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Effect of salt stress on the metabolism of ethanolamine and choline in leaves of the betaine-producing mangrove species *Avicennia marina*

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Abstract

Glycinebetaine synthesis from [methyl-^{14}C]choline and [1,2-^{14}C]ethanolamine in leaf disks of *Avicennia marina*, was increased by salt stress (250 and 500 mM NaCl). After 18 h incubation with [methyl-^{14}C]choline, phosphocholine and CO_2 were found to be heavily labelled. Phosphocholine contained 39% of the total radioactivity taken up by non-salinised (control) leaf disks and 15% of the total for salinised leaf disks stressed with 500 mM NaCl. Eighteen and 49% of the radioactivity absorbed by control and salinised disks, respectively, were released as CO_2. Metabolic studies of [1,2-^{14}C]ethanolamine revealed that the radioactivity taken up by the leaf disks was recovered as the following compounds after 18 h: phosphorylated compounds (mainly phosphoethanolamine, phosphodimethylethanolamine and phosphocholine) (40–50%); choline (1–2%); glycinebetaine (3–5%); lipids (20–28%); CO_2 (6–10%). Unlike glycinebetaine, incorporation into phosphorylated compounds and lipids were reduced by salt stress. Incorporation of [methyl-^{14}C]*S*-adenosyl-L-methionine (SAM) into choline, phosphocholine and glycinebetaine in leaf disks was stimulated by salt stress. In vitro activities of adenosine kinase and adenosine nucleosidase, which are implicated in stimulating the SAM regeneration cycle, increased after the leaf disks were incubated with 250 and 500 mM NaCl for 18 h. Changes in metabolism involving choline and glycinebetaine due to salt stress are discussed.

Keywords: *Avicennia marina*; Avicenniaceae; Grey mangrove; Biosynthesis; Choline; Degradation; Ethanolamine; Glycinebetaine; Salt stress

1. Introduction

Avicennia marina is a halophytic mangrove shrub that grows in tidal swamps in Iriomote Island, Okinawa, Japan. It accumulates considerable amounts of glycinebetaine, asparagine and stachyose in its leaves and roots (Ashihara et al., 1997). The glycinebetaine (**10**) content increased up to two-fold when leaf disks of *A. marina* were incubated with NaCl (Ashihara et al., 1997; Hibino et al., 2001). This quaternary ammonium compound (**10**) therefore seems to function as a compatible solute in leaves of *Avicennia marina*.

In plants, glycinebetaine (**10**) is synthesized from choline (**8**) through betaine aldehyde (**9**) via two enzyme reactions, catalysed by choline monooxygenase (EC 1.1.3.17) (Rathinasabapathi et al., 1997) and betaine aldehyde dehydrogenase (EC 1.2.1.8) (Rhodes and Hanson, 1993; Rhodes et al., 2002) respectively. Choline (**8**) is a direct precursor of glycinebetaine (**10**) and is produced from ethanolamine (**1**) via *N*-methyltransferase reactions. Different pathways for entry of methyl groups into methylated derivatives of ethanolamine (**1**) have been proposed (Summers and Weretilnyk, 1993; Datko and Mudd, 1988). In spinach and tobacco, phosphoethanolamine (PEA) (**2**) was methylated three times to produce phosphocholine (**5**), followed by the release of the phosphate group to form choline (**8**) (Weretilnyk et al., 1995). Nuccio et al. (2000) reported that a single enzyme PEA *N*-methyltransferase catalysed all three

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methylation reactions. Aside from glycinebetaine (**10**) synthesis, ethanolamine (**1**) is also involved in the synthesis of phosphatidylcholine (**7**), which is a dominant constituent of membrane phospholipids (Datko and Mudd, 1988; Mudd and Datko, 1986), and choline-*O*-sulphate, another compatible solute (Hanson et al., 1991).

Recently, Hibino et al. (2001) cloned a cDNA encoding salt-inducible betaine-aldehyde dehydrogenase (BADH) from *A. marina*. This was homologous to the spinach chloroplast BADH. The transcript level of this gene increased at high salinity.

In the present study, the metabolic fate of [1,2-^{14}C]ethanolamine (**1**) and [methyl-^{14}C]choline (**8**) in leaf disks of *A. marina* under different conditions of salt stress was investigated. Our data indicate that salt stress increases glycinebetaine (**10**) synthesis from both ethanolamine (**1**) and choline (**8**). The supply of *S*-adenosyl-L-methionine (SAM), which acts as a methyl donor for the synthesis for choline (**8**), also seems to be increased by salt stress. Weretinyk et al. (2001) reported that salt stress stimulates the regeneration of SAM by activating adenosine kinase (EC 2.7.1.20). We confirm that the adenosine kinase level increases in *A. marina* leaf disks with salt stress, and that levels of adenosine nucleosidase (EC 3.2.2.7) and adenine phosphoribosyltransferase (EC 2.4.2.7) also increase. The present study also found new evidence that salt stress increases the catabolism of choline (**8**). Nearly 50% of the radioactivity from [methyl-^{14}C]choline (**8**) was released as $^{14}CO_2$ when leaf disks were incubated with 500 mM NaCl. The catabolic pathway is discussed and its possible role in salinised leaf disks of *A. marina* is considered.

