Changes in protein methylation associated with the elicitation response in cell cultures of alfalfa (*Medicago sativa* L.)

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Abstract The methylation of endogenous proteins increased in alfalfa cell suspension cultures following treatment with a fungal elicitor. Carboxyl methylation, a post-translational modification associated with controlling the localisation and longevity of proteins, was the dominant form of protein methylation in both elicited and unelicited cells. Protein methylation was restricted to a limited number of peptides prior to elicitor treatment but as elicitation progressed the number of endogenous substrates increased. Increases resulted from a combination of an elicitor-dependent increase in the activity of a protein carboxyl methyltransferase and the accumulation of preferred endogenous substrates in the latter stages of elicitation.

Key words: Protein methylation; Carboxyl methylation; Elicitor; Suspension culture; *Medicago sativa* L.

1. Introduction

Protein methylation is a commonly observed post-translational modification in both prokaryotes and eukaryotes and may be divided into reversible methyl-esterification of carboxylic acids and irreversible methylation of amino and sulfydryl groups [1]. The functions of protein carboxyl methylation include cell signalling, protein targeting and assembly and the repair of deamidated aspartate residues [1]. The role of methylation of amino and sulfydryl groups in proteins is less well understood but modified proteins may demonstrate altered functional activity, localisation and increased turnover times [1]. Various protein methylation reactions have been demonstrated in plants most notably the *N*-methylation of lysine residues in cytochrome *c* [2] and histone [3] in wheat germ, calmodulin [4,5] in carrots and peas, and the large subunit of ribulose-1,5-bisphosphate carboxylase in a number of species [6]. Although in most instances the effects of these post-translational modifications are unknown, in peas the extent of methylation of the lysine-115 residue of calmodulin has been shown to be higher in mature differentiated tissues than in dividing tissues [4]. The importance of calmodulin methylation in plant development has been confirmed by the impaired growth and development observed in tobacco expressing a foreign calmodulin which could not undergo lysine methylation [7]. It has been proposed that methylation of calmodulin modifies its ubiquitin-dependent turnover [5] and its activation of NAD kinase [4]. The importance of protein methylation in cellular regulation in plants may also be inferred from the specific changes in carboxyl methylation and *N*-methylation which occur in peptides of the thylakoid and stroma respectively in isolated chloroplasts following illumination [8]. Protein carboxyl methyltransferase (PCMT) activities have been reported in wheat germ [9,10]. Of particular interest has been the observation that wheat seeds contain an active L-isoaspartyl methyltransferase catalysing the first step in the conversion of damaged isoaspartate residues back to the corresponding aspartates [10]. It has been demonstrated that such a mechanism can restore function in age-damaged protein [1] with the suggestion that this may account for the longevity of key enzymes in seeds [10].

The response of plant cells to fungal elicitors results in the extensive synthesis of new proteins [11] and presumably their targetting to subcellular compartments and is associated with oxidative conditions which can result in the damage to macromolecules [12]. Since in animals protein methylation has been shown to be involved in all of these processes [1] it was of interest to determine whether the elicitation response modifies protein methylation in plant cells, particularly as it has been reported that transcripts encoding enzymes involved in supporting methylation reactions are induced in response to elicitor treatment [13]. As part of a study investigating the importance of methylation in plant defence reactions we now report on the effect on protein methylation of treating alfalfa cell cultures with a fungal elicitor.

2. Materials and methods

2.1. Plant material and chemicals

Seedlings and suspension cultures of alfalfa cv. Europe were obtained and maintained as described previously [14]. Fungal elicitor was prepared from a yeast cell wall fraction [15] and used at a final concentration of 125 μg glucose equivalents · ml^{-1} cell culture medium. All chemicals were obtained from Sigma and [^{3}H-methyl]methionine (2.79 GBq · mmol^{-1}), [^{35}S]methionine (37 GBq · mmol^{-1}), *S*-adenosyl-[^{3}H-methyl]L-methionine ([^{3}H]SAM, 3.07 TBq · mmol^{-1}) and *S*-adenosyl-[^{14}C-methyl]L-methionine ([^{14}C]SAM, 2.07 GBq · mmol^{-1}) from Amersham Int., Aylesbury, Bucks, UK

2.2. Labelling studies with cell cultures

Suspension cultures (50 ml) in mid-logarithmic growth phase were treated with either 0.25 ml elicitor or 0.25 ml sterile water and at timed intervals 2 ml samples transferred aseptically to a sterile 50 ml plastic centrifuge tube and cycloheximide added to a final concentration of 35 μM. After 30 min incubation on an orbital shaker (130 rpm, 25°C in the dark) 1.48 MBq of either [^{35}S]methionine or [^{3}H-methyl]methionine was added. After a 2 h incubation with radiolabel the cells were harvested by centrifugation (900 × *g*, 10 min, room temp.) and frozen in liquid N_2. To confirm that the cultures were showing an elicitation response parallel incubations were carried out with the radiolabel omitted and the medium analysed for the phytoalexin medicarpin [15]. Radiolabelled cells were homogenised with a Polytron homogeniser (Kinematica, Lucerne, Switzerland) in 4 v/w ice-cold 20 mM Tris-HCl, pH 7.5, buffer containing 14 mM 2-mercaptoethanol, 5 mM EDTA and

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Abbreviations: SC, liquid scintillation counting; PMT, protein methyltransferase; PCMT, protein carboxyl methyltransferase; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; SDS-PAGE, SDS-polyacylamide gel electrophoresis.

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5 mM PMSF. After centrifugation (17000 × g, 15 min, 4°C) the protein content of the supernatant was determined using the BioRad dye-binding assay reagent with γ-globulin as the reference standard and the total radioactivity present by liquid scintillation counting. To determine the incorporation of radioactivity into protein 10% v/v of a solution of bovine serum albumin (10 mg · ml^{-1}) was added to a portion of the supernatant and the sample precipitated with an equal volume of ice-cold TCA (20% w/v) containing 6.7 mM L-methionine. After washing the pellet with 1 ml of 10% TCA containing 6.7 mM L-methionine esterified radioactive residues were hydrolysed by treating the pellet with 100 mM NaOH (150 μl) at 30°C for 15 min in a tightly capped tube. The volatile nature of the released [^{3}H]methanol was confirmed as required by distillation assay [10]. After cooling on ice the solution was acidified with 5 μl concentrated HCl and protein precipitated by adjusting to 10% (w/v) TCA. The protein pellet was then solubilised in 88% formic acid (150 μl) and the radioactivity determined by LSC. For the determination of total incorporated radioactivity the base hydrolysis step was omitted.

To identify methylated peptides 250 μg of protein from the cell free supernatant was precipitated in TCA (10% w/v) and analysed by SDS-PAGE on a 12% acrylamide slab gel [16] prior to fluorography using Amplify (Amersham) as recommended by the manufacturer. As required membrane bound proteins were analysed after preparing a microsomal preparation from the cell free supernatant by incubating with 50 mM $MgCl_2$ and collecting the precipitate by centrifugation [17].

2.3. Assay of protein methyltransferase (PMT) activity

Frozen plant tissue was homogenized with a Polytron in 3 v/w 0.1 M Tris-HCl (pH 7.5) containing 2 mM DTT, 2 mM EDTA and 1 mM PMSF and after centrifuging (17,000 × g, 20 min, 4°C) the protein in the supernatant precipitated by adjusting to 80% saturation with $(NH_4)_2SO_4$. The protein precipitates were then dissolved and dialysed against 50 mM Tris-HCl (pH 8.0) containing 5 mM DTT and 1 mM PMSF for 16 h prior to assay. For quantitative determinations incubations were normalised for protein content (2 mg · ml^{-1}) and 100 μl enzyme, 5 μl [^{14}C]SAM (1.9 kBq) and 10 μl of potential protein substrates (100 μg) incubated at 37°C for 30 min. The reaction was then stopped with 1 v/v 20% TCA containing 6.7 mM L-methionine and the radioactive protein analysed as described for the cell labelling studies. For the identification of methylated proteins a similar assay was used except 5 μl [^{3}H]SAM (185 kBq) was used in place of [^{14}C]SAM and the reaction products analysed by SDS-PAGE and fluorography.

3. Results

The total PMT activities in crude ammonium sulphate precipitated preparations from control cells and cells treated with elicitor for 48 h were optimised with [^{14}C]SAM serving as methyl-donor. When tested with the endogenous substrates present in the enzyme preparations the PMT activity in control and elicited cells appeared to be similar in nature with both enzymes stimulated two-fold by the inclusion of reducing agents and having a pH optimum of pH 8.0. The incorporation of radioactivity into protein was strictly dependent on the protein content of the assay up to (10 mg protein · ml^{-1}) and was time-dependent up to 30 min, after which the activity was rapidly lost at 37°C. In contrast EDTA and divalent cations had no effect on total activity. Having optimised conditions for measuring total PMT activity the activities responsible for incorporation of base labile radioactivity (carboxyl methylation) and base stable incorporation (predominantly amino methylation) were characterised separately in alfalfa plants and cell cultures (Table 1). In the absence of exogenous substrates the PMT specific activities were in the order roots > leaves > unelicited cell cultures for carboxyl methylation and leaves > roots > unelicited cultures for alternative forms of methylation. In roots and cell cultures carboxyl methylation was two times more extensive than *N*-methylation while in leaves *N*-methylation pre-dominated. Treatment of cell cultures with an elicitor for 12 h increased the specific activities of the two PMT activities two-fold (Table 1). To determine whether this increase in activity was due to increased PMT activity or greater availability of endogenous protein substrate in the elicited extracts equal amounts of protein from elicited and unelicited cells were heat-treated at 60°C for 5 min to inactivate the endogenous PMT activity and then incubated with active PMT preparation from control and elicited cells. The PMT from the elicited cells was over twice as active toward the heat-treated elicited protein as compared to the control substrate preparations with all of the increase in incorporated radioactivity being base labile. In the PMT preparation from the control cells this difference was not observed. It was concluded that the increase in carboxyl methylation in the elicited extracts was due in part to the greater availability of endogenous protein substrates. The PMT preparation from elicited cells was incubated with a range known protein substrates of PMTs from other sources [9]. Inclusion of calmodulin, cytochrome C, gelatin, γ-globulin and ovalbumin increased incorporation of base-labile radioactivity into protein. To confirm that the incorporation of radioactivity into the proteins was due to a SAM-dependent methyltransferase *S*-adenosylhomocysteine (SAH) was used as a competitive inhibitor [9] in the PMT assays (Table 1). SAH was a more effective inhibitor of *N*-methylation than carboxy methylation when tested at a range of concentrations.

To determine the kinetics of the elicitation of PMT activities alfalfa cultures were harvested at timed intervals after elicitor treatment and the two methyltransferases assayed. When incubated with γ-globulin as substrate the specific activities of the PCMT increased significantly within 4h of elicitor treatment and reached maximal levels at 36 h (Fig. 1A). However, in this experiment elicitation had a negligible effect on the specific activities of the *N*-methyltransferase (Fig. 1B). In a further study PMT preparations from a elicited time course were incubated with [^{3}H]SAM and the nature of the proteins methylated in vitro examined by SDS-PAGE and fluorography (Fig. 2). Up to 4 h after elicitor treatment the pattern of protein methylation remained identical with a doublet of peptides of M_r 35 kDa and

Table 1
PMT activities in alfalfa plants and cell cultures

Plant source	Addition	(nKats · kg^{-1} protein)	
		Base labile methylation	Base stable methylation
Plant (30 days)			
Leaves	–	12.2 ± 0.7	18.4 ± 1.0
Roots	–	22.5 ± 0.3	10.1 ± 3.0
Cell cultures (7 days)			
Unelicited	–	7.7 ± 0.9	3.9 ± 1.5
Elicited	–	15.8 ± 1.9	7.5 ± 1.1
	Histone	17.9 ± 1.6	6.0 ± 0.9
	Calmodulin	16.6 ± 0.4	11.3 ± 1.3
	Cytochrome C	31.0 ± 2.2	6.3 ± 1.4
	Gelatin	29.7 ± 3.7	10.5 ± 2.1
	Ovalbumin	32.4 ± 0.1	7.5 ± 2.4
	γ Globulin	37.1 ± 0.6	8.0 ± 0.2
	BSA	16.3 ± 0.0	7.7 ± 0.8
	250 μM SAH	8.3 ± 0.8	2.7 ± 1.6

Values refer to means of duplicate determinations ± variation in the replicates. Protein added to incubation at a final concentration of 1 mg · ml^{-1}.

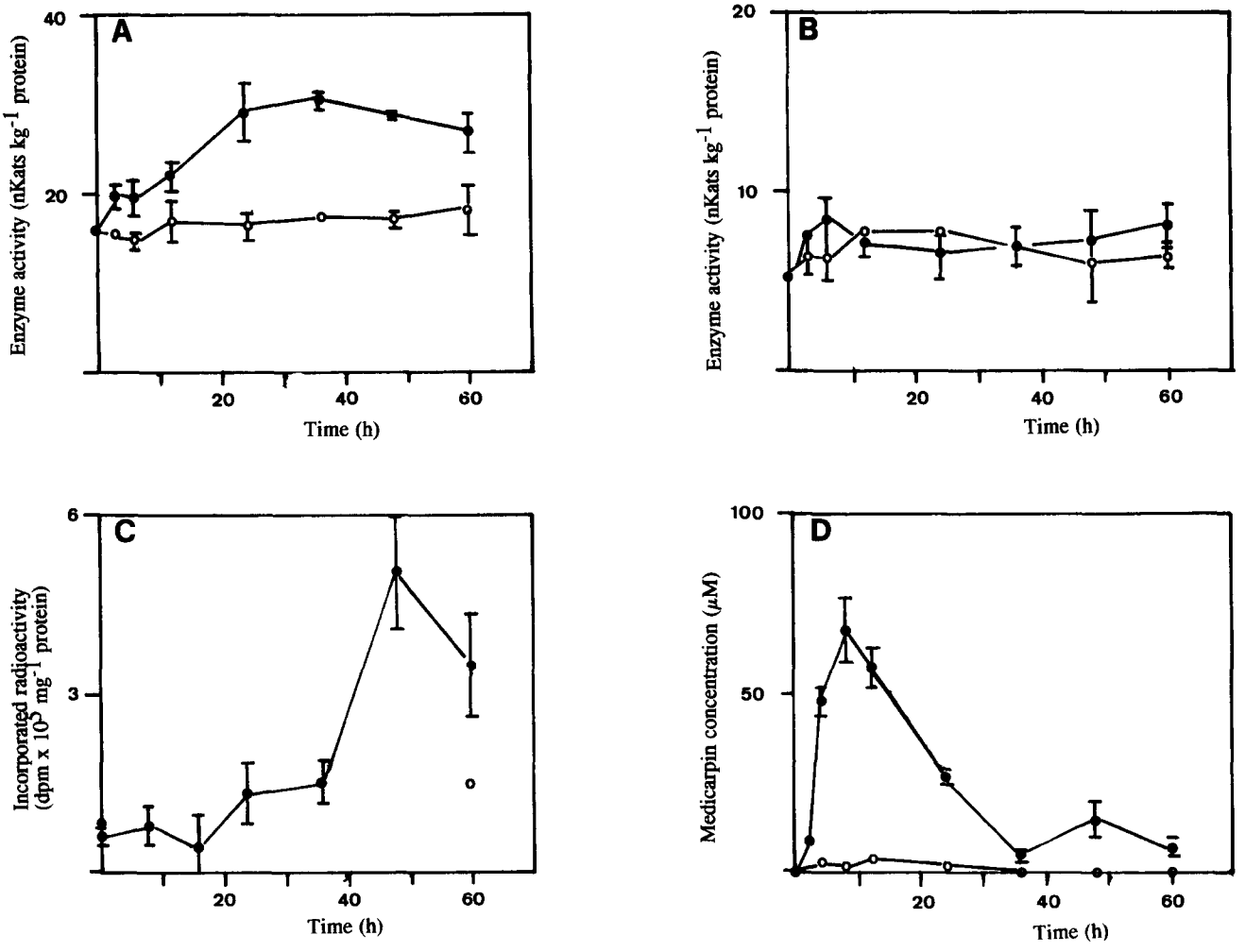

Fig. 1. The effect of treating alfalfa cell suspension cultures with a fungal elicitor on: (A) extractable protein carboxyl methyltransferase activity; (B) extractable protein N-methyltransferase activity; (C) incorporation of [^{3}H-methyl]methionine into protein in cycloheximide treated cells; (D) the accumulatiuon of the phytoalexin medicarpin in the medium. Data points represent mean values with error bars showing the variation in duplicate determinations in control (○) and elicitor treated (●) cells.

37 kDa and a peptide of 17 kDa being highly labelled. The in vitro methylation of the 35 kDa and 37 kDa peptides was also observed in extracts from alfalfa leaves in addition to a peptide of 50 kDa tentatively identified on the basis of its high abundance as the large subunit of ribulose 1,5-bisphoshate carboxylase [6] (data not shown). Between 8 h and 24 h after elicitor treatment the intensity of the labelling of the 17 kDa, 35 kDa and 37 kDa peptides declined with the appearance of radiolabel in multiple peptides. The methylation of mutiple peptides persisted at 48 h but was accompanied by increased incorporation into the 35 kDa and 37 kDa peptides.

To study protein methylation in vivo it was first necessary to inhibit the incorporation of [^{3}H-methyl]methionine into protein using cycloheximide. As determined from monitoring the incorporation of [^{35}S]methionine into protein a 30 min pretreatment with 35 μM cycloheximide inhibited 73 ± 11% (mean ± S.D., n = 8) of protein synthesis in both control and