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S-ADENOSYL-L-METHIONINE METABOLISM IN ALFALFA CELL CULTURES FOLLOWING TREATMENT WITH FUNGAL ELICITORS

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Key Word Index—*Medicago sativa*; Leguminosae; alfalfa; methylation; plant cell cultures; elicitors; *S*-adenosyl-L-methionine synthetase; *S*-adenosyl-L-homocysteine hydrolase; adenosine deaminase; adenosine nucleosidase.

Abstract—The activities of the enzymes *S*-adenosyl-L-methionine synthetase (SAM synthetase), *S*-adenosyl-L-homocysteine hydrolase (SAH hydrolase), adenosine nucleosidase and adenosine deaminase, all of which may be involved in maintaining an active pool of SAM for transmethylation reactions, have been determined in seedlings and cell cultures of alfalfa. The expected reaction products were observed with all the assays, but when SAH formation was determined using ^{14}C-adenosine and homocysteine as substrates, an unknown metabolite derived from SAH was also observed. The specific activities of the enzymes were in the order root>leaf = cultures for SAM synthetase, cultures = root > leaf for SAH hydrolase, root >> leaf > cultures for adenosine nucleosidase, with low activities being determined in all tissues with adenosine deaminase. Elicitation of the phytoalexin response in suspension cultures using a yeast cell wall preparation resulted in a sustained six-fold increase in SAM synthetase activity, a rapid transient two-fold increase in SAH hydrolase and adenosine nucleosidase activity, and a delayed two-fold induction in adenosine deaminase activity. The relative importance of these enzymes in the active methyl cycle during the elicitation response in alfalfa is discussed. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

After ATP, *S*-adenosyl-L-methionine (SAM) is the major high energy intermediate in the cell [1], and in plants SAM is used as the principle methyl-donor of a wide range of biomolecules including secondary products, proteins, nucleic acids, sugars and chlorophyll [2]. Surprisingly little is known regarding the regulation of SAM availability and utilization in transmethylation reactions in plants [3]. In common with animals, SAM is synthesized from L-methionine and ATP by the action of the enzyme *S*-adenosyl-L-methionine synthetase (SAM synthetase). On donation of the *S*-methyl group to an acceptor molecule, SAM is converted to *S*-adenosyl-L-homocysteine (SAH). The relative concentrations of SAM and SAH appear to be important in regulating methylation reactions as the various methylases which catalyse these reactions are differentially sensitive to competitive inhibition by SAH [3]. In order to sustain methylation in the cell it is therefore necessary to remove the accumulating SAH, and this is achieved by its reversible hydrolysis to L-homocysteine and adenosine, by the action of the enzyme *S*-adenosyl-L-homocysteine hydrolase (SAH hydrolase). It is believed that, under normal conditions, the reaction is displaced in favour of SAH hydrolysis by the removal of adenosine from the cycle by adenosine nucleosidase, though the involvement of other enzymes such as adenosine deaminase and adenosine kinase cannot be discounted [4, 5]. The homocysteine derived from SAH is re-utilized to form L-methionine [6], thus completing the 'activated methyl' cycle (Fig. 1).

Recently it has been demonstrated that mRNAs encoding SAM synthetase and SAH hydrolase accumulate in plants during developmental changes and stress treatments. Thus, transcripts encoding SAM synthetase accumulate in actively growing tissues [7, 8], particularly those undergoing lignification [8], and in response to salt stress [9] and drought stress [10]. Similarly, mRNAs encoding SAH hydrolase in cell cultures of *Catharanthus roseus* are responsive to changes in growth conditions and heat shock [11]. A major increase in mRNAs encoding both SAM synthetase and SAH hydrolase was determined in parsley leaves and cell cultures treated with a fungal elicitor [6]. This suggested that the defence response in parsley was associated with increases in the corresponding enzyme activities and increased SAM turnover and methylation. The induction of SAH hydrolase activity by fungal elicitors was confirmed, but changes in other enzymes of the methylation cycle were not reported. In the case of SAM synthetase this is particularly significant, as it does not necessarily follow that increased levels of transcripts relate directly to changes in SAM synthetase activity in view of the complex post-transcriptional and post-translational regulation of this

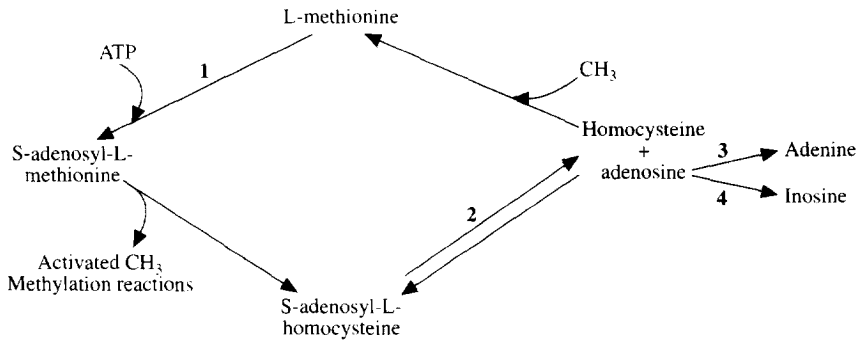

Fig. 1. Synthesis and turnover of *S*-adenosyl-L-methionine in plants. The numbered reactions are catalysed by: 1, SAM synthetase; 2, SAH hydrolase; 3, adenosine nucleosidase; 4, adenosine deaminase.

enzyme in plants [12] and animals [1]. Furthermore, it is also intriguing to determine whether changes in SAH hydrolase activities are associated with corresponding changes in other enzymes, like adenosine nucleosidase and adenosine deaminase, which can remove adenosine from the cycle and thus promote methylation by promoting SAH degradation [3].

As part of a study investigating the importance of methylation reactions in the defence response of plants to pathogenic fungi, the activities of several enzymes of the activated methyl cycle have been determined in elicitor-treated suspension cultures of alfalfa. Alfalfa cell cultures were selected in view of their relatively well characterized response to fungal elicitors [13] and the earlier observation that the elicitation response in these cells was associated with the accumulation of transcripts encoding SAM synthetase [14].

RESULTS

Activities of enzymes of the activated methyl cycle in alfalfa plants

Assays for SAM synthetase, SAH hydrolase, adenosine deaminase and adenosine nucleosidase were developed from published procedures [4, 5, 15] for use in alfalfa. The activities of the four enzymes in the roots and foliage of 14-day-old alfalfa seedlings are shown in Table 1. In the case of SAM synthetase attempts to measure the enzyme activity using a procedure developed for the enzyme from peas [15] proved unsuccessful, with the formation of radioactive product bound to the phosphocellulose paper being independent of incubation time and protein content. By a combination of altering the pH of the assay to pH 8, increasing the specific radioactivity of the [^{3}H-methyl]-L-methionine used, and binding the reaction products to the phosphocellulose paper in the presence of perchloric acid, it was possible to develop a sensitive assay for SAM synthetase. To confirm that *S*-adenosyl-[^{3}H-methyl]-L-methionine was the reaction product, the radioactive material remaining bound to the paper after washing with K-Pi buffer was eluted with 6 M HCl and analysed using HPLC [16]. Using an enzyme preparation derived form the leaves 84±13% (mean±S.D., $n = 3$) of the bound radioactivity co-chromatographed with an authentic sample of SAM, confirming the specificity of the assay. In the presence of an enzyme extract from alfalfa leaves the formation of *S*-adenosyl-[^{14}C-methyl]-L-methionine was directly proportional to the amount of protein present, over the range 0–0.25 mg protein per assay, and remained linear over the 10 min incubation period. Reduced glutathione is commonly included in SAM synthetase assays [1, 15] but in the assays of alfalfa extracts this thiol resulted in only a modest 7% increase in activity. Using the modified SAM synthetase assay, activities were higher in alfalfa roots than in the foliage (Table 1), though the possibility that the enzyme was more highly expressed in the stems than in the leaves was not investigated [8].

SAH hydrolase activity was monitored by measuring the reverse reaction, namely the synthesis of SAH from adenosine and homocysteine using a procedure adapted from that of Poulton and Butt [4]. The formation of SAH could be readily determined in plant extracts. However, when using crude extracts, autoradiography of the TLC plates revealed the presence of two reaction products in addition to the expected SAH. To increase

Table 1. Activities of SAM synthetase, SAH hydrolase, adenosine nucleosidase and adenosine deaminase in the roots and foliage of 14-day-old alfalfa plants

		Enzyme activity (nkat g^{-1} protein)		
Tissue	SAM synthetase*	SAH hydrolase	Adenosine nucleosidase	Adenosine deaminase
Leaf	57±4	81±2	573±72	4±1
Root	112±10	190±13	3280±90	5±2

Values refer to the means of triplicate determinations ±S.D.
* pkat g^{-1} protein.

the formation of the reaction products, extracts from roots and leaves were incubated for 60 min in the presence and absence of DL-homocysteine, to determine whether the additional reaction products were derived directly from adenosine or indirectly from SAH (Table 2). In the presence of homocysteine the majority of the adenosine had been consumed in extracts from both tissues over 60 min. SAH was the major reaction product in leaves, while in roots almost equal quantities of SAH and the unknown metabolite had accumulated. In addition a small quantity of adenine was also determined. When homocysteine was omitted from the incubations, adenine accumulated as major metabolite and the unknown metabolite was not observed. Using root extracts it was shown that the adenine was formed by the action of adenosine nucleosidase, which at pH 8.5 shows 18% of its optimal activity at pH 4.6. Since the unknown metabolite only appeared in the presence of homocysteine, it appeared that this reaction product was either a derivative of SAH composed of a metabolite of homocysteine or it was derived from the metabolism of SAH. In view of the known specificity of SAH hydrolase for homocysteine the former explanation seemed unlikely [17]. The derivation of the unknown metabolite from the SAH was confirmed by recovering [^{14}C]-SAH from the plate and incubating it with a crude root preparation. After 60 min, the unknown metabolite and SAH were present in a ratio of 1:1. Although the identity of radioactive SAH and adenine could be confirmed by HPLC with authentic standards [16] the nature of the unknown metabolite could not be determined and the reaction leading to its formation was not investigated further in this study. Since the unknown metabolite was derived from SAH synthesis, in all subsequent assays the true activity of SAH hydrolase was calculated by summing the formation of both SAH and the unknown compound. In alfalfa plants SAH hydrolase activity specific activities were two-fold higher in the roots than the foliage (Table 1) and in both tissues the formation of SAH and the related metabolite was dependent on protein content and incubation time (up to 30 min).

Using cellulose as the stationary phase it was possible to clearly resolve inosine and adenine by TLC [5] and determine the activities of adenosine deaminase and adenosine nucleosidase, respectively. The resolution of the two reaction products was important due to the appreciable activity of the nucleosidase under the assay conditions used to measure adenosine deaminase. Both activities were strictly dependent on protein content under the assay conditions used. On the basis of enzyme specific activities adenosine nucleosidase was far more active than adenosine deaminase in both roots and foliage. While adenosine nucleosidase showed highest activities in the roots, adenosine deaminase showed similar activities in both tissues.

Changes in the activities of enzymes of the activated methyl cycle following treatment with fungal elicitors

Cell suspension cultures were treated with a fungal elicitor derived from baker's yeast and the defence response monitored by assaying for phenylalanine ammonia lyase (PAL) activity and the accumulation of the phytoalexin medicarpin (Fig. 2). The specific activity of PAL began to increase within 2 hr of elicitor treatment and this was associated with the accumulation of medicarpin. PAL activity was maximal 24 hr after elicitor treatment, while the phytoalexin continued to accumulate up to 32 hr before declining. These elicitor-mediated changes were similar to those determined in other experiments with alfalfa cell cultures [13], and clearly demonstrated that the cells were undergoing a rapid phytoalexin response. Therefore, the cells were also examined for associated changes in the specific activities of SAM synthetase, SAH hydrolase, adenosine nucleosidase and the adenosine deaminase (Fig. 3). The specific activities of all of the enzymes of the activated methyl cycle were differentially altered following elicitor treatment. The specific activity of SAM synthetase activity began to increase 4 hr after elicitor treatment, and by 24 hr was six times higher than in the untreated cells (Fig. 3(A)). Activity then declined but remained appreciably higher than that determined in the control cells between 24 and 48 hr. The increase in the activity of SAH hydrolase (Fig. 3(B)) and adenosine

Table 2. TLC analysis of reaction products formed from ^{14}C-adenosine in SAH hydrolase assays of alfalfa roots and foliage in the presence and absence of DL-homocysteine

Metabolite	R_f	Homocysteine (−/+)	% Applied radioactivity* Leaf	Root
SAH	0.16	−	0±0	0±0
		+	51.3±2.7	38.1±4.6
Unknown	0.35	−	0±0	0±0
		+	10.7±3.1	39.5±0.5
Adenine	0.39	−	19.8±0.4	62.1±9.8
		+	10.4±1.1	8.9±0.07
Adenosine	0.42	−	80.2±6.7	37.9±6.8
		+	27.6±2.2	13.5±4.0

Values represent the means of triplicate determinations ±S.D.

*Radioactivity applied for each analysis = 29 900 dpm±2160 dpm (n = 12).

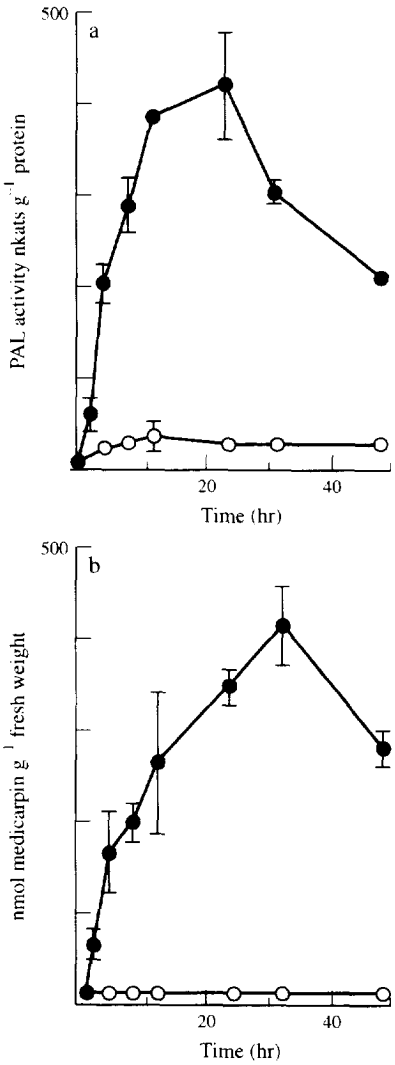

Fig. 2. The elicitation response in alfalfa suspension cultures treated with (●) and without (○) fungal elicitor showing A. Specific activities of PAL and B. accumulation of the phytoalexin medicarpin. Data points refer to the mean of triplicate determinations with the error bars showing the standard deviations. Where no error bars are visible they lie within the extent of the graphical symbol.

nucleosidase (Fig. 3(C)) was more rapid and transient than that determined with SAM synthetase with the activities of both enzymes being almost doubled within 4 hr of elicitor treatment. However, by 8 hr the activities of both enzymes had returned to similar levels to those determined in untreated cells. In the case of adenosine nucleosidase, enzyme activities continued to decline in the elicitor treated cultures to well below the respective activities determined in the control cells. The activities of adenosine deaminase (Fig. 3(D)) were considerably lower than those of adenosine nucleosidase, with the enzyme specific activity only increasing 32 hr after elicitor treatment.

DISCUSSION

The activities of SAM synthetase, SAH hydrolase, adenosine deaminase and adenosine nucleosidase could all be readily determined in crude extracts from both alfalfa seedlings and cell cultures. When SAH hydrolase was assayed for the formation of SAH from adenosine and homocysteine, appreciable quantities of an unknown reaction product accumulated in addition to the expected SAH. This metabolite was formed from SAH, and its characterization warrants further attention as it may represent an as yet undescribed route of SAH catabolism in plants. In all tissues the activity of SAH hydrolase, adenosine nucleosidase and adenosine deaminase activities could be directly compared, as they were all performed using similar concentrations of substrate. However, the substrate concentration used in the SAM synthetase assay was far lower than that employed in the other assays and this contributed to the 1000-field lower specific activity determined with this enzyme as compared with the others. The specific activities of the enzymes in the various tissues tested were in the order root > leaf = cell cultures for SAM synthetase, cell cultures = root > leaf for SAH hydrolase, root >> leaf >> cell cultures for adenosine nucleosidase, with the activities toward adenosine deaminase being low and similar in all tissues. Relatively little is known regarding the factors regulating the developmental expression of these enzymes in plants. mRNAs encoding SAM synthetase are highly expressed in the roots and stems of *Arabidopsis thaliana* and in the stems appreciable activities of the respective enzyme could be determined [8]. SAH hydrolase activity has been found in leaves [4, 6], seeds [17], germinating cotyledons [18], and shoots [19], with little reference to the activity in the roots. Similarly, the few studies on adenosine nucleosidase have concentrated on leaves [5] and germinating seeds [18], and the very high activities of the enzyme in healthy roots have not been reported. Adenosine deaminase has not received much attention in plants, though its low enzyme activities in plant tissues have been remarked on previously [4].

Although the individual contribution that the various enzymes make to regulating the activated methyl cycle can only be assessed by carrying out detailed metabolism studies, some conclusions may be drawn from the changes in the activities of the various enzymes during a period of increased cellular methylation. Thus, the elicitation response in alfalfa cell cultures is associated with an overall four-fold increase in the methylation of cellular components, notably phytoalexins [20], and a two-fold accumulation of SAM and SAH [16]. In the elicitor-treated cells the only enzyme activity whose induction mirrored that of the increase in cellular methylation [20] and changes in SAM and SAH concentrations [16] was SAM synthetase. The transient induction of SAH hydrolase and adenosine nucleosidase occurred before major changes in cellular methylation, and the elevation of adenosine deaminase occurred