2. Results and discussion

2.1. Metabolism of [methyl-^{14}C]choline (8)

Table 1 shows the overall metabolism of [methyl-^{14}C]-choline (**8**) by leaf disks of *A. marina* in the presence of 0, 250 or 500 mM NaCl. During an 18 h incubation, radioactivity from [methyl-^{14}C]choline (**8**) was found mainly in choline (**8**), P-choline (**5**), glycinebetaine (**10**), monomethylethanolamine (**15**) (MEA), dimethylethanolamine (**14**) (DEA), lipid, and CO_2. Approximately 4% of [^{14}C]choline (**8**) taken up by *A. marina* leaf disks was converted to [^{14}C]glycinebetaine (**10**) in control leaf disks, and that rates were up to two times greater in salinised leaf disks (Table 1). These results support the hypothesis that glycinebetaine (**10**) is synthesized from choline (**8**), and this compound acts as compatible solute in *A. marina*. It is well known that glycinebetaine (**10**) is synthesized from choline (**8**) in many glycinebetaine (**10**) accumulating plant species, especially Chenopodiaceae (spinach and sugar beet) and Gramineae (barley) (Weretilnyk et al., 1989). However, glycinebetaine (**10**) is not a major product of choline (**8**) metabolism in *A. marina*. The most heavily labelled compound from [methyl-^{14}C] choline (**8**) in control disks was P-choline (**5**), containing nearly 40% of radioactivity; in salinised discs, however, radioactivity from this compound was low. Instead, release of $^{14}CO_2$ from [methyl-^{14}C] choline (**8**) was extremely high in leaf disks treated with 250 or 500 mM NaCl, for which 40–50% of total radioactivity was recovered as $^{14}CO_2$. Here, choline (**8**) metabolism was studied in darkness to eliminate the re-fixation of $^{14}CO_2$ released from ^{14}C-labelled compounds by photosynthetic CO_2 fixation. Since *A. marina* plants sink below the sea surface when the tide is high, compatible solute synthesis might take place even at night. However, synthesis of glycinebetaine (**10**) may be stimulated by light; it has been reported that in other plants choline monooxygenase is a ferredoxin-dependent enzyme localized in chloroplasts (Brouquisse et al., 1989). If *A. marina* choline monooxygenase has similar location and properties, glycinebetaine (**10**) formation induced by salt might be underestimated in the present study.

Little is known about the catabolic pathway of choline (**8**) in plants, and no plant enzymes that participate in choline (**8**) and glycinebetaine (**10**) catabolism have

Table 1
Overall metabolism of 9.0 μM [methyl-^{14}C]choline (specific activity 2.04 GBq mmol^{-1}) by leaf disks of *Avicennia marina* in the presence and absence of 250 or 500 mM NaCl[a]

Metabolite	0 mM		250 mM		500 mM	
PCA-soluble compounds	118.2±3.6	(77.0)	81.3±0.83	(51.0)	63.7±10.7	(45.2)
Choline **8**	35.2±1.3	(22.9)	30.1±1.4	(18.9)	27.5±5.8	(19.5)
P-Choline **5**	60.2±1.3	(39.3)	34.6±0.1	(21.7)	21.0±4.0	(14.8)
Glycinebetaine **10**	6.3±0.1	(4.1)	8.0±0.4	(5.0)	10.0±0.1	(7.1)
DEA **14** + MEA **15**	15.9±3.2	(10.3)	8.0±0.4	(5.0)	5.0±0.8	(3.5)
PCA-insoluble compounds	8.0±0.3	(5.2)	9.5±0.6	(6.0)	7.9±1.2	(5.6)
Lipids	5.2±0.4	(3.4)	6.8±0.7	(4.2)	5.3±1.1	(3.7)
Carbon dioxide	27.3±2.1	(17.8)	68.7±1.5	(43.1)	68.5±5.7	(49.2)
Total uptake	153.4±1.8	(100.0)	159.5±0.1	(100.0)	140.2±6.2	(100.0)

[a] Incorporation of radioactivity into metabolites is expressed as kBq g^{-1} fresh weight. and sd and percentage of total radioactivity taken up by the samples (parentheses).

been reported. In several mammals, glycinebetaine (**10**) is demethylated by betaine-homocysteine *S*-methyltransferase (EC 2.1.1.5) (Finkelstein et al., 1972), and the resulting dimethylglycine (**11**) is further demethylated to glycine (**13**) via monomethylglycine (**12**) (sarcosine) (Hayashi et al., 1980). However, glycinebetaine (**10**) does not seem to be actively catabolised in plants (Rhodes and Hanson, 1993), so that catabolism of choline (**8**) via glycinebetaine (**10**) is not likely to occur in *A. marina*.

A possible path for CO_2 formation from choline (**8**) is direct demethylation of choline (**8**), in which the three methyl groups are converted to CO_2 via formaldehyde and formate. Similar catabolic routes exist for trimethylamine in some bacteria (Colby and Zatman, 1974; Meiberg and Harder, 1979). NAD-dependent formaldehyde dehydrogenase (EC 1.2.1.46) (Ando et al., 1979) and formate dehydrogenase (EC 1.2.1.2) (Kanamori and Suzuki, 1968) have been observed in various organisms. If this pathway operates, 6 mols of NADH should be produced when 1 mol of choline (**8**) is catabolised. Furthermore, if NADH is linked to ATP synthesis by the mitochondrial electron transport chain, this mechanism may be involved in the increased demand for ATP in salt tolerant plants. Further studies would be needed to test this hypothesis.

2.2. Metabolism of [1,2-^{14}C]ethanolamine (*1*)

Table 2 shows the overall metabolism of [1,2-^{14}C]ethanolamine (**1**) by leaf disks of *A. marina*. Uptake of labelled compound from the incubation medium by the leaf disks was not significantly influenced by NaCl, and [1,2-^{14}C]ethanolamine (**1**) was readily metabolised to other compounds. In contrast, uptake of [1,2-^{14}C]ethanolamine (**1**) by spinach leaf disks was significantly reduced by 100 mM NaCl (Summers and Weretilnyk, 1993). This discrepancy may derive from the difference in materials used and from the difference in experimental methods; the total uptake quoted by Summers and Weretilnyk did not include radioactivity released as $^{14}CO_2$, and they incubated their leaf disks under fluorescent light. In *A. marina* leaf disks, more than 60% of the radioactivity was distributed as PCA-soluble small molecular weight compounds, and most of the radioactivity of this fraction was found in phosphorylated compounds, mainly PEA (**2**), P-choline (**5**) and P-*N*-methylethanolamine (**3**) (PMEA). Only trace amounts of radioactivity were observed in P-*N*,*N*-dimethylethanolamine (**4**) (PDEA). It has been reported that most [1,2-^{14}C]ethanolamine (**1**) supplied exogenously to spinach leaf disks is converted to these same phosphorylated compounds (Summer and Weretilnyk, 1993). The rate of incorporation into glycinebetaine (**10**) was higher in salinised leaf disks than in control disks. Compared to P-choline (**5**), choline (**8**) exhibited low radioactivity, but its level was slightly increased in salinised disks. These metabolic profiles of [1,2-^{14}C]ethanolamine (**1**) were similar to those in non-salinised and salinised spinach leaf disks reported by Summers and Weretilnyk, but different from other data obtained from classical work using other plant materials (Miedema and Richardson, 1966). Our results suggest that a major route of choline (**8**) synthesis from ethanolamine (**1**) in *A. marina* is a "phospho-base route" as classified by Summer and Weretilnyk.

Radioactivity from [1,2-^{14}C]ethanolamine (**1**) was also found in the PCA-insoluble fraction. Almost all radioactivity in this fraction was in EtOH–Et_2O-soluble lipids. The major labelled compounds in this fraction were the phosphatidyl (Ptd) derivatives, Ptd-ethanolamine, Ptd-choline, lyzo Ptd-ethanolamine and lyzo Ptd-choline. Salt stress reduced the formation of these phosphatidyl derivatives from [1,2-^{14}C]ethanolamine (**1**).