elicited cells over a 2 h labelling period. In elicitor treated cells the cycloheximide treatment had no significant effect on medicarpin accumulation as compared with the cells treated with elicitor alone. Higher concentrations of cycloheximide or longer pre-incubation times gave only a slight improvement in the inhibition of incorporation of [^{35}S]methionine into protein. Since the inhibitory effect of cycloheximide on protein synthesis was uniform in both control and elicited cells at all time points differences in the incorporation of L-[^{3}H-methyl]methionine into protein during elicitation could be ascribed to protein methylation. To confirm this elicited cells were exposed to 100 μM deazaadenosine or 100 μM sinefungin, both potent inhibitors of transmethylation reactions in cells [19], in addition to cycloheximide. In cells treated with elicitor for 12 h, the results with deazaadenosine and sinefungin confirmed that relative to cells treated with cycloheximide alone that the incorporation of [^{3}H-methyl]methionine was reduced by 53% and 34% respectively confirming that incorporation was arising from methylation reactions. Using the in vivo labelling procedure total protein methylation increased in the cell cultures only after a 36 h treatment with elicitor (Fig. 1C) with a five-fold increase being observed at 48 h relative to the unelicited controls. In contrast the accumulation of the phytoalexin medicarpin was maximal at 8 h (Fig. 1D). Analysis of the incorporated radioactivity confirmed the results of the in vitro methylation experi-

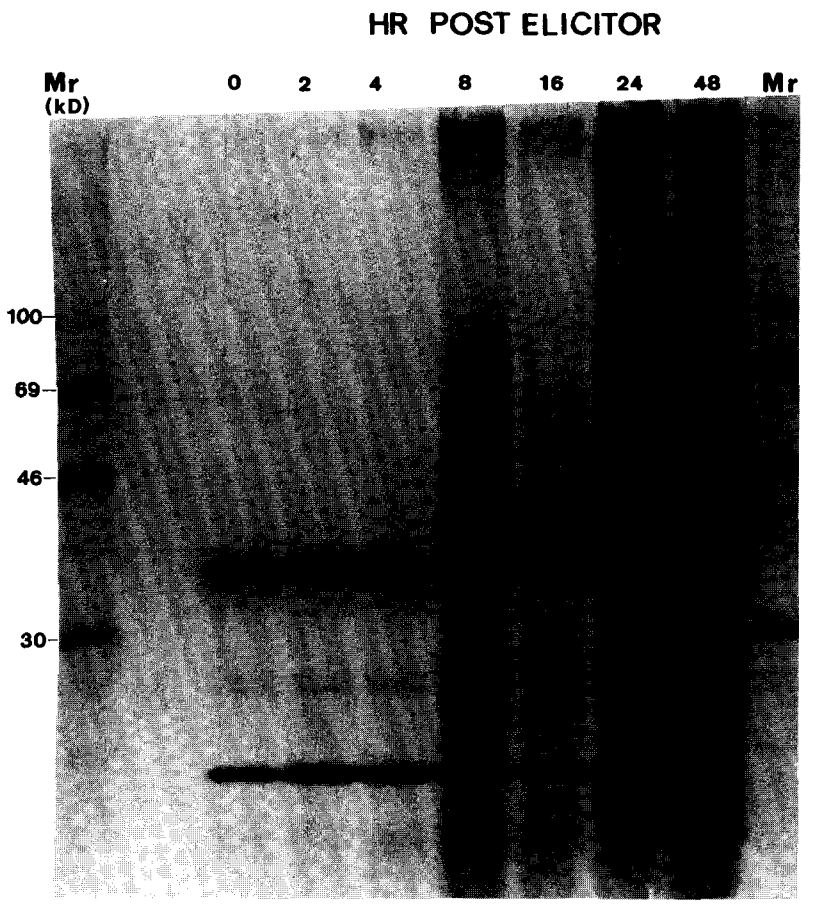

Fig. 2. Fluorograph of protein extracts from elicitor treated cells incubated with [^{3}H]SAM and then analysed by SDS-PAGE. Proteins where labelled under identical conditions and 50 μg applied to a 12% gel with the molecular masses of standard proteins indicated.

ments with 68 ± 16% (mean ± S.D., n = 8) of the incorporated radioactivity being base labile in both elicited and unelicited protein preparations. Despite numerous attempts the specific activities of the proteins labelled in vivo was far lower than that observed in vitro and as a result fluorographs required lengthy exposures to visualise the labelled peptides (Fig. 3). To confirm that the changes in the labelling of the peptides with [^{3}H-methyl]methionine following elicitor treatment resulted from methylation rather than residual protein synthesis a batch of elicitor treated cells was labelled with [^{35}S]methionine in the presence of cycloheximide. At all time points the incorporation of label appeared identical with a diffuse smear of radiolabelled peptides being observed and no specific elicitor-mediated changes in the incorporation of [^{35}S]methionine into distinct peptides determined (data not shown). It was therefore concluded that the specific changes in incorporation of [^{3}H-methyl]methionine into proteins (Fig 3) resulted from changes in protein methylation. No specific peptides were methylated in the unelicited cells with two peptides of M_r 45 kDa and 23 kDa transiently labelled between 0 h and 2 h, and 8 h and 10 h, respectively after addition of elicitor. Between 16 h and 38 h a peptide of M_r 31 kDa was labelled and after 48 h additional peptides of M_r 33 kDa, and 36 kDa, and finaly 23 kDa and 45 kDa were also observed. The autoradiograph confirmed that after 36 h of elicitor treatment these increases in peptide labelling were consistent with the quantitative increases previously observed (Fig. 1C). Subcellular fractionation studies of the labelled cell extracts from cells treated with elicitor for 48 h demonstrated that proteins in both the cytosol and the microsomes were methylated with respectively five-fold and three- fold higher specific activities than the corresponding fractions from control cells.

4. Discussion

These studies suggest that alfalfa cell cultures and plants contain at least two PMTs with activities toward endogenous protein substrates. The less active PMT catalyses the formation of uncharacterised base-stable methylated proteins and is presumabley an *N*-methyltransferase analogous to the enzymes present in tobacco [18] and peas [4] which have activities toward the large subunit of ribulose 1,5-bisphosphate carboxylase and calmodulin, respectively. Significantly the large subunit of ribulose 1,5-bisphosphate carboxylase appears to be a substrate for protein methylation in alfalfa leaves. The more active PMT had activities toward carboxy-groups on proteins and based on its substrate specificity resembled the protein methylase II in wheat seeds [9] which appears to be predominantly an L-isoaspartyl methyltransferase, which has also been identified in alfalfa [10].

Both in vivo and in vitro labelling studies showed that protein carboxyl methylation increases in alfalfa cell cultures as a late response to elicitor treatment. These changes are associated with increased PCMT activity with the other PMT activities

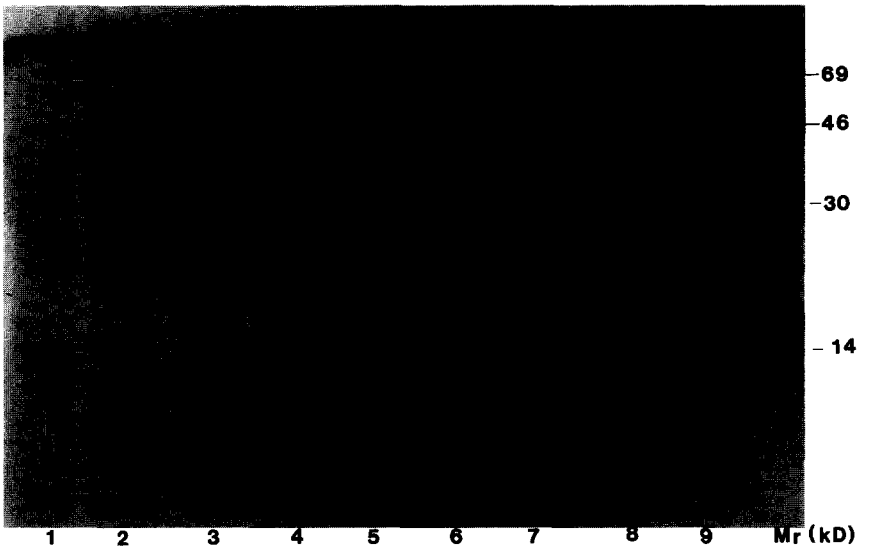

Fig. 3. Fluorograph of extracts (100 μg protein) from cell cultures labelled with [^{3}H-methyl]methionine in the presence of cycloheximide at: 0–2 h (lane 2), 8–10 h (lane 3), 16–18 h (lane 4), 24–26 h (lane 5), 36–38 h (lane 6), 48–50 h (lane 7), and 60–62 h (lane 8) after the addition of elicitor. Labelled extracts from cells labelled at time 0–2 h and 60–62 h after the addition of water are shown in lanes 1 and 9, respectively. Samples were applied to an SDS-PAGE minigel. The molecular masses of standard proteins are shown, with peptides referred to in the text indicated (►).

being unaffected. The observation that PCMT activity was induced before protein methylation increased in vivo suggested that substrate availability also played a part in regulating protein methylation. The relative availability of *S*-adenosylmethionine and *S*-adenosylhomocysteine are known to regulate transmethylation reactions in eukaryotes but our studies have shown that the changes in the concentrations of these compounds during elicitation are quite modest and unlikely to regulate protein methylation [20]. In contrast the mixing experiment in which protein from elicited and non-elicited cell extracts was incubated with the PMT peparations suggested that protein from elicited cells was indeed preferentially methylated. Such changes may result from the accumulation of novel elicitor-inducible proteins [11] containing increased numbers of carboxyl-groups suitable for methylation. In this instance the function of protein methylation may be to regulate protein turnover and targeting to membranes [1]. Our recent results (data not shown) have also demonstrated that elicitation does result in increased methylation of membrane bound proteins and labelling studies with [^{3}H]mevalonic acid suggest that this methylation is associated with prenylation [21]. A 48 h elicitor treatment resulting in a four fold increase in the accumulation of prenylated microsomal proteins. Alternatively increased protein methylation in the latter stages of elicitation may result from an accumulation of proteins damaged by the oxidative conditions associated with elicitation with the resulting isoaspartate residues serving as preferred substrates for the L-isoaspartyl methyltransferase [1,10]. The further determination of the function of protein methylation in the elicitation response in plant cells and the identification of specific substrates is currently in progress.

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