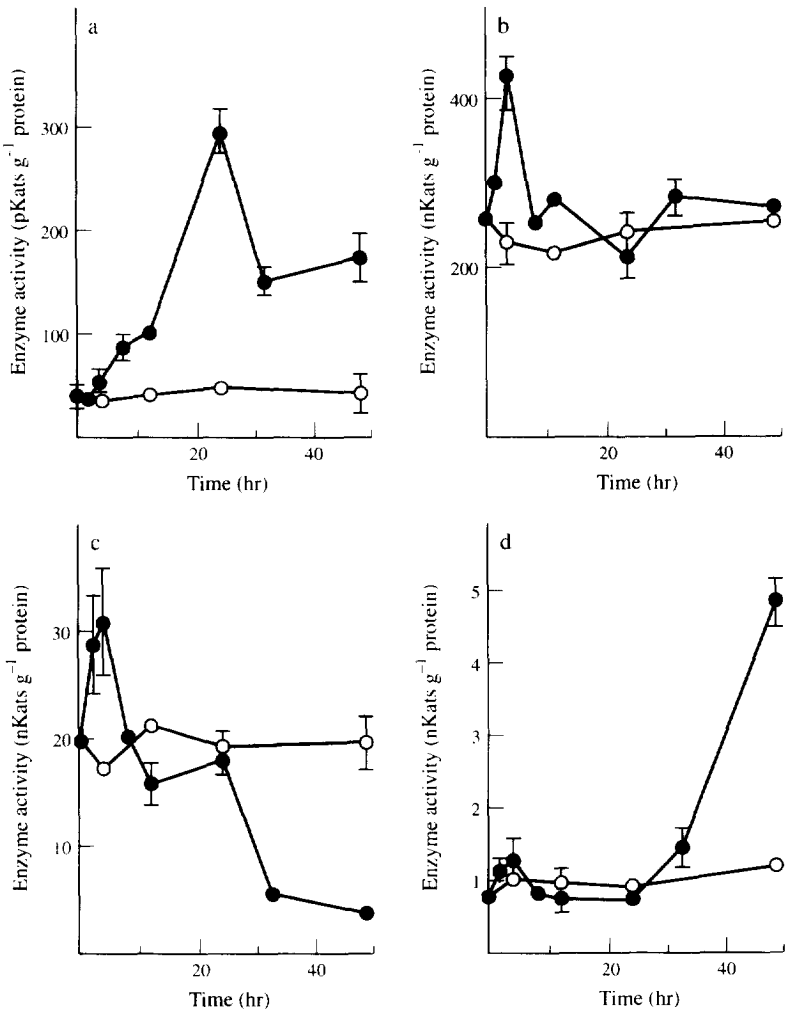

Fig. 3. Specific activities of (A) SAM-synthetase; (B) SAH hydrolase; (C) adenosine nucleosidase; and (D) adenosine deaminase, in cultures treated with (●) or without (○) fungal elicitor. Data points refer to means of triplicate determinations with the error bars showing the standard deviations. Where no error bars are visible they lie within the extent of the graphical symbol.

at a time when total methylation was declining [20]. Significantly, between 8 and 24 hr after elicitor treatment, when total methylating activity was maximal, the activities of SAH hydrolase, adenosine nucleosidase and adenosine deaminase were similar to, or even less than, the corresponding activities in the untreated cells. Since SAH does not accumulate during this period [16], this suggests that the basal activities of these three enzymes are sufficient to cope with the increased turnover of SAM during elicitation. Certainly, in view of the very low activities of adenosine deaminase in all the plant tissues tested it seems unlikely that this enzyme plays a significant role in SAH catabolism.

The results of this study confirm that the accumulation of transcripts encoding SAM synthetase in elicitor-treated plant cells [6, 14] corresponded to increases in the respective enzyme activity. This was an important point to resolve, as studies in wheat had suggested that regulation of SAM synthetase activity appears to be post-transcriptional [12], and in animals its enzymic activity is subject to post-translational control by reducing agents and phosphorylation [1]. SAM synthetase occupies a key role in regulating plant metabolism [21] and this is reflected by its complex regulation by a diverse range of factors including plant development [8], environmental stress [9, 10], infection [6, 14] and plant growth regulators such as gibberellins [15, 22] and auxins [23]. Molecular genetic studies have also implicated SAH hydrolase as a regulator of transmethylation reactions in both photosynthetic bacteria [24] and plants [25]. However, the results of this study suggest that although some plant species, such as parsley may need to induce the expression of both SAM synthetase and SAH hydrolase to sustain increases in methylation [6], in other species, such as alfalfa, up-regulation of SAM synthetase is sufficient. It

will now be of interest to study other factors which regulate the turnover of SAM, and in particular determine how adenosine is removed from the cycle.

EXPERIMENTAL

Chemicals and plant material. SAM, SAH, adenosine and adenine were obtained from Sigma and all radiochemicals from Amersham. Alfalfa seedlings cv. Euver, were grown in vermiculite for 14 days, without inoculation with *Rhizobium meliloti*, as described previously [26]. After separating roots and foliage, weighed tissue was frozen in liquid N_2 and stored at −80°. Suspension-cultured cells of alfalfa cv. Europe were treated with or without an elicitor preparation derived from yeast cell walls and the cells harvested at timed intervals and frozen prior to enzyme assay and extraction and quantification of phytoalexins [27].

Enzyme assays. Frozen cells were homogenized in 2 v/w 0.1 M Tris–HCl pH 7.6 containing 10 mM $MgCl_2$, 0.1 mM EDTA and 2 mM DTT using a Polytron (Kinematica, Lucerne, Switzerland). After centrifugation at 10 000 g (30 min, 4°) the supernatant was adjusted to 80% satn with respect to $(NH_4)_2SO_4$ and the ppt. divided into a number of tubes and collected by centrifugation prior to storage at -80°.