Table 2
Overall metabolism of 4.5 μM [1,2-^{14}C]ethanolamine (specific activity 4.07 GBq mmol^{-1}) by leaf disks of *Avicennia marina* in the presence and absence of 250 or 500 mM NaCl[a]

Metabolite	0 mM		250 mM		500 mM	
PCA-soluble compounds	67.4±1.4	(63.1)	70.1±1.5	(64.2)	69.8±2.8	(67.6)
Ethanolamine **1**	5.7±0.7	(5.3)	6.4±0.4	(5.9)	9.2±1.2	(9.0)
PMEA **3**	8.4±2.4	(7.9)	5.8±1.3	(5.3)	7.7±1.7	(7.5)
P-Choline **5**	17.6±1.9	(16.5)	19.3±0.4	(21.5)	17.7±4.0	(17.0)
Choline **8**	1.3±0.2	(1.2)	1.9±0.1	(1.7)	2.2±0.0	(2.2)
Glycinebetaine **10**	4.0±0.5	(3.7)	4.8±0.6	(4.4)	5.3±0.0	(5.1)
Unidentified	0.5±0.1	(0.5)	0.4±0.1	(0.4)	0.8±0.0	(0.8)
PCA-insoluble compounds	32.8±0.4	(27.9)	28.1±0.5	(25.7)	23.2±3.4	(22.4)
Lipids	29.8±1.3	(27.9)	26.1±0.5	(23.9)	21.0±1.1	(20.2)
Carbon dioxide	6.6±0.7	(6.2)	11.0±1.3	(10.1)	10.2±1.1	(10.0)
Total uptake	106.8±2.4	(100.0)	109.3±0.7	(100.0)	103.2±5.1	(100.0)

[a] Incorporation of radioactivity into metabolites is expressed as kBq g^{-1} fresh weight. and sd and percentage of total radioactivity taken up by the samples (parentheses).

A portion of the radioactivity from [1,2-^{14}C]ethanolamine (**1**) was observed in CO_2. The incorporation of ^{14}C into these compounds was higher in salinised leaves than in control leaves. Release of $^{14}CO_2$ from [1,2-^{14}C]ethanolamine (**1**) has been detected in some plant species, including oat, tobacco, apple, wheat and pea; glycolate (**17**) may be an intermediate of ethanolamine (**1**) catabolism, although the detailed pathway has not yet been identified (Miedema and Richardson, 1966).

2.3. Supply of methyl group from SAM

Three methyl group transfer reactions by a single *N*-methyltransferase take place in the synthesis of P-choline (**5**) from PEA (**2**) (Smith et al. 2000; Nuccio et al. 2000). Since the methyl group is supplied by SAM, incorporation of radioactivity from [methyl-^{14}C]SAM into P-choline (**5**), choline (**8**) and glycinebetaine (**10**) was studied in salinised and non-salinised leaf disks (Fig. 1). Incorporation of radioactivity into P-choline (**5**) plus choline (**8**) and into glycinebetaine (**10**) fractions was increased by salt stress. Virtually complete incorporation of radioactivity from [methyl-^{14}C]SAM into glycinebetaine (**10**) was found in disks incubated with 500 mM NaCl. These results suggest that the increased rate of PEA (**2**) methylation with SAM is closely related to the increased level of glycinebetaine (**10**) induced by the salt stress.

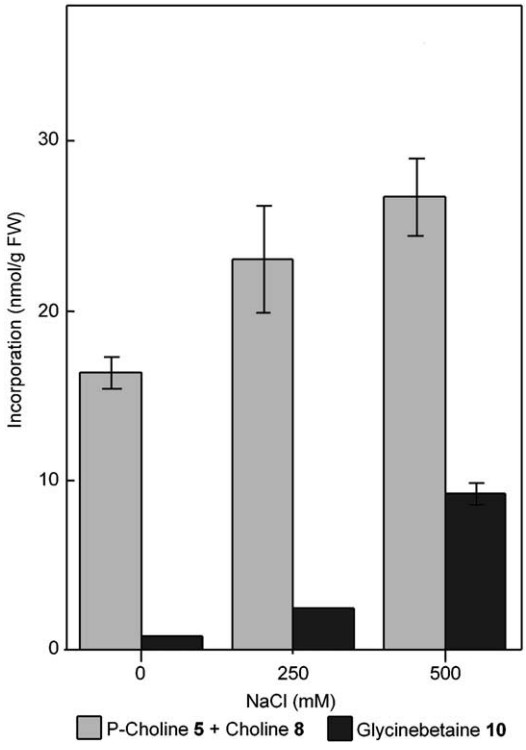

Fig. 1. Incorporation of 9 μM[methyl-^{14}C]SAM (specific activity 2.2 GBq/mmol) into choline (**8**) plus P-choline **5** and into glycinebetaine (**10**) fractions in leaf disks of *A. marina* incubated for 18 h with 0, 250, and 500 mM NaCl. Incorporation of ^{14}C is expressed as nmol/g fresh weight ± S.D. ($n = 3$).

2.4. Activities of adenosine kinase, adenosine nucleosidase and adenine phosphoribosyltransferase

SAM is converted to SAH by donation of its methyl groups to PEA (**2**), PMEA (**3**) and PDEA (**4**). Since SAH is a strong inhibitor of methyltransferases, its removal by SAH hydrolase is essential to allow methylation reactions to continue, including P-choline (**5**) biosynthesis from PEA (**2**). The SAH hydrolase reaction is reversible and the equilibrium is heavily in favour of SAH synthesis from adenosine and homocysteine (Poulton and Butt, 1976). Removal of adenosine and homocysteine is therefore essential for the effective hydrolysis of SAH. It has been suggested that removal of adenosine is more important than removal of homocysteine for SAH hydrolase activity (Poulton and Butt). Removal of adenosine therefore stimulates the conversion of homocysteine to methionine, which is further converted to SAM to complete the SAM regeneration cycle.

The four enzymes, adenosine kinase, adenosine nucleosidase, adenosine deaminase (EC 3.5.4.4), and purine nucleoside phosphorylase (EC 2.4.2.1) might participate in the removal of adenosine. In higher plants, however, only adenosine kinase and adenosine nucleosidase are significant in adenosine metabolism (Ashihara and Crozier, 1999; Moffatt and Ashihara, 2002). One product of adenosine kinase is AMP, whereas adenine is a product of adenosine nucleosidase. Adenine is salvaged by adenine phosphoribosyltransferase to form AMP. Fig. 2 shows the activities of adenosine kinase, adenosine nucleosidase and adenine phosphoribosyltransferase in extracts from control and salinised *A. marina* leaf disks. Salt stress stimulated the in vitro activities of all three enzymes. The rate of increase was ordered as: adenosine kinase > adenosine nucleosidase > adenine phosphoribosyltransferase. These results support the claim that adenosine kinase is involved in maintaining methylation activities during salt stress in plants (Weretilnyk et al., 2001). Not only adenosine kinase, but also adenosine nucleosidase and possibly adenine phosphoribosyltransferase may be involved. The activities of adenosine kinase, adenosine nucleosidase and adenine phosphoribosylransferase in control leaf disks were respectively 8.3 ± 0.7, 220 ± 14, and 12 ± 0.7 pkat per mg protein. In vitro activity of adenosine nucleosidase with adenosine at saturated concentration was therefore at least 25 times higher than that of adenosine kinase. When [8-^{14}C]adenosine was administered to leaf disks of *A. marina*, a significant amount of radioactivity was recovered in adenine and adenine nucleotides (Ashihara et al., 2003). This latter route of adenosine metabolism is therefore important in *A. marina*.

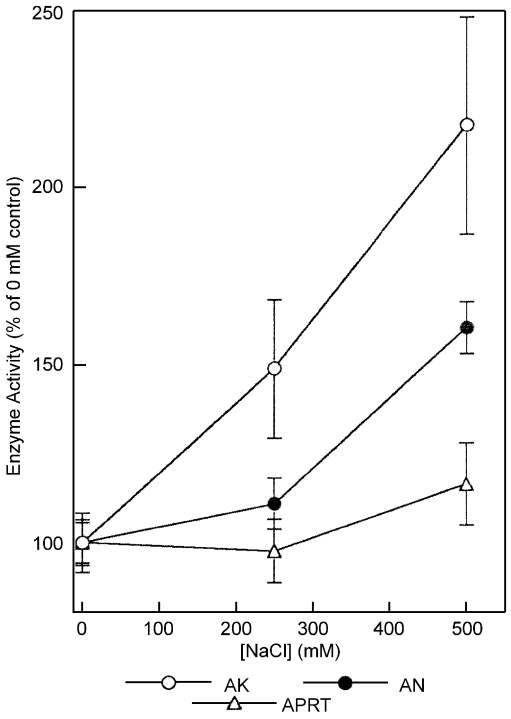

Fig. 2. Effect of salt stress on the activities of adenosine kinase, adenosine nucleosidase, adenine phosphoribosyltransferase in *A. marina*. After the leaf disks were incubated in 0, 250 and 500 mM NaCl for 18 h a crude enzyme preparation was obtained from leaf disk homogenate. Enzyme activities are expressed as percentage of enzyme activity obtained from control leaf disks and S.D. ($n=4$). The activities of adenosine kinase (AK), adenosine nucleosidase (AN) and adenine phosphoribosyltransferase (APRT) in the control sample (0 mM NaCl) were 8.3 ± 0.7, 220.3 ± 14.3 and 12.3 ± 0.7 pkat mg^{-1} protein, respectively.

2.5. Concluding remarks

In relation to the synthesis of compatible solutes in mangrove plants, we have investigated the overall metabolism of choline (**8**) and ethanolamine (**1**) using leaf disks of *A. marina* (Fig. 3). Glycinebetaine (**10**) synthesis from ethanolamine (**1**) and choline (**8**) was increased by the salt stress. Our results suggest that the pathway of ethanolamine (**1**) to glycinebetaine (**10**) via PEA (**2**), PMEA (**3**), PDEA (**4**), P-choline (**5**), choline (**8**) and betaine aldehyde (**9**), is activated by salt. This is probably due to an increase in the expression of genes that encode the related enzymes. In particular, mRNA for *A. marina* betaine-aldehyde dehydrogenase has been induced at high salinity (Hibino et al., 2001). Salt induction of genes for enzymes, PEA *N*-methyltransferase and choline monooxygenase has also been reported in other plant materials (Weretilnyk and Hanson, 1989, 1990; Russell et al., 1998; Weretilnyk et al., 2001). In addition to stimulating the enzymes of the biosynthetic pathway of choline (**8**) and glycinebetaine (**10**), salt increases the level of enzymes that contribute to the regeneration of SAM, which is a methyl donor for choline (**8**) synthesis (Fig. 4). The role of adenosine kinase in the SAM-regeneration cycle has been reported in glycinebetaine (**10**) accumulators, spinach and sugar beet (Weretilnyk et al., 2001). In *A. marina*, adenosine nucleosidase also participates to remove adenosine from the cycle, as observed in tea leaves in which caffeine (trimethylxanthine) biosynthesis occurs (Koshiishi et al., 2001).

Catabolism of choline (**8**) is greatly stimulated by salt; the possible routes are shown in Fig. 3. NADH formation, which is linked by electron transfer chain to ATP synthesis, may be involved in demethylation of choline (**8**). However, enzymes of choline (**8**) catabolism have not yet been clearly identified in plants or any other organisms. Further investigation is necessary to clarify the salt-induced catabolism of choline (**8**).

3. Experimental

3.1. Plant materials

The leaves used in this study were obtained from field-grown shrubs of *Avicennia marina* (Forssk.) Vierh. in Iriomote Island, Okinawa, Japan. Young pale green leaves, recently emerged (ca. 40 mm in length, 20 mm in width, 400 mg fresh weight) were selected and used in the experiments.

3.2. Chemicals

[Methyl-^{14}C]choline (**8**) and [methyl-^{14}C]SAM were purchased from Amersham International plc. (Amersham, Bucks., UK) and [1,2-^{14}C]ethanolamine (**1**), [8-^{14}C]adenosine and [8-^{14}C]adenine came from Moravek Biochemicals Inc. (Irvine, CA). Biochemicals were obtained from Sigma (St. Louis, MO).

3.3. Administration of radiolabelled precursors

[Methyl-^{14}C]choline (**8**), [1,2-^{14}C]ethanolamine (**1**) and [methyl-^{14}C]SAM were administered as described by Fukushima et al. (1997). In summary, leaf disks (3-mm squares, 200 mg fresh weight) and 2.0 ml of Linsmaire and Skoog (1965) medium supplemented with NaCl and 37 kBq of ^{14}C-labelled substrate were placed in the main compartment of a 30-ml Erlenmeyer flask fitted with a glass tube in a centre well. This tube contained a piece of filter paper impregnated with 0.1 ml of 20% KOH. Each reaction was started by the addition of labelled compounds (10 μl) to the main compartment of the flask. Flasks were incubated in an oscillating water bath at 27 °C. After incubation, the glass tube was removed from the centre well and placed in a 50-ml Erlenmeyer flask containing 10 ml of distilled water. Meanwhile the leaf disks were harvested, washed with

distilled water, and frozen with liquid nitrogen to be stored at −80 °C until extraction. Potassium bicarbonate, which had been absorbed by the filter paper, was allowed to diffuse into the distilled water overnight, and aliquots of the resultant solution (0.5 ml) were used in the determination of radioactivity. Radioactivity was measured with a liquid scintillation counter.

3.4. Analysis of radiolabelled metabolites

Frozen leaf disks were extracted successively with cold 6% perchloric acid (PCA) and a mixture of EtOH–Et_2O (1:1, v/v) as specified by Ashihara et al. (2003). After each extraction procedure, the mixture was centrifuged at 20,000 *g* for 7 min, the resulting supernatant was collected, and the precipitate was resuspended in the same extraction reagent and washed by centrifugation. The PCA-soluble fraction was neutralised with KOH. After the precipitated potassium perchlorate was removed by brief centrifugation, the supernatant was evaporated to dryness in vacuo at 37 °C. The cold PCA-soluble metabolites (small molecular weight metabolites) were then fractionated with microcrystalline cellulose TLC plates, using *n*-BuOH–HOAc–H_2O (4:1:2, v/v) as a solvent system. To identify phosphorylated metabolites, parts of the samples were treated with alkaline phosphatase (Sigma) in 100 mM Tris–HCl buffer (pH 9.8) at 37 °C for 3 h.

The radioactivity observed in the EtOH–Et_2O-soluble fraction was regarded as the radioactivity incorporated into lipids. This fraction was evaporated and then dissolved in small amounts of $CHCl_3$. Lipids were fractionated with two-dimensional TLC using $CHCl_3$–MeOH–H_2O (65:25:4, v/v) and $CHCl_3$–MeOH–isopropyl amine–NH_3 (65:35:0.5:5, v/v) as solvent systems (Ashihara and Tokoro, 1985). The radioactivity of ^{14}C on the TLC sheet was determined using a Bio-Imaging Analyser (Type, FLA-2000, Fuji Photo Film Co., Ltd. Tokyo, Japan).

3.5. Preparation of enzymes

After the leaf disks (ca. 500 mg fresh weight) were incubated in 0, 250 and 500 mM NaCl for 18 h, they were homogenised in extraction medium using a mortar and pestle on ice. The extraction medium consisted of 50 mM Tris–HCl buffer (pH 7.5), 2 mM NaEDTA, 0.1% (w/v) 2-mercaptoethanol, 0.5% (w/v) sodium ascorbate and 0.2% (w/v) polyvinylpolypyrrolidone. The homogenate (ca. 10 ml) was centrifuged at 20,000 *g*

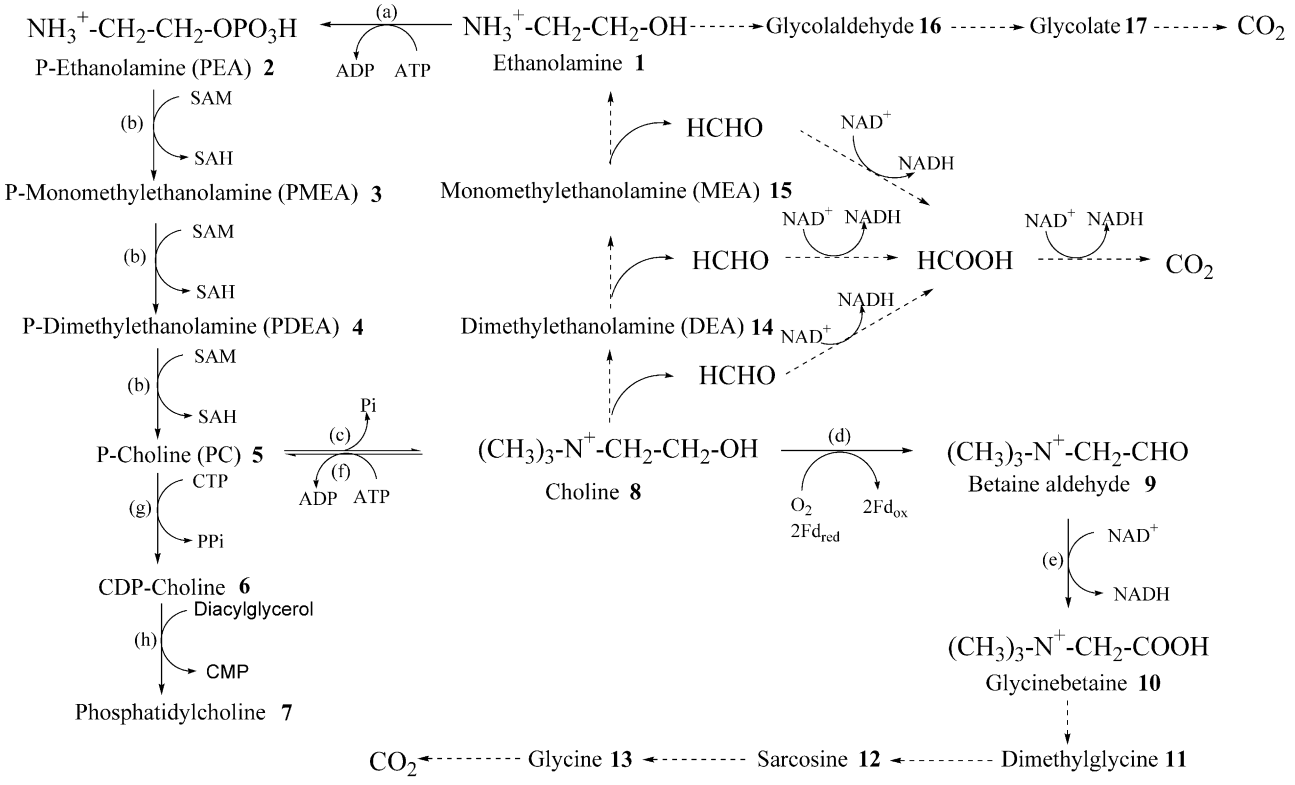

Fig. 3. Possible metabolic routes of ethanolamine (**1**) and choline (**8**) in *A. marina* leaves. Solid arrows indicate established routes in plants, and dotted arrows are speculative routes. Enzymes in established routes are: (a) EA kinase, (b) PEA *N*-methyltransferase, (c) P-choline phosphatase, (d) choline monooxygenase, (e) betaine-aldehyde dehydrogenase, (f) choline kinase, (g) CTP: P-choline cytidyltransferase, (h) CDP-choline:1,2-diacylglycerol choline phosphotransferase.

