt-responsive increases in SAHH and ADK activities and protein whereas tobacco (*Nicotiana tabacum*) and canola (*Brassica napus*), which do not accumulate Gly betaine, did not show comparable changes in these enzymes. In spinach, subcellular localization positions SAHH and ADK in the cytosol with the phospho-base *N*-methyltransferase activities. Because SAHH activity is inhibited by its products, we propose that ADK is not a stress-responsive enzyme per se, but plays a pivotal role in sustaining transmethylation reactions in general by serving as a coarse metabolic control to reduce the cellular concentration of free adenosine. In support of this model, we grew Arabidopsis under a short-day photoperiod that promotes secondary cell wall development and found both ADK activity and transcript levels to increase severalfold.

With salt stress, spinach (*Spinacia oleracea*) plants accumulate the compatible osmolyte Gly betaine (Rhodes and Hanson, 1993). This accumulation is the consequence of an increased rate of oxidation of choline to Gly betaine that is accompanied by the increased rate of synthesis of the immediate precursor to choline, phosphocholine (Summers and Weretilnyk, 1993). Phosphocholine is synthesized by three successive *S*-adenosyl-Met (SAM)-dependent *N*-methylations of the phospho-base phosphoethanolamine as shown in Figure 1. The enzyme that catalyzes the first methyl transfer, phosphoethanolamine *N*-methyltransferase (P-EAMeT; EC 2.1.1.103), also catalyzes the remaining methylation reactions (Smith et al., 2000). As is true for plants that accumulate Gly betaine or other methylated compounds produced by methyl transfer from SAM, periods of high methyl demand require that the substrate SAM be furnished in sufficient quantity and that the product of these reactions, *S*-adenosylhomo-Cys (SAH), be removed or catabolized to prevent feedback inhibition of SAM-dependent methyltransferases (Poulton, 1981) including P-EAMeT (Smith et al., 2000).

There is currently no compelling evidence to suggest that SAM levels actually limit transmethylation activity. This is possible presumably due to the very active resynthesis of SAM from ATP and Met made possible by recycling adenosine and homo-Cys derived from SAH (Fig. 1). Thus the catabolism of SAH not only alleviates its inhibition of methyltransferases, SAH should be considered as being an important intermediate in restoring SAM levels. The enzyme SAH hydrolase (SAHH; EC 3.3.1.1) catalyzes the hydrolysis of SAH (Fig. 1). Chiang and Cantoni (1979) identified a key in vivo role for SAHH in regulating transmethylation reactions when they found that the livers of rats injected with an inhibitor of SAHH showed reduced rates of three transmethylation reactions. They hypothesized that the ratio of SAM to SAH played a critical regulatory role over these transmethylation activities. Based upon in vitro enzyme assays, Poulton and Butt (1975) also suggested that the ratio of SAM to SAH could regulate caffeic acid O-methyltransferase activity in leaves of sugar beet (*Beta vulgaris*). Thus it would appear that rates of transmethylation reactions are reported to be sensitive to the ratio of SAM to SAH as opposed to the absolute levels of either. In his review, Poulton (1981) proposed that SAHH would perform a key function in reducing levels of SAH relative to those

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[2] These authors contributed equally to this work.

* Corresponding author; e-mail weretil@mcmaster.ca; fax 905–522–6066.

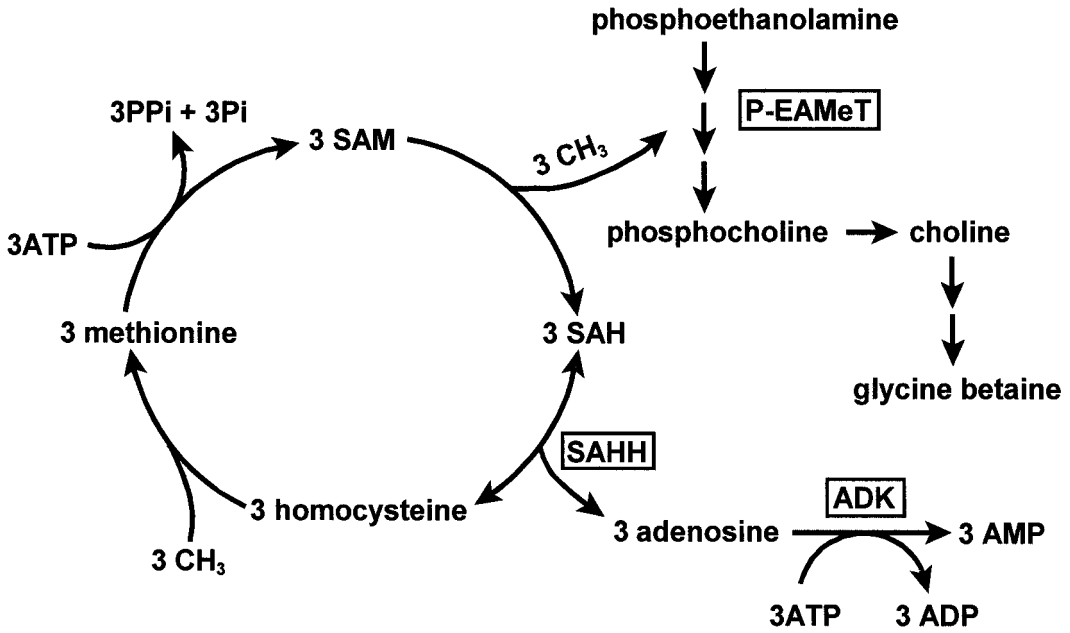

Figure 1. Schematic representation of Met methyl group metabolism in relation to phosphocholine and Gly betaine synthesis.

for SAM that, in turn, would help maintain a ratio of SAM to SAH favoring the continued activity of SAM-dependent methyltransferases. With respect to regulation of SAHH activity, it has been reported that SAHH activity is significantly inhibited by its products (de la Haba and Cantoni, 1959; Poulton and Butt, 1976b; Guranowski and Pawelkiewicz, 1977). The logical extension of this observation is that either adenosine or homo-Cys must be removed to relieve inhibition of SAHH. Poulton and Butt (1976b) showed that relative to adenosine, the removal of homo-Cys is of lesser importance to in vitro SAHH activity and that the removal of adenosine during the SAHH assay by the addition of adenosine deaminase leads to linear enzyme activity rates over time.

There are three possible enzymatic routes for adenosine removal: adenosine nucleosidase, adenosine deaminase, and adenosine kinase (ADK; EC 2.7.1.20). In humans the predominant route for adenosine catabolism is by deamination (Kredich and Martin, 1977) but there are comparatively low levels of this enzyme in plants (Dancer et al., 1997), raising the suggestion that this enzyme is unlikely to play a significant role in SAH metabolism (Edwards, 1996). Adenosine nucleosidase activity in plants is described as being present or absent depending on the plant (Poulton and Butt, 1976a; Leszczynska et al., 1984; Edwards, 1996), whereas ADK activity has been documented for several plants (Schomberg and Stephan, 1997).

In this study, we have subjected two Gly betaine-accumulating plants to a salt-stress treatment, a condition under which phosphocholine levels increase due to higher P-EAMeT activity (Summers and Weretilnyk, 1993). Extracts of these plants were then assayed for P-EAMeT, SAHH, and ADK activities. Here we report that all three enzymes increase in activity with salinity, whereas these activities did not change comparably for two species known to be non-accumulators of Gly betaine (Weretilnyk et al., 1989; Selvaraj et al., 1995). The increased levels of enzyme activity and protein for SAHH and ADK suggest that these enzymes are limiting when plants require higher levels of methylated products. Moreover, the increased SAHH and ADK activities associated with salt stress are accompanied by elevated levels of transcripts for each enzyme, raising the intriguing possibility that transcription of the genes encoding these enzymes is coordinately regulated to respond to methylation requirements.

RESULTS AND DISCUSSION

Increases in Methyl Demand and SAH Catabolism with Salt Stress

The enzyme that likely catalyzes the committing step for the synthesis of phosphocholine is P-EAMeT (Datko and Mudd, 1988; Fig. 1) and its activity is salt-responsive and up-regulated in salinized spinach plants (Weretilnyk et al., 1995). Operation of this methylation sequence places a high demand for methyl groups derived from Met, but the outcome with possibly more deleterious metabolic effects is the concomitant production of SAH. For each mole of phosphocholine synthesized, 3 mol of SAM are required to supply the necessary methyl groups transferred resulting in the production of 3 mol of SAH, a known inhibitor

of SAM-dependent methyltransferases (Poulton, 1981). Thus a Gly betaine-accumulating plant such as spinach provides a suitable model system to examine the response of downstream enzymes to an increased production of SAH provoked as the plant undergoes osmotic adjustment in response to saline conditions. It should be noted, however, that the importance of phosphocholine synthesis in utilizing high amounts of methyl groups from Met is not unique to Gly betaine-accumulating plants like spinach. This series of transmethylations was described by Mudd and Datko (1986) as quantitatively the major route by which methyl groups derived from Met are used in *Lemna paucicostata*, a plant that is not a Gly betaine accumulator, but nonetheless uses this route for phosphatidylcholine synthesis.

Exposing spinach plants to an increasing level of salt results in an increase of P-EAMeT activity in extracts of leaves (Fig. 2A). This determination ensured that the plants were undergoing the same salt-responsive changes in choline metabolism as previously reported (Weretilnyk et al., 1995). We then directed our attention to the activities of enzymes involved in SAH catabolism. The enzyme responsible for catabolism of SAH, SAHH, catalyzes a reversible reaction that is favored to proceed in the direction of SAH synthesis (Fig. 1). Only the continuous removal of both reaction products, adenosine and homo-Cys, draws the reaction in the direction of SAH hydrolysis (de la Haba and Cantoni, 1959; Poulton, 1981). Given that the synthesis of Gly betaine in salinized spinach would provide an addi-

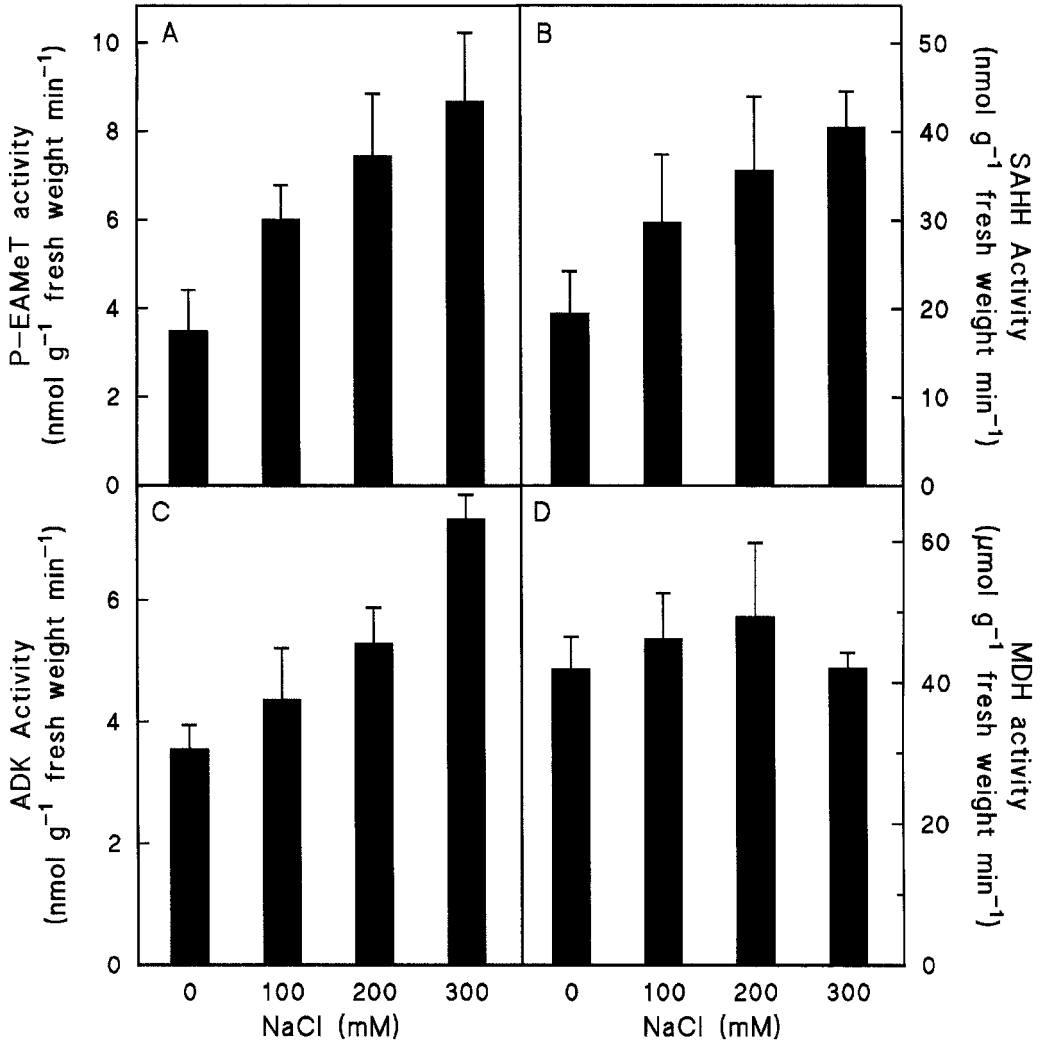

Figure 2. In vitro enzyme activity measurements for P-EAMeT (A), SAHH (B), ADK (C), and malate dehydrogenase (MDH; D) in extracts prepared from leaves of control (0 mM NaCl) spinach plants and spinach plants irrigated with a nutrient solution containing 100, 200, or 300 mM NaCl. SAHH activity was determined in the direction of SAH hydrolysis. Crude extracts were desalted and suitably diluted for enzyme activity measurements to ensure linearity of the assay. The entire experiment was repeated three times (data are given as means ± SE, $n = 3$). Details regarding desalting of extracts and assay conditions are given in "Materials and Methods."

tional requirement for SAH hydrolysis to prevent feedback inhibition of P-EAMeT activity, we determined SAHH activity in extracts from our salinized plants. Figure 2B shows that SAHH activity increased with salt stress and that the fold increases for SAHH at the salinity levels used were comparable to those of P-EAMeT (Fig. 2A).

ADK Activities in Salinized Spinach

Figure 1 shows that one product of SAH hydrolysis, homo-Cys, can be recycled to Met by SAM-independent transfer of methyl groups to homo-Cys (Giovanelli et al., 1980). In contrast, the enzymes contributing to the removal of adenosine are less well-described and so we investigated the activity of ADK in the salinized spinach plants. As shown in Figure 2C, ADK activity in salinized spinach plants increased in a stepwise manner with increasing salt stress, closely mirroring the changes in P-EAMeT and SAHH activities in the same plants. These salt-responsive increases are not found for all enzymes as exemplified by MDH activities measured in the same extracts (Fig. 2D).

SAHH and ADK Activities in Different Plants

We sought to determine whether P-EAMeT, SAHH, and ADK activities would also change in other plants, particularly in response to salt stress. The plants selected for salinization included a second species that accumulates Gly betaine, sugar beet, and two species that are reported to be nonaccumulators of Gly betaine, tobacco (*Nicotiana tabacum*) and canola (*Brassica napus*; Weretilnyk et al., 1989; Selvaraj et al., 1995). The level of salt used for salinization treatments varied between the species to minimize any salt-related injury to the more salt-sensitive tobacco and canola. None of the plants used in this study showed visible signs of wilting as a result of salinization and chlorophyll levels of salinized plants never decreased by more than 20% relative to chlorophyll levels of their respective unsalinized controls (data not shown).

