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Enzymes of Choline Synthesis in Spinach[1]

Response of Phospho-Base N-Methyltransferase Activities to Light and Salinity

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In spinach (*Spinacia oleracea* L.), choline is synthesized by the sequential *N*-methylation of phosphoethanolamine → phosphomono- → phosphodi- → phosphotrimethylethanolamine (i.e. phosphocholine) followed by hydrolysis to release choline. Differential centrifugation of spinach leaf extracts shows that enzymes catalyzing the three *N*-methylations are cytosolic. These enzymes were assayed in leaf extracts prepared from plants growing under various light/dark periods. Under a diurnal, 8-h light/16-h dark photoperiod, the activity of the enzyme catalyzing the *N*-methylation of phosphoethanolamine is highest at the end of the light period and lowest following the dark period. Prolonged dark periods (exceeding 16 h) lead to a further reduction in the activity of this enzyme, although activity is restored when plants are reexposed to light. In contrast, the activity of the enzyme(s) catalyzing the *N*-methylations of phosphomono- and phosphodimethylethanolamine does not undergo comparable changes in response to light/dark treatments. Salt shock of plants with 200 mM NaCl results in a 2-fold increase in all three *N*-methylation activities relative to nonsalinized controls but only in plants exposed to light. Thus, light is required for the salt-responsive up-regulation of choline synthesis in spinach.

In spinach (*Spinacia oleracea* L.), choline is found in the form of phosphatidylcholine and it also serves as a precursor for the osmolyte Gly betaine (betaine) (Coughlan and Wyn Jones, 1982). For spinach and sugar beet, both members of Chenopodiaceae, radiotracer studies have shown that choline is synthesized by a pathway involving water-soluble, phospho-base intermediates (Hanson and Rhodes, 1983; Summers and Weretilnyk, 1993). In this pathway, ethanolamine is phosphorylated by the enzyme ethanolamine kinase to form PEA, and then PEA is sequentially *N*-methylated three times to form PCho. The conversion of PEA to PCho is catalyzed by SAM:phospho-base *N*-methyltransferase enzymes, although the exact number of enzymes catalyzing the *N*-methylation reactions is uncertain. The product PCho can serve in the synthesis of phospholipids, or alternatively, it can be hydrolyzed, presumably by PCho phosphatase, to release choline, which can be oxidized to betaine. Whereas the two enzymes responsible for choline oxidation to betaine are found in chloroplasts (Hanson et al., 1985), the subcellular distribution of the other choline biosynthetic enzymes is unknown.

Several studies of choline metabolism in higher plants suggest that the rate of choline synthesis is tightly regulated. For example, Hanson and Rhodes (1983) proposed that the *N*-methylation of PEA to PCho is likely the site regulating choline synthesis in salinized sugar beet. Thus, any or indeed all of the enzymes responsible for the conversion of PEA to PCho, the SAM:phospho-base *N*-methyltransferases, may play a potentially critical regulatory role in furnishing choline for betaine synthesis. More recently, Mudd and Datko (1989a, 1989b) provided evidence that the *N*-methylation of PEA may also regulate choline metabolism with respect to phosphatidylcholine synthesis in plants that do not accumulate betaine. In these studies, Mudd and Datko (1989a, 1989b) showed that growth of *Lemna paucicostata* or soybean and carrot cultures in the presence of choline leads to a down-regulation in the rate of choline synthesis. In examining the activity of the SAM: phospho-base *N*-methyltransferases, they provided evidence that the enzyme *N*-methylating PEA (PEA → PMEA), PEAMeT, contributes to this down-regulation and that the regulatory step for choline synthesis resides at the site catalyzed by this enzyme. Although this enzyme may play a regulatory role for choline synthesis in these plants, its distribution does not appear to be universal. For example, Prud'homme and Moore (1992) found no evidence for an enzyme capable of *N*-methylating PEA in castor bean endosperm. The presence or absence of an enzyme catalyzing a putative regulatory step suggests that divergence exists with respect to the mechanism(s) by which choline synthesis is regulated among different plants or plant tissues. Given this apparent diversity, more information and, in particular, more detailed biochemical information about the pathway(s) and enzymes responsible for choline synthesis is required. This information would help identify key regulatory steps in choline synthesis and determine whether the enzymes involved in the synthesis of choline for phospholipids and osmotic stress tolerance exhibit comparable, possibly identical, physical and regulatory properties.

We have been studying the enzymes responsible for choline synthesis in spinach plants. Earlier, we reported

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Abbreviations: PEA, PMEA, PDEA, and PCho, phosphate esters of ethanolamine, *N*-methylethanolamine, *N,N*-dimethylethanolamine, and choline, respectively; PEAMeT, PMEAMeT, and PDEAMeT, the *N*-methyltransferases using PEA, PMEA, and PDEA as substrates; PEPC, PEP carboxylase; SAM, *S*-adenosyl-L-methionine.

that enzyme activities associated with all three *N*-methylations (PEA → PMEA → PDEA → PCho) increase 2- to 3-fold in leaves of salinized spinach plants (Summers and Weretilnyk, 1993). In this study, we show that exposure to light is an important component in the regulation of choline metabolism and, particularly, in the salt-responsive increase of all three SAM:phospho-base *N*-methyltransferase activities. Although all three activities are associated with cytosolic enzymes, the first enzyme, PEAMeT, shows apparent diurnal fluctuations in activity, whereas the enzyme(s) catalyzing the subsequent two *N*-methylations do not. This observation is consistent with at least two *N*-methyltransferase enzymes catalyzing the *N*-methylation of PEA to PCho in spinach.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea* L. cv Savoy Hybrid 612) plants were grown under the controlled environment conditions described previously (Summers and Weretilnyk, 1993). In several experiments the plants were exposed to dark periods exceeding 16 h, and reference to these plants is qualified by the term "dark adapted." For the duration of the light and dark experimental treatments, growth cabinet conditions were adjusted to provide a uniform temperature of 22°C. Salt shock of plants was carried out 4 to 6 weeks after emergence and was completed by irrigating plants at least twice daily during the experimental period with a one-half-strength Hoagland solution (Hoagland and Arnon, 1950) containing 200 mM NaCl.

Extract Preparation

For each treatment, deveined leaf tissue was harvested from at least two individual plants. Total leaf extracts for SAM:phospho-base *N*-methyltransferase activities were prepared by grinding deveined leaf tissue with a mortar and pestle, followed by centrifugation to remove sand and cell debris using the method described by Summers and Weretilnyk (1993). Aliquots of the supernatant were desalted by centrifugation through Sephadex G-25 columns prior to assay (Summers and Weretilnyk, 1993). Alternatively, enzyme activity in the supernatant was concentrated by fractionation with $(NH_4)_2SO_4$. The concentration of the supernatant was first increased to 1.4 M $(NH_4)_2SO_4$ using an appropriate volume of 3.8 M $(NH_4)_2SO_4$, pH 7.5 (Wood, 1976), allowed to equilibrate on ice for 20 min with gentle agitation, and then centrifuged at 10,000*g* for 20 min at 4°C. The pellet was discarded, and the the concentration of the supernatant was increased to 3.0 M $(NH_4)_2SO_4$, equilibrated as earlier, and recentrifuged. The pellet was resuspended in a minimal volume of 100 mM Hepes-KOH (pH 7.8), 5 mM DTT, and 1 mM Na_2EDTA and was desalted by centrifugation through Sephadex G-25 prior to assay. This concentration step enabled more sensitive enzyme assay measurements in extracts prepared from tissues with very low *N*-methyltransferase activities and also purified the activities of interest approximately 3- to 5-fold over those found in crude, desalted extracts.

Subcellular Fractionation

Leaves were harvested from plants that were exposed to at least 4 h of light following their normal 16-h dark period. The leaves were deribbed, coarsely chopped with a razor blade, and then 100 g fresh weight were homogenized with 3 rapid (3 s) bursts of a Waring blender using 300 mL of ice-cold buffer containing 330 mM sorbitol, 50 mM Tricine-KOH (pH 7.9), 2 mM Na_2EDTA, 1 mM $MgCl_2$, and 0.1% (w/v) BSA (Mills and Joy, 1980). The homogenate was gently squeezed through three layers of cheesecloth and one layer of Miracloth (Calbiochem). An aliquot of the filtrate was removed to measure enzyme activities in "total extract," and the remainder was centrifuged at 1,000*g* at 4°C for 15 min. The pellet was resuspended with resuspension buffer containing 50 mM Tricine-KOH (pH 7.9), 1 mM Na_2EDTA, 1 mM $MgCl_2$, and 0.1% (w/v) BSA. This fraction was designated the "1,000*g* pellet." The supernatant was recentrifuged at 20,000*g* for 10 min, yielding a "20,000*g* supernatant" fraction, and the pellet was resuspended with resuspension buffer to provide a "20,000*g* pellet" fraction. All fractions were used for enzyme activity measurements. The final volumes were determined for the total extract and each of the fractions to quantify recovery of the enzymes assayed. To ensure lysis of intact organelles present in the total extract and the fractions, all samples were sonicated with an Ultrasonic Processor XL (Heat Systems, Inc., Farmingdale, NY) for 6 min at 4°C set to cycle 30 s on and 20 s off at setting 0.8. Sonication did not lead to a reduction in activity for any of the enzymes tested.

Enzyme Assays

SAM:phospho-base *N*-methyltransferase activities were assayed as described previously using PEA (Sigma), PMEA, or PDEA as substrates (Summers and Weretilnyk, 1993). NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (Lunn et al., 1990) was used as a chloroplast stromal marker, fumarase (Winter et al., 1982) was used as a mitochondrial matrix marker, and PEPC was assayed under optimized assay conditions (Kalt-Torres et al., 1987) as a cytosolic marker. Chl concentration was determined using the method of Arnon (1949). Protein was estimated by the method of Bradford (1976) with BSA as the standard.

Solute Potential Measurements and Leaf Na^+ Content

Leaf solute potential was measured with an HR-33T Dewpoint Microvoltmeter using C-52 sample chambers (Wescor, Logan, UT). Three leaf discs (0.6 cm diameter) were excised from leaves, wrapped in foil, rapidly frozen in liquid nitrogen, and then permitted to thaw before being placed in the sample chamber. Measurements were made on leaf discs selected from leaves 5 and 6 (leaves 1 and 2 being the oldest) from at least two 5-week-old plants.

Leaf Na^+ ion content was measured in aqueous extracts of leaf tissue using a flame photometer (Evans Electroselenium Ltd., Halstead, UK). A 4-g fresh weight sample of leaf material was ground with an acid-washed mortar and pestle using 2 volumes of deionized H_2O. The slurry was centrifuged at 10,000*g* for 10 min at 4°C to remove cell

debris, and an aliquot of the supernatant was microfuged at 16,000*g* for 2 min at 22°C before dilution for Na^+ quantification.

RESULTS

SAM:phospho-base *N*-methyltransferase activities were measured in leaf extracts prepared from plants harvested at various times during a 2-d period. Preliminary experiments showed that the activity of PEAMeT, the enzyme that catalyzes the *N*-methyl transfer of PEA → PMEA, was lowest following the 16-h dark period and increased throughout the course of the 8-h light period. Figure 1A shows the times associated with maximal and minimal daily activities. The level of PEAMeT activity decreased to approximately half of the maximal light activity level during the course of the 16-h dark period. The effect of a prolonged exposure to dark conditions on PEAMeT activity is shown in Figure 1B. If plants were kept in the dark for 40 or 48 h, PEAMeT activity decreased to a level that was at the detection limits of our radioassay method. If, however, plants maintained in the dark for up to 40 h were placed under lights, PEAMeT activity measured in crude leaf extracts prepared from these plants showed an increase with time.