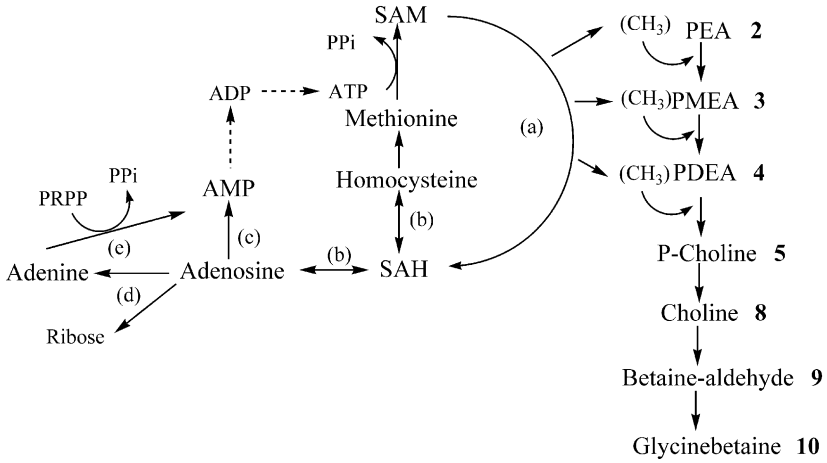

Fig. 4. Pathways for the generation and utilization of SAM in *A. marina* leaves. Three mols of SAM are used to convert PEA (**2**) to P-choline **5**. *S*-adenosylhomocysteine (SAH), which is a product of *N*-methyltransferase reaction (a), is hydrolysed by SAH hydrolase (b). Homocysteine, one of the products of SAH hydrolase, is utilized for the regeneration of SAM; adenosine is directly salvaged by adenosine kinase (c) or hydrolysed by adenosine nucleosidase (d). Adenine, which is a product of adenosine nucleosidase, is salvaged by adenine phosphoribosyltransferase (APRT) (e). Ribose, a further product, may be converted to phosphoribosylpyrophosphate via ribose-5-P, and utilized for APRT reaction (Ashihara and Crozier, 1999).

for 25 min at 2 °C. The supernatant was treated with finely ground solid ammonium sulphate. The protein fraction precipitated by 80% saturation was collected by centrifugation (12,000 *g* for 20 min), and was dissolved in 2.5 ml of the same extraction medium and applied on a pre-packed column of Sephadex G-25 (PD10 Columns, Amersham Pharmacia Biotech, Uppsala, Sweden). The eluted protein fraction (3.5 ml) was used as the enzyme preparation.

3.6. Determination of enzyme activity

Activity of enzymes was determined using labelled substrates (Ashihara et al., 2000). Reaction mixtures (100 µl) were incubated at 30 °C, and terminated by addition of 60% PCA (10 µl) at 2, 5 and 10 min after initiation. The reaction mixture was neutralised with KOH as described above. After removal of the precipitate, the neutralised samples were evaporated to dryness, the pellets were dissolved in H_2O:EtOH (1:1, v/v, 55 µl), and an aliquot (7 µl) was loaded onto the TLC plate. The labelled substrate and product were separated by TLC, using *n*-BuOH–HOAc–H_2O (4:1:2, v/v) (adenosine kinase and adenine phosphoribosyltransferase) or distilled water (adenosine nucleosidase) as a solvent system. The radioactivity of ^{14}C on the TLC sheet was determined as described above.

The reaction mixture for adenine phosphoribosyltransferases consisted of 30 mM HEPES–NaOH buffer (pH 7.6), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.6 mM 5-phosphoribosyl-1-pyrophosphate and 45 µM [8-^{14}C]adenine (specific activity 0.36 MBq µmol^{-1}) . The mixtures for adenosine kinase contained 30 mM HEPES–NaOH buffer (pH 7.6), 10 mM $MgCl_2$, 1 mM DTT, 3.75 mM ATP and 45 µM [8-^{14}C]adenosine (specific activity 0.30 MBq µmol^{-1}). The composition of the reaction mixture for adenosine nucleosidase was the same as for adenosine kinase, except that ATP was excluded.

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