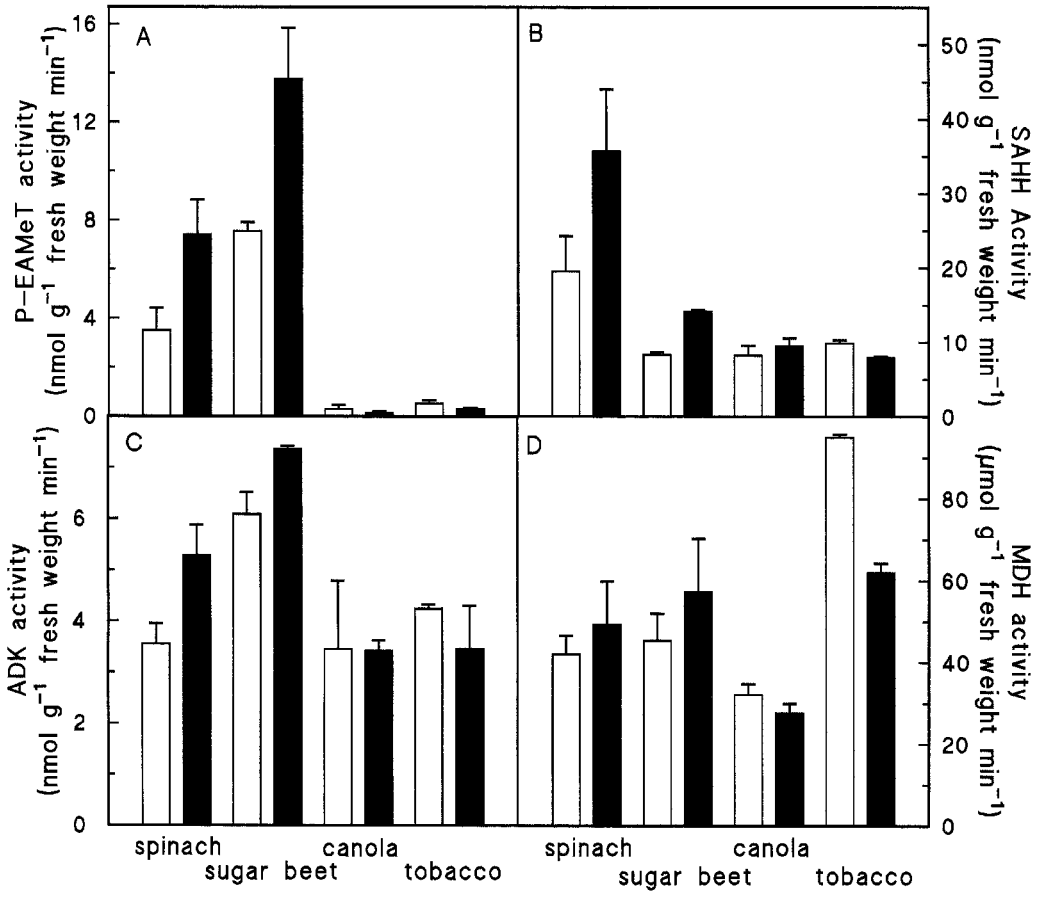

Figure 3. The effects of salt stress on in vitro activity of P-EAMeT (A), SAHH (B), ADK (C), and MDH (D) in crude, desalted leaf extracts prepared from various plants. Where necessary, extracts were diluted to ensure linearity of the assay. Plants were grown in the absence (white bars) or presence (black bars) of salt. The final salinity levels reached during treatment of the plants were as follows: 200 mM NaCl for spinach, 350 mM NaCl for sugar beet, and 150 mM for tobacco and canola. The data are given as means ± SE (n = minimum of 2).

The absolute levels of activity for the various enzymes assayed varied among the four species examined (Fig. 3). Data for spinach (unsalinized and salinized to 200 mM with NaCl) is reported in this figure for ease of comparison. Sugar beet, as shown for spinach, showed increases in P-EAMeT, SAHH, and ADK activities relative to the unsalinized control plants whereas neither canola nor tobacco showed increases for any of these activities with exposure to salt stress. We also assayed SAHH activity in the direction of SAH synthesis (Fig. 1) and, although absolute enzyme activity rates are approximately 3-fold higher (data not shown), the results did not differ qualitatively from those depicted in Figure 3B. A comparison between the plants tested shows that spinach and sugar beet had significantly higher rates of in vitro P-EAMeT activity than tobacco or canola even in the absence of salt stress. Furthermore, sugar beet had the highest level of ADK activity and spinach SAHH activity rates were greater than those estimated for the other plants. The only significant change in MDH activity was in tobacco where activity decreased with salinity. It is worth noting that expressing enzyme activity rates on a per milligram protein or per milligram chlorophyll basis did not change the outcome in the comparison of these activities, particularly those comparisons between the salinized plants and their respective unsalinized controls (data not shown).

Photoaffinity cross-linking studies have been used to identify a salt-responsive polypeptide that binds to SAM and copurifies with P-EAMeT activity in extracts of spinach leaves. This experimental approach has provided evidence that P-EAMeT protein levels likely increase in plants salinized to the same extent as those described here (Smith et al., 2000). To determine whether increases in SAHH and ADK activities could also be ascribed to increased protein levels, the same extracts used for enzyme assays were used to quantify ADK and SAHH by immunodetection. As shown in Figure 4, using an antibody raised against tobacco SAHH, the protein level associated with SAHH increased in extracts prepared from salinized spinach plants relative to extracts from unsalinized plants. The increases in SAHH protein are incremental with the increased salt concentrations used to irrigate the plants and the changes in intensity seen on the immunoblot are comparable to the fold increases in SAHH activity. The same pattern of stepwise changes is also shown for ADK activity and protein (Fig. 4) using an antibody prepared against Arabidopsis ADK. Comparable increases for SAHH and ADK activity and protein are also found for sugar beet salinized to 350 mM NaCl (Fig. 5). The changes for SAHH and ADK protein levels in spinach and sugar beet quantified from the immunoblots by phosphorimager analysis were 1.5-fold for spinach SAHH or ADK and 2- and 2.8-fold for sugar beet SAHH and ADK, respectively. These fold changes

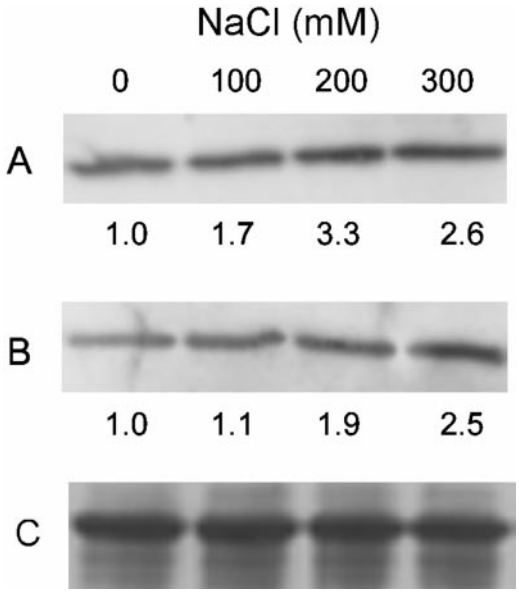

Figure 4. Immunoblotting of crude leaf extracts prepared from control (0 mM NaCl) spinach and spinach salinized stepwise to final concentrations of 100, 200, or 300 mM NaCl using anti-SAHH serum (A) and anti-ADK serum (B). C, A polypeptide corresponding to the position of Rubisco large subunit in a Coomassie Brilliant Blue-stained gel run in parallel to demonstrate that equal loading (10 μg of crude, desalted protein) was achieved for all samples. The numbers below A and B denote the fold increases in SAHH (A) or ADK (B) enzyme activities associated with the sample used for immunodetection analysis relative to the activity level detected in the 0 mM NaCl control sample. The experiment was carried out twice with different samples and the immunoblots showed the same results.

are similar to the increases in enzyme activity in the extracts used for immunoblot analysis (Fig. 5). In contrast, no increases were observed for protein levels of either SAHH or ADK in leaf extracts prepared from salt-stressed canola or tobacco relative to their respective unsalinized controls (Fig. 5).