The activities catalyzed by PMEAMeT (PMEA → PDEA) and PDEAMeT (PDEA → PCho) are shown in Figure 1C. Although fluctuations in the activities of these enzymes were observed during the 48-h experimental period, neither enzyme activity showed any changes that could be correlated readily to changes in ambient light conditions. In contrast to PEAMeT, the activities of PMEAMeT and PDEAMeT did not undergo a continuous decline upon exposure to prolonged dark periods, nor did exposure to lights following a 40-h dark period have any apparent affect on their activities (Fig. 1D).

PEAMeT activity was then measured using leaf extracts from plants dark adapted for 42 h and then exposed to a prolonged light cycle in the presence or absence of a 200 mM NaCl salt-shock treatment (Fig. 2). As shown in Figure 1, A and B, PEAMeT activity increased when plants were exposed to light. However, this increase was greater in plants that were also salt shocked. Within 24 h of initiating the salt treatment, PEAMeT activity was 2.1-fold higher in salt-shocked plants than in the control plants exposed to light for a comparable time. Salt-shocked plants maintained a higher level of PEAMeT activity relative to that of control plants during the 72 h of the experiment. For both control and salt-shocked plants, exposure to continuous light for 72 h led to a slight decline in PEAMeT activity, when compared to the measurement taken at 48 h (Fig. 2). This decline appeared negligible but was not likely spurious, since it was reproducible between experiments. Also, we found that prolonged exposure of nonsalinized plants to continuous light (up to several weeks) eventually led to an almost complete loss of PEAMeT activity (data not shown). This observation suggests that exposure to light alone did not lead to sustained PEAMeT activity. Although intriguing, this observation is difficult to interpret without establishing whether plants exposed to these conditions were synthesizing choline.

The response of PEAMeT, PMEAMeT, and PDEAMeT activities to light and salinity was examined in more detail. The activities of these enzymes were measured in extracts prepared from control plants growing under normal diurnal conditions (8 h light/16 h dark) and compared to those in extracts prepared from plants dark adapted for 42 h (Fig. 3A). In this experiment, PEAMeT activity in the dark-

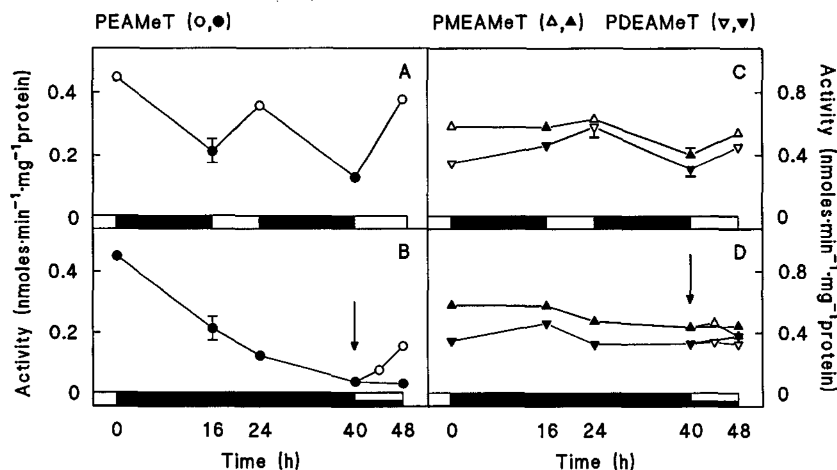

Figure 1. SAM:phospho-base *N*-methyltransferase activities in crude, desalted leaf extracts of plants growing under varying light/dark periods. A and B, PEAMeT (○, ●) activity. C and D, PMEAMeT (△, ▲) and PDEAMeT (▽, ▼) activities. Open symbols, Leaves harvested when plants were exposed to lights. Closed symbols, Leaves harvested from plants kept in the dark. Plants were initially harvested at the end of a normal 8-h photoperiod and periodically during a subsequent 48-h interval. The arrow designates the time when one-half of the plants were transferred to continuous light and the remainder were kept in the dark. Each point represents the mean ± SE of duplicate measurements. When absent, the SE bars fall within the dimensions of the symbol. The entire experiment was repeated twice. The solid bar at the bottom of each panel indicates darkness and the open bar indicates light.

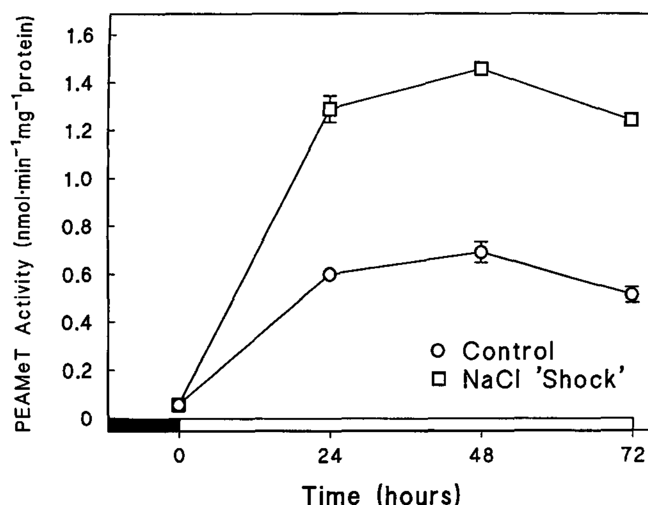

Figure 2. PEAMeT activity in crude, desalted leaf extracts of non-salinized plants (○) and plants salt shocked with 200 mM NaCl (□). Plants were dark adapted for 42 h and transferred to light conditions, and then leaves were harvested following various periods of exposure to continuous light. Each point represents the mean ± SE of duplicate measurements. When absent, the SE bars fall within the dimensions of the symbol. The entire experiment was repeated three times.

adapted plants was about 25% that found in extracts of control plants harvested at the same time, whereas PMEAMeT and PDEAMeT activities were 86 and 108% of control values, respectively. Figure 3B shows what happened to the activities of all three reactions when plants that had been dark-adapted for 42 h were placed under lights for 24 h or kept in the dark for an additional 24 h, either with or without salt-shock treatment. Following a 24-h exposure to light, PEAMeT activity increased approximately 21-fold, whereas PMEAMeT and PDEAMeT activities were almost equivalent and only approximately 3-fold higher than levels detected at the onset of the light period (42 h dark). If dark-adapted plants were placed under lights and salt shocked, the activities associated with all three *N*-methyltransferases increased 1.7- to 2.2-fold over those measured in extracts of plants exposed to 24 h of light but not subjected to a salt-shock treatment. In contrast, plants that were dark-adapted for 42 h and subsequently kept in the dark for an additional 24 h showed a further reduction in PEAMeT activity, whereas PMEAMeT and PDEAMeT activities showed little decrease over 42-h dark or control values. Salt-shock treatment under these prolonged dark conditions did not increase the activity of any of the three *N*-methylation reactions. Thus, none of the SAM:phospho-base *N*-methyltransferase enzymes showed a salt-responsive increase in activity in the absence of light.

To determine the subcellular location(s) of the SAM: phospho-base *N*-methyltransferases, spinach leaves were homogenized in isotonic medium, and fractions enriched in chloroplasts, mitochondria, and cytosol were prepared by differential centrifugation (Table I). The 1,000*g* pellet contained approximately 32% of the chloroplasts and less than 1% of the SAM:phospho-base *N*-methyltransferase activity. The 20,000*g* pellet contained approximately 32% of the mitochondria and 2% or less of the total activity associated with each of the SAM:phospho-base *N*-methyltransferase enzymes. About 84% of the cytosolic marker, PEPC, was found in the 20,000*g* supernatant, and 91.2, 90.4, and 89.3% of PEAMeT, PMEAMeT, and PDEAMeT activities, respectively, could be accounted for by this fraction.

DISCUSSION

The enzymes that catalyze the sequential *N*-methylation of PEA → PMEA → PDEA → PCho show a differential response to ambient light conditions. Leaf extracts of plants grown under normal diurnal light/dark cycles were used to show that the activity of the first enzyme, PEAMeT, gradually increases during the light periods and decreases during dark periods. No apparent diurnal changes in the activity of the enzyme(s) catalyzing the subsequent two *N*-methylation reactions were observed. This is consistent with at least two different enzymes that operate in the conversion of PEA to PCho, each possessing different regulatory properties. This evidence corroborates an earlier proposal that at least two enzymes catalyze this sequence in spinach based on the differential distribution of the three activities in shoots and roots, i.e. PEAMeT activity is found in extracts of leaves but not roots, whereas PMEAMeT and PDEAMeT activities are found in leaf and root extracts (Weretilnyk and Summers, 1992).

The presence of at least two distinct enzymes catalyzing this sequential *N*-methylation of PEA to PCho is not likely a unique property of spinach. For example, Datko and Mudd (1988) examined choline synthesis in *L. paucicostata*, soybean, and carrot. They observed that all of these species

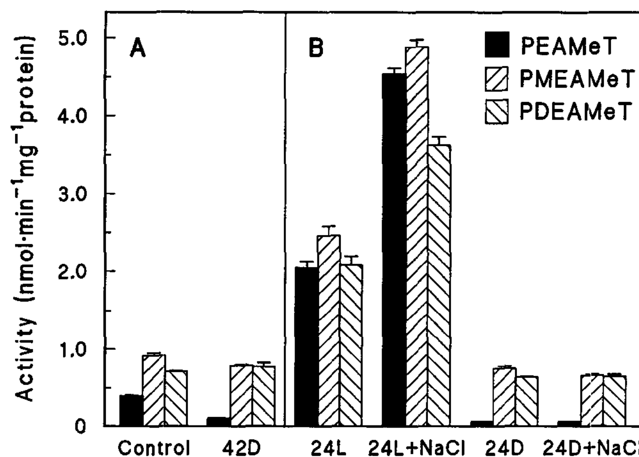

Figure 3. SAM:phospho-base *N*-methyltransferase activities in leaf extracts concentrated by $(NH_4)_2SO_4$ fractionation and desalted prior to assay. A, Plants were grown under a normal diurnal photoperiod of 8 h light/16 h dark and harvested 2 h into the light period (Control) or continuous dark for 42 h (42D). B, Plants exposed to 42 h of darkness (42D) were then exposed to an additional 24-h period of continuous light (24L), light and salt-shock treatment (24L + NaCl), continuous dark (24D), or continuous dark and a salt-shock treatment (24D + NaCl). Data represent the means ± SE of duplicate measurements. The entire experiment was repeated three times.

Table I. *Localization of PEAMeT, PMEAMeT, and PDEAMeT by differential centrifugation of leaf extract*

Spinach leaves were briefly homogenized in isotonic medium and filtered. This total extract was centrifuged for 15 min at 1,000*g*, and then the supernatant was centrifuged again for 10 min at 20,000*g*. In the total extract used for fractionation, the activities of the enzymes assayed were 459, 48, 119, 0.725, 0.992, and 1.046 nmol min^{-1} mL^{-1} for NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH), fumarase, PEPC, PEAMeT, PMEAMeT, and PDEAMeT, respectively. The Chl concentration of the total extract was 135.5 μg mL^{-1}. The entire experiment was repeated twice.

Fraction	Enzyme Activity (Percent of activity recovered from total extract)					
	NADP-GAPDH	Fumarase	PEPC	PEAMeT	PMEAMeT	PDEAMeT
1,000*g* pellet	31.6	6.1	1.9	0.4	0.5	0.9
20,000*g* pellet	0.8	31.9	3.4	2.0	1.7	1.6
20,000*g* supernatant	54.9	33.7	84.1	91.2	90.4	89.3

possess the capacity to convert PEA to PMEA, but the route by which phosphatidylcholine was synthesized from PMEA diverged among the plants they studied. For example, *Lemna* appears to have enzymes capable of catalyzing all three phospho-base *N*-methylations, whereas soybean appears to possess only the first *N*-methyltransferase converting PEA to PMEA. In soybean, subsequent *N*-methylations of PMEA occur at the level of phosphatidyl-bases, eventually producing phosphatidylcholine. Furthermore, Prud'homme and Moore (1992) found no evidence for an enzyme converting PEA to PMEA but did show that PMEA could be converted to PCho in castor bean endosperm. It is interesting that this study used castor bean seeds that were germinated in the dark. It may be that tissues such as spinach roots or castor bean endosperm, both dark-grown tissue, lack PEAMeT activity because there is no PEAMeT enzyme or isozyme that is synthesized or, if synthesized, stably maintained or enzymatically competent in the absence of light.

One explanation could be that spinach roots or the leaves from dark-adapted plants possess an inhibitor that inhibits the PEAMeT enzyme present in these tissues. We ground a mixture composed of leaf samples from light-grown plants, with either leaf tissue from dark-adapted plants or roots, and found that PEAMeT activity assayed from these mixed grinds was additive (data not shown). Therefore, if such an inhibitor exists in dark-adapted tissue, it does not become associated with an enzyme contributed from the light-grown tissue during the grinding procedure. Also, an inhibitor would have to be tightly bound to the enzyme so that it cannot be removed by desalting extracts through Sephadex G-25. Alternatively, levels of PEAMeT activity may reflect the level of PEAMeT protein present in the samples. This would be explained if PEAMeT is degraded gradually in the dark. However, since this enzyme has not been well studied, an explanation of the loss of PEAMeT activity upon exposure to dark conditions awaits further characterization and very likely the purification of this enzyme.

Salinity has been shown previously to increase the activity of the three SAM:phospho-base *N*-methyltransferase activities (Summers and Weretilnyk, 1993). Figure 3 shows that light is essential for the salt-responsive increase in the activity of these enzymes. We determined leaf solute potential from plants subjected to the various light/dark treatments both with and without salt shock. No signs of wilting were observed under any of the treatment conditions used. However, when the leaf solute potential of the treated plants was compared to that of plants growing under a normal diurnal cycle (control plants), we found that all of the plants exhibited a significant decrease in solute potential regardless of the treatment. For example, leaf solute potential for control plants was −0.76 MPa, whereas the value obtained for plants dark-adapted for 42 h was −1.23 MPa. When dark-adapted plants were transferred to 24 h of light or 24 h of light and salt shocked, leaf solute potential declined to −1.5 and −1.6 MPa, respectively. Therefore, leaf solute potential did not provide a good indication of NaCl uptake with salt-shock treatment in the light or under dark conditions. To address the possibility that the plants might not have taken up salt from the irrigating medium, particularly under dark conditions, we measured leaf Na^+ content. The Na^+ level of plants salt shocked in the dark and light was 13.3 and 19.4 μmol g^{-1} fresh weight, respectively. In contrast, plants of all other treatments had leaf Na^+ contents between 4.3 and 5.0 μmol g^{-1} fresh weight. Thus, there is no apparent correlation between leaf solute potential measurements or Na^+ ion content and the in vitro enzyme activity associated with the three SAM:phospho-base *N*-methylation reactions. Rather, the presence of light is the critical component leading to the salt-responsive increase in the activities for all three *N*-methylation reactions.

All three SAM:phospho-base *N*-methyltransferase activities appear to be cytosolic (Table I). This is in contrast to the two enzymes catalyzing the oxidation of choline to betaine, both of which are found in chloroplasts (Hanson et al., 1985). Despite the cytosolic location, PEAMeT displays light-responsive changes in activity. Light regulation of enzyme activity is also shared by a number of other cytosolic enzymes in plants, including nitrate reductase, Suc phosphate synthase, and PEPC (Huber et al., 1994). For these latter enzymes, a picture of a mechanism involving reversible phosphorylation is emerging, and these studies are providing some insight into the role of how light activates or deactivates enzymes. Exactly how light affects the activity of PEAMeT or, indeed, how light and salinity combine to up-regulate the activity of all three SAM:phospho-base *N*-methyltransferases is, at present, unknown.

In contrast to choline synthesis, it is perhaps easier to envision how light might regulate choline oxidation to betaine. In spinach, the enzyme choline monooxygenase

uses reduced Fd as a cofactor to oxidize choline to betaine aldehyde in chloroplasts (Brouquisse et al., 1989). This reaction would provide a direct link between photosynthetic electron transport activity and choline metabolism. Since choline utilization for betaine synthesis is likely light dependent, it is interesting that light also plays a role in regulating choline synthesis, particularly through the activity of the first and likely committing step of PEA conversion to PCho. It is possible that the role of light in mediating the conversion of PEA to PMEA is indirect and that choline monooxygenase activity provides the link between choline synthesis and the availability of light. With light, choline monooxygenase might deplete the cell of choline or possible regulatory intermediates that might inhibit choline synthesis, whereas in the dark the reduced oxidation of choline might lead to the accumulation of an intermediate that inhibits SAM:phospho-base *N*-methyltransferase activity. Although such an inhibitory metabolite, if cytosolic, might effectively regulate all three *N*-methylation reactions in vivo, the diminished in vitro activity of the first enzyme in desalted leaf extracts of dark-grown plants suggests that a distinct mechanism operates in regulating the activity of this enzyme.

In proposing a methyl budget for leaves of a betaine-accumulating C_4 grass, Hanson et al. (1995) estimated that the greatest demand for methyl groups in mature, salinized plants lies potentially in the synthesis of betaine. Thus, down-regulation of this pathway would prevent the use of the 3 mol of SAM required to *N*-methylate the PEA to PCho and the associated metabolic expense of ATP required for the regeneration of SAM. Under conditions in which choline use is reduced, such as in the dark, the down-regulation of the first *N*-methyltransferase would impose the most stringent limitation on the possible level of PCho, and hence choline, synthesized de novo from PEA.

The down-regulation of choline biosynthetic enzymes may not be a unique property of dark-adapted plants, in that the down-regulation of choline synthesis has been documented in other plant systems. The use of a series of near isogenic lines of maize that either contained or were deficient for betaine also show that choline synthesis can be down-regulated in nonsalinized or salinized plants growing under greenhouse growth conditions (Yang et al., 1995). In these plants, the diminished capacity of betaine-deficient lines to oxidize choline to betaine did not lead to an expanded pool of choline, whose size was commensurate with the difference in the betaine pool size between the two lines. In an earlier study, vacuum infiltration of leaf discs from salinized sugar beet with PCho led to a significantly decreased rate of incorporation of [1,2-^{14}C]ethanolamine into betaine (Hanson and Rhodes, 1983). These studies point toward the down-regulation of choline synthesis in betaine-accumulating plants when perturbations are introduced in either the rate of choline utilization, in the case of maize, or the pool size of choline biosynthetic intermediates, as shown with sugar beet. Among plants that do not accumulate betaine, the addition of exogenous choline to *L. paucicostata* (Mudd and Datko, 1989a) and soybean or carrot cultures (Mudd and Datko, 1989b) was shown to down-regulate choline synthesis. For these plants, as with our own extracts from dark-adapted spinach, diminished PEAMeT activity was found.

Mudd and Datko (1989a) estimated that choline synthesis in *Lemna* could account for 55% of the methyl groups derived from Met used in transmethylation. This suggests that the synthesis of choline should place a significant demand on the methyl budget of plants that do not accumulate betaine. As discussed earlier, this demand is likely to be intensified among betaine-accumulating plants growing under saline conditions (Hanson et al., 1995). Given the apparent importance of choline synthesis to the methyl budget of a plant, it is perhaps not surprising that the various plants studied to date display somewhat similar patterns of regulation over choline synthesis. However, more detailed studies of the biochemical properties of the enzymes responsible for choline metabolism are required to determine whether the same mechanism(s) operate to regulate choline synthesis among all of the phylogenetically diverged plant systems just described or whether several, possibly distinct, mechanisms might serve to fine-tune choline synthesis in response to its utilization for growth or osmotic adjustment.

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