For the assay of PAL and SAM synthetase the protein pellets were redissolved and desalted by dialysis in 0.1 M Tris–HCl pH 8.0 containing 20 mM $MgCl_2$, 70 mM KCl and 5 mM DTT. PAL was assayed spectrophotometrically by monitoring the formation of cinnamic acid [27]. For SAM synthetase assays 75 μl enzyme was incubated with 10 μl 80 mM reduced glutathione, 10 μl 125 mM ATP and 5 μl [^{3}H-methyl]-methionine (18.5 kBq, specific activity 23.6 GBq mmol^{-1}) at 30° for 10 min. The reaction was then stopped with 20 μl 2 M $HClO_4$ containing 5 mM L-methionine, and after removing the protein ppt. by centrifugation, 60 μl of the supernatant was spotted onto a 3 cm^2 strip of P81 phosphocellulose paper (Whatman, H^+ form). The paper was then washed ×2 with 25 ml 5 mM K-Pi buffer pH 7.0 prior to eluting the [^{3}H-methyl]-SAM with 0.3 ml 6 M HCl and radioassaying the eluant by scintillation counting.

SAH hydrolase activity was assayed after desalting the protein ppt. in 62.5 mM Tris–HCl pH 8.0 containing 5 mM DTT. The enzyme prepn (80 μl) was then incubated with 10 μl 100 mM DL-homocysteine and 10 μl [8-^{14}C]-adenosine (9.25 kBq, sp. act. 88 MBq mmol^{-1}) at 30° for 30 min. The reaction was terminated with 10 μl 50% w/v CCl_3 COOH (TCA) and after removing precipitated protein by centrifugation, 20 μl of the supernatant was over-spotted onto a sample of authentic SAH (2.5 μg) previously applied to a plastic-backed silica gel TLC plate containing fluorescent indicator. The plate was developed with *n*-BuOH–HOAc–H_2O (12:3:5) and radioactive metabolites visualized by autoradiography using X-ray film (Fuji). The UV absorbing region corresponding to SAH was cut out with a pair of scissors and placed in a 5 ml plastic scintillation vial. MeOH (0.3 ml) was added and after vortex mixing 4 ml scintillant was added and the vial assayed for radioactivity by scintillation counting.

For the assay of adenosine deaminase, the protein ppt. was taken up and dialysed with 62.5 mM Tris–HCl pH 7.5. The enzyme prepn (90 μl) was then incubated with 10 μl [8-^{14}C]-adenosine (9.25 kBq, sp. act. 88 MBq mmol^{-1}) at 30° for 30 min. The reaction was then stopped as described for the SAH hydrolase assay, and 20 μl of the deproteinized supernatant over-spotted onto a sample of inosine (2.5 μg) on a plastic-backed TLC plate coated with cellulose (CEL 300, Whatman). The inosine was then resolved from adenosine by developing the plate with H_2O. The radioactive metabolites were then identified by autoradiography and [^{14}C]-inosine quantified by scintillation counting as described for SAH hydrolase.

For adenosine nucleosidase assays the protein extract was dialysed in 50 mM NaOAc buffer pH 4.6 and 80 μl of the enzyme incubated with 10 μl 10 mg ml^{-1} BSA and 10 μl [8-^{14}C]-adenosine (3.7 kBq, 37 MBq mmol^{-1}) for 15 min at 30°. After stopping the reaction with 10 μl 50% w/v TCA, 10 μl of the de-proteinized supernatant was over-spotted onto 1.25 μg of adenine on a cellulose TLC plate. After developing in H_2O the material co-chromatographing with adenine was identified and quantified by radioassay as described for SAH hydrolase. In all assays protein content was determined using the BioRad dye binding reagent with γ-globulin for calibration, as recommended by the manufacturer.

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REFERENCES

1. Pajares, M. A. Corrales, F., Durán, C., Mato, J. M. and Alvarez, L. (1992) *FEBS Letters* **309**, 1.
2. Mudd, S. H. and Datko, A. H. (1986) *Plant Physiol.* **81**, 103.
3. Poulton, J. E. (1981) in *The Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds) Vol. 7, p. 667. Academic Press, New York.
4. Poulton, J. E. and Butt, V. S. (1976) *Arch. Biochem. Biophys.* **172**, 135.
5. Poulton, J. E. and Butt, V. S. (1976) *Planta* **131**, 179.
6. Kawalleck, P., Plesch, G., Hahlbrock, K. and Somssich, I. E. (1992) *Proc. Natl Acad. Sci. USA* **89**, 4713.
7. Izhaki, A., Shoseyov, O. and Weiss, D. (1995) *Plant Physiol.* **108**, 841.
8. Peleman, J., Boerjan, W., Engler, G. Seurinck, J., Botterman, J., Alliotte, T., Van Montagu, M. and Inze, D. (1989) *Plant Cell* **1**, 81.
9. Espartero, J., Pintor-Toro, J. A. and Pardo, J. M.

(1994) *Plant Mol. Biol.* **25**, 217.
10. Van Breusegem, F., Dekeyser, R., Gielen, J., Van Montagu, M. and Caplan, A. (1994) *Plant Physiol.* **105**, 1463.
11. Schröder, G., Waitz, A., Hotze, M. and Schröder, J. (1994) *Plant Physiol.* **104**, 1099.
12. Mathur, M., Saluja, D. and Sachar, A. C. (1991) *Biochim. Biophys. Acta* **1078**, 161.
13. Dixon, R. A., Choudhary, A. D., Dalkin, K., Edwards, R., Fahrendorf, T., Gowri, G., Harrison, M. J., Lamb, C. J., Loake, G. J., Maxwell, C. A., Orr, J. and Paiva, N. L. (1992) In *Phenolic Metabolism in Plants* (Stafford, H. A. and Ibrahim, R. K., eds), p. 91. Plenum Press, New York.
14. Gowri, G., Bugos, R. C., Campbell, W. H., Maxwell, C. A. and Dixon, R. A. (1991) *Plant Physiol.* **97**, 7.
15. Mathur, M. and Sachar, R. C. (1991) *FEBS Letters* **287**, 113.
16. Edwards, R. (1994) *Phytochemical Analysis* **6**, 25.
17. Guranowski, A. and Pawelkiewicz, J. (1977) *Eur. J. Biochem.* **80**, 517.
18. Guranowski, A. and Pawelkiewicz, J. (1978) *Planta* **139**, 245.
19. Romanov, G. A. and Dietrich, A. (1995) *Plant Sci.* **107**, 77.
20. Edwards, R., Parry, A. D., Gregory, A. C. E., Tiller, S. A. and Daniell, T. J. (1994) *Acta Hort.* **381**, 214.
21. Boerjan, W., Bauw, G., Van Montagu, M. and Inze, D. (1994) *Plant Cell* **6**, 1401.
22. Mathur, M., Satpathy, M. and Sachar, R. C. (1992) *Biochim. Biophys. Acta* **1137**, 338.
23. Munksgaard, D., Mattsson, O. and Okkels, F. T. (1995) *Physiol. Plant.* **93**, 5.
24. Sganga, M. W., Aksamit, R. R., Cantoni, G. L. and Bauer, C. E. (1992) *Proc. Natl Acad. Sci. USA* **89**, 6328.
25. Masuta, C., Tanaka, H., Uehara, K., Kuwata, S., Koiwai, A. and Noma, M. (1995) *Proc. Natl Acad. Sci. USA* **92**, 6117.
26. Tiller, S. A., Parry, A. D. and Edwards, R. (1994) *Physiol. Plant.* **91**, 27.
27. Edwards, R. and Kessmann, H. (1992) in *Molecular Plant Pathology. A Practical Approach* (Gurr, S. J., McPherson, M. J. and Bowles, D. J., eds) Vol. 2, p. 45. IRL Press, Oxford.