To determine whether the changes in SAHH and ADK activities and protein levels were associated with increased transcript levels we performed RNA blot hybridizations using poly(A^+) RNA isolated from spinach plants grown in the absence of added NaCl (control) or plants growing under the lowest (100 mM) and highest (300 mM) levels of NaCl used. Transcripts corresponding to ADK and SAHH genes were detected with cDNA probes that hybridized to yield bands at positions consistent with the anticipated size for their respective mRNA. Figure 6 shows that transcripts hybridizing to either the SAHH or ADK cDNA probes are faint but present in the lane containing poly(A^+) RNA from control plants and both show increased intensity in response to salinization. It is notable that the fold changes in transcript abundance as estimated by phosphorimager analysis are comparable in magnitude to those changes found for enzyme activity (Fig. 2, B and C) and protein levels (Fig. 4) with increasing salt stress.

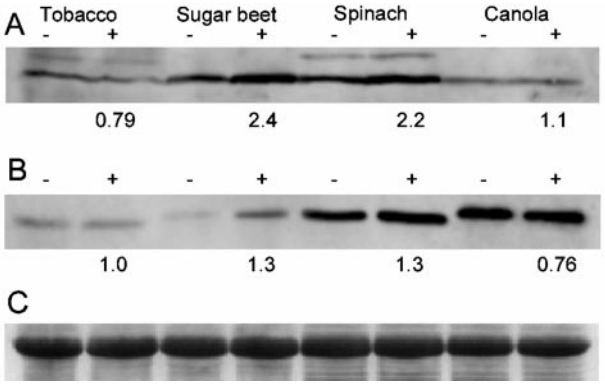

Figure 5. Immunoblotting of crude leaf extracts prepared from control and salinized plants using anti-SAHH (A) and anti-ADK (B). The numbers below A and B denote the fold increases in SAHH (A) or ADK (B) enzyme activities detected in the sample from the salinized plants (+) relative to the activity level measured in their respective 0 mM NaCl control (-) sample. The final salinity levels reached during treatment of the plants were as follows: 200 mM NaCl for spinach, 350 mM NaCl for sugar beet, and 150 mM for tobacco and canola. C, A polypeptide corresponding to the position of Rubisco large subunit in a Coomassie Brilliant Blue-stained gel run in parallel to demonstrate that equal loading (10 μg of crude, desalted protein) was achieved for all samples. The experiment was carried out at least twice with samples from different salinization experiments and the immunoblots gave similar results.

Subcellular Localization for SAHH and ADK

P-EAMeT activity has been localized to the cytosol of spinach plants (Weretilnyk et al., 1995) and it has been proposed that SAHH and SAM synthetase (SAMS) are similarly positioned in the cytosol (Wasternack et al., 1985; Ravanel et al., 1998). Figure 7 shows that SAHH, ADK, and SAMS proteins are detected in total leaf extracts of spinach by immunoblot analysis and that the signals associated with these enzymes are only barely visible when the source of extract is a "1,000*g* pellet" fraction prepared from spinach leaves. Using marker enzyme assays, the "1,000*g* pellet" fraction was found to contain 14% of the total chloroplastic NADP-dependent glyceraldehyde-3-phosphate dehydrogenase activity and less than 1% contamination of the cytosolic enzyme phosphoenolpyruvate carboxylase. SAHH and SAMS protein as well as ADK protein and activity are absent when stromal extracts from Percoll-purified chloroplasts of spinach are used. We also obtained a sample of soluble proteins prepared from purified mitochondria of Arabidopsis and were unable to detect SAHH or ADK protein in this subcellular fraction (data not shown). These observations are consistent with a shared, cytosolic location for SAMS, SAHH, and ADK and pertinent to this study, both SAHH and ADK reside in the same compartment as the source of SAH generated by transmethylation of phosphoethanolamine to phosphocholine (Fig. 1).

Regulation of SAHH and ADK Activities

A decreased capacity to synthesize SAM by SAMS and an increased capacity to catabolize SAH by SAHH has been reported in conjunction with D-ononitol accumulation in water-stressed *Vigna umbellata* (Wanek and Richter, 1997). The SAH is generated by the transmethylation of *myo*-inositol to form D-ononitol and this synthesis occurs primarily in stems and not leaves. The authors showed that the activity of the enzyme catalyzing this reaction is sensitive to the ratio of SAM to SAH and they described three means by which D-ononitol levels increase: a reduced use of SAM by other transmethylases that ultimately reduces SAH production, higher *myo*-inositol levels, and an increased SAHH activity in stems. We now report that SAHH activity in extracts of leaves from salinized spinach and sugar beet increases, and that the changes in the SAHH activity resemble the observed increased rate of P-EAMeT activity. However, an in vivo capacity for increased SAH hydrolysis can only be maintained if there is

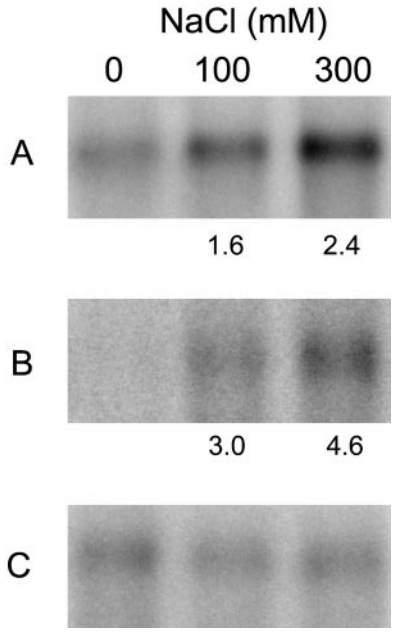

Figure 6. Northern-blot hybridization for transcripts encoding SAHH (A) or ADK (B) using poly(A^+) RNA isolated from leaves of unsalinized (0 mM NaCl) and salinized (100 or 300 mM NaCl) spinach plants. Each lane contained 2 μg poly(A^+) RNA. The numbers below each blot indicate the intensity of the hybridization signal as quantified by phosphorimager analysis and reported relative to the signal obtained for the corresponding band in the 0 mM NaCl sample corrected for background. The blot shown is a representative outcome of three separate experiments where poly(A^+) RNA was isolated from non-salinized and salt-stressed plants then subjected to northern-blot hybridization. Plasmids bearing cDNA inserts encoding SAHH and ADK were random primed to generate ^{32}P-labeled probes for hybridization and blots shown in A and B were washed as described in "Materials and Methods." C, A constitutively expressed transcript from the same blot to illustrate the equal loading of poly(A^+) RNA in each lane.

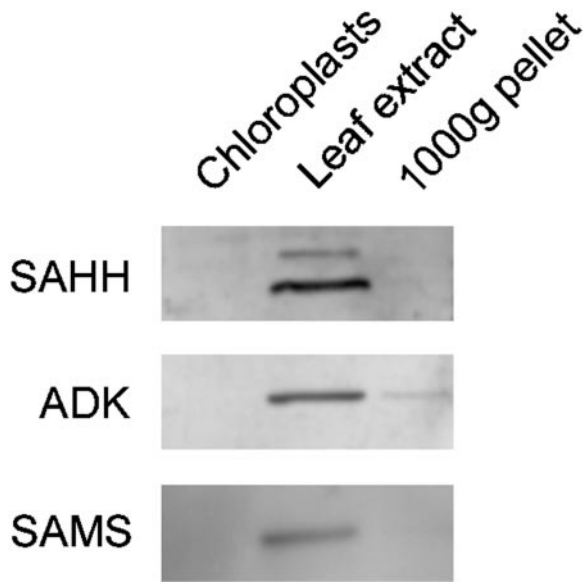

Figure 7. Localization of SAMS, ADK, and SAHH in fractions prepared from spinach leaves. Fractions tested by immunoblotting with anti-SAMS, anti-SAHH, and anti-ADK include stromal extracts of purified chloroplasts (Chloroplasts), crude homogenate of leaves (Leaf Extract), and a plastid-enriched fraction of the homogenate (1,000*g* Pellet). Each lane was loaded with equivalent levels (10 μg) of crude, desalted protein. The immunoblot analysis was repeated and showed similar results.

continued removal of its reaction products, particularly adenosine. Thus an increased capacity to catabolize SAH in response to salinization, thereby allowing an increased rate of phosphocholine synthesis, is not unexpected. However, the involvement of ADK has not been experimentally shown to be important in maintaining rates of transmethylation under periods of methyl demand although it was proposed to offer an energetically conservative route for adenosine removal (Poulton, 1981). The incremental increase in ADK activity and protein, which mimicked the increases for both P-EAMeT and SAHH activities (Figs. 2 and 3), strongly suggests that ADK activity would otherwise be limiting under conditions that lead to Gly betaine accumulation. In keeping with this hypothesis, tobacco and canola, plants for which an osmotically generated increase in the methyl demand due to Gly betaine accumulation would not accompany salt stress (Weretilnyk et al., 1989; Selvaraj et al., 1995), showed no change in either SAHH or ADK activity or protein levels (Figs. 3 and 5). Thus, the role of ADK should not be viewed as a generalized stress response, but rather as a coarse control for transmethylation activity and the subsequent regeneration of SAM. In this regard, it is interesting to note that the coarse control mechanism in operation for salt-stressed spinach involves increased transcript levels for SAHH and ADK and that the change in transcript abundance appears to be salt-responsive (Fig. 6).

To explore whether other conditions known to require increased methylation are associated with changes in SAHH and ADK, we investigated their expression and activities in Arabidopsis undergoing increased lignification of secondary cell walls. Arabidopsis plants grown under a short day photoperiod (8 h light/16 h dark) for extended periods develop lignified secondary cell walls in the hypocotyl (S. Regan, personal communication). We analyzed leaf tissue from Arabidopsis plants grown for 5 months under short days and found ADK activity to increase approximately 3-fold ($n = 3$) relative to plants of a comparable developmental age but grown under long days. Steady-state transcript levels of ADK increased (Fig. 8) in leaves exposed to short days as did those encoding SAHH. This is the first report of transcriptional induction of ADK in response to methyl demand, although both ADK and SAHH transcripts are found to be enriched in cambial tissue of poplar (Sterky et al., 1998). Exactly how this induction is mediated remains to be elucidated. It may be that the metabolic adjustments associated with methyl demand, whether invoked

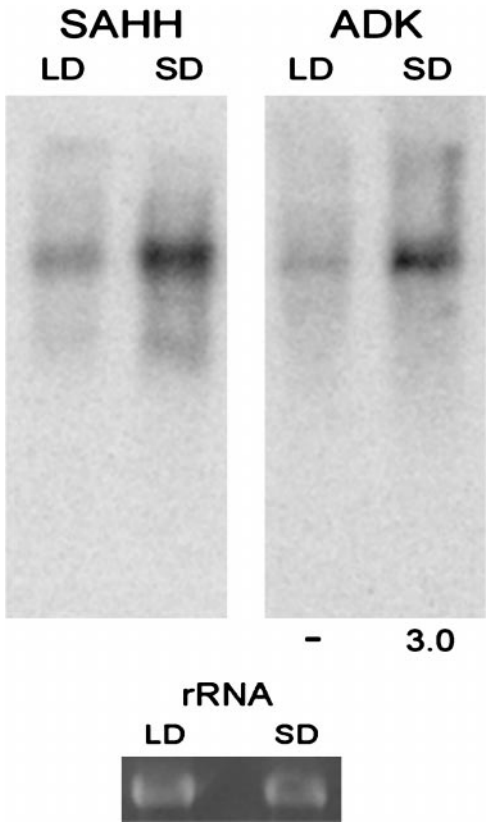

Figure 8. Northern-blot hybridization with total RNA isolated from leaves of Arabidopsis grown under a long-day (LD) or short-day (SD) photoperiod. Each lane contained 10 μg RNA and equal loading is confirmed by the comparable intensity of the 18S rRNA bands when stained with ethidium bromide. Plasmids bearing cDNA inserts encoding SAHH and ADK were random primed to generate ^{32}P-labeled probes for hybridization and blots were washed as described in "Materials and Methods."

by Gly betaine accumulation or by development, respond to a common inducer that acts as a global governor of methylation activities. If this type of regulation exists, metabolic engineering of traits that require elevated transmethylation activities should be accommodated regardless of the plant species, providing a sufficient supply of substrate(s) to be methylated is available and SAM regeneration is unimpeded.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All plants were grown in controlled environment chambers. Spinach (*Spinacia oleracea* cv Savoy hybrid 612) plants were grown at 22°C under an 8-h-light (300 μmol m^{-2} s^{-1} photosynthetic photon flux density)/16-h-dark photoperiod. Sugar beet (*Beta vulgaris* cv HM 8282), tobacco (*Nicotiana tabacum* cv Wisconsin 38), and canola (*Brassica napus* cv OAC Summit) plants were grown at 22°C with a 16-h-light (300 μmol m^{-2} s^{-1} photosynthetic photon flux density)/8-h-dark photoperiod. Plants were grown individually in pots containing coarse-grade vermiculite (spinach and sugar beet) or a potting mixture of peat:perilite:vermiculite, 1:1:1 (tobacco and canola) and all plants were watered daily with half-strength Hoagland solution (Hoagland and Arnon, 1950). Plants were routinely grown until the third or fourth true leaf was beginning to emerge (generally 4 to 8 weeks post-germination depending on the species) and then watering was either continued with half-strength Hoagland solution alone (control plants) or supplemented with NaCl (salinized plants). Salinization was started with 50 mM NaCl and the salinity level in the irrigating medium was increased in increments of 50 mM NaCl every 3 d to variable final concentrations for spinach (100, 200, or 300 mM NaCl) or final concentrations of 350 mM for sugar beet or 150 mM for tobacco and canola plants.

Preparation of Leaf Extracts

Following harvest of leaf tissue, all subsequent operations were completed on ice or at 4°C. To prepare extracts for analysis, fully expanded and expanding leaves from at least two individual plants were harvested, coarsely chopped, and then mixed before a sample was removed for grinding. The chopped leaf tissue was ground in a chilled mortar with a pestle, a small quantity of sea sand, and grinding buffer (2 mL g^{-1} fresh weight). For spinach leaves, a grinding buffer containing 50 mM HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid])-KOH (pH 7.8), 5 mM dithiothreitol (DTT), and 1 mM Na_2EDTA was used but this buffer was modified to include 5 mM ascorbic acid, 10 mM boric acid, and 20 mM Na-metabisulfite for sugar beet and canola with a further addition of polyvinylpyrrolidone at 4% (w/v) to the grinding buffer used for tobacco. In each case, the brei was passed through four layers of cheesecloth and one layer of Miracloth (Calbiochem, La Jolla, CA) and the filtrate centrifuged at 10,000*g* for 10 min. Aliquots of the supernatant were then desalted by centrifugation through Sephadex G-25 Medium (Amersham-Pharmacia, Baie d' Urfé, QC) as described by Summers and Weretilnyk (1993) using beads equilibrated in a buffer of appropriate pH and composition for the different enzymes assayed. The equilibration buffer used to desalt extracts for P-EAMeT and MDH assays was composed of 50 mM HEPES-KOH (pH 7.8), 5 mM DTT, and 1 mM Na_2EDTA; this buffer was adjusted to pH 7.5 for the SAHH activity determinations although DTT was omitted for the SAHH spectrophotometric assay. Extracts used for ADK determinations were desalted with beads equilibrated with 50 mM HEPES-KOH (pH 7.2) and 2 mM DTT. For the enzymes assayed as part of this study, we determined that crude, undesalted extracts could be flash-frozen with liquid nitrogen and then stored at −80°C for at least several weeks prior to assay without apparent loss of activity.

Subcellular Fractionation

Spinach leaves (25 g) were homogenized and fractionated by centrifugation to yield three subcellular fractions designated "1,000*g* pellet," "20,000*g* pellet," and "20,000*g* supernatant" as reported previously (Weretilnyk et al., 1995). Purified chloroplasts were prepared from a homogenate of spinach leaves following the centrifugation of isolated plastids through a 40% (v/v) Percoll cushion (Amersham-Pharmacia; Mills and Joy, 1980). The Percoll-purified plastids were resuspended with a buffer composed of 50 mM HEPES-KOH (pH 7.2), 5 mM DTT, and 1 mM Na_2EDTA and the resuspended organelles were ground with a small quantity of sea sand to ensure complete release of stromal enzymes. The chloroplast suspension was then centrifuged at 16,000*g* for 5 min, the supernatant desalted as described above, and the desalted extract used for ADK activity measurements or immunoblot analysis.

Enzyme Assays

P-EAMeT activity was assayed using P-EA as the phospho-base substrate following the radiometric assay described previously (Summers and Weretilnyk, 1993). SAHH activity was measured spectrophotometrically in the direction of SAH hydrolysis by monitoring the change in A_{412}. The 1.0-mL assay included 50 mM HEPES-KOH (pH 7.5), 1 mM Na_2EDTA, 2 μL mL^{-1} adenosine deaminase (Roche Diagnostics, Laval, QC; 400 units mL^{-1}), and 0.2 mM 5,5′-dithio-bis(-2-nitrobenzoic acid) (Roche Diagnostics). SAHH activity was also measured in the direction of SAH synthesis using a radiometric assay carried out at 30°C with a buffer containing 50 mM HEPES-KOH (pH 7.5), 2 mM DTT followed by thin-layer chromatography separation on silica Gel-G 60 (Macherey-Nagel, Düren, Germany) to identify and quantify the reaction products as outlined by Guranowski and Jakubowski (1987). ADK activity was measured by a radioisotope assay as the synthesis of [^{3}H]AMP from [2,8 ^{3}H]adenosine (ICN, Costa Mesa, CA; 2.7 μM, 3 nCi $pmol^{-1}$) and ATP (8 mM) in 50 mM HEPES-

KOH (pH 7.2), 30 mM NaF, 2.5 mM deoxycoformycin (Pentostatin, Warner-Lambert, Morris Plains, NJ), 2 mM $MgCl_2$, and 1 mg mL^{-1} bovine serum albumin in a 50-μL assay volume. ADK assays were incubated at 30°C for 5 min and then reactions were stopped by placing the tubes in boiling water for 1 min. The stopped reactions were then spotted onto DE-81 filter discs (Whatman, Clifton, NJ) from which any remaining adenosine was removed by washing with 1 mM ammonium formate (Lukey and Snyder, 1980). Discs were air dried, transferred to vials containing Ready Safe fluor (Beckman-Coulter, Mississauga, ON), and [^{3}H]AMP bound to discs was quantified by liquid scintillation counting. MDH activity was assayed in 0.1 M HEPES-KOH (pH 7.8) with 2.5 mM oxaloacetate, 1 mM Na_2EDTA, and 0.16 mM NADH by monitoring the change in A_{340} associated with the oxaloacetate-dependent oxidation of NADH. Enzyme activity measurements of marker enzymes used in the subcellular fractionation study as well as determinations completed for protein and chlorophyll concentrations were all performed as described previously (Weretilnyk et al., 1995).

PAGE and Immunodetection

SDS-PAGE separation of polypeptides (10 μg protein/lane) was performed as described by Laemmli (1970) with a 12.5% (w/v) acrylamide gel. The gels were either stained with Coomassie Brilliant Blue or they were electophoretically transferred to a polyvinylidene difluoride membrane using a semidry transfer apparatus (Bio-Rad, Mississauga, ON) for 30 min at 20 V. The membranes were probed with affinity-purified polyclonal anti-ADK antibodies diluted 1:5000, polyclonal anti-SAHH antibodies diluted 1:2000 (Mitsui et al., 1993), or polyclonal anti-SAMS antibodies diluted 1:1500 (Schröder et al., 1997). The anti-ADK was prepared against Arabidopsis ADK (Moffatt et al., 2000), anti-SAHH against tobacco SAHH (a kind gift from Dr. M. Sugiura, Center for Gene Research, Nagoya University, Japan), and anti-SAMS against *Catharanthus roseus* (a kind gift from Dr. J. Schröder, Institut für Biologie II, University of Freiburg, Germany). Immunocomplexes were detected by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, St. Louis) for ADK and SAHH detection or anti-chicken IgG (Sigma) for SAMS detection and bound antibody revealed by reaction with enhanced chemifluorescence (Amersham-Pharmacia) and quantified using a Storm 860 Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RNA Isolation and Electrophoresis

Total RNA and poly(A^+) RNA were isolated from leaves of control and salinized spinach plants as described previously (Weretilnyk and Hanson, 1989). Total RNA was prepared from Arabidopsis leaves of plants grown for either short days for 5 months (8-h light) or long days for 5 weeks (16-h day) following the procedure described by Logemann et al. (1987). Poly(A^+) RNA (2 μg) or total RNA (10 μg) were denatured and subjected to electrophoresis on a 1.5% (w/v) agarose-formaldehyde gel and then transferred to a nylon membrane. Hybridization was carried out overnight at 55°C in 5× SSC, 10× Denhardt's solution, 0.1% (w/v) SDS, 50 mM sodium phosphate (pH 7.0), and 100 μg mL^{-1} tRNA with probes ^{32}P-labeled by random priming SAHH cDNA (GenBank accession no. T45811) or ADK1 cDNA (GenBank accession no. R30128). The blots with spinach poly(A^+) RNA were washed at 55°C to a final stringency of 5× SSC, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate for 30 min for the SAHH blot or 3× SSC, 0.1% (w/v) SDS, and 0.1% (w/v) sodium pyrophosphate for 15 min for the ADK blot. These blots also included total RNA (10 μg) isolated from Arabidopsis leaves as a control lane to ensure that conditions of hybridization and washes were adequate and sufficiently specific for ADK or SAHH transcript detection (data not shown). For the analysis of total RNA from Arabidopsis, the blots were incubated in 50% (v/v) formamide hybridization buffer at 65°C with either ^{32}P-labeled ADK1 cDNA or SAHH cDNA and then washed to a final stringency of 0.1× SSC, 0.1% (w/v) SDS at 65°C for 30 min. Bound probe was detected using a Storm 860 Phosphorimager following overnight exposure to the storage phosphor screen.

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