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INDUCTION OF EARLY MEVALONATE PATHWAY ENZYMES AND BIOSYNTHESIS OF END PRODUCTS IN POTATO (*SOLANUM TUBEROSUM*) TUBERS BY WOUNDING AND ELICITATION

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Key Word Index—3-Hydroxy-3-methylglutaryl coenzyme A reductase; *Solanum tuberosum*; isoprenoid metabolism; mevalonate kinase; phosphomevalonate kinase; biosynthesis; steroid glycoalkaloids; sesquiterpenoid phytoalexins; protease inhibitors.

Abstract—In plants, several important classes of terpenoid compounds are synthesized via the mevalonate pathway. In addition to essential constitutive metabolites, potato (*Solanum tuberosum* L.) tubers synthesize antifungal sesquiterpenoid phytoalexins in response to fungal infection or arachidonic acid elicitation, and toxic steroid glycoalkaloids in response to wounding. The activity of the early pathway enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) has previously been shown to increase rapidly and then decrease in response to these stimuli. During an investigation of the possible post-translational control of this enzyme, it was found that the inclusion of the cysteine protease inhibitors leupeptin and E-64 {*N*-[*N*-(L-3-*trans*-carboxyran-2-carbonyl)-L-leucyl]agmatine} in the enzyme extraction buffer increased nine-fold the total HMGR activity recovered in the microsomal fraction and greatly increased the ratio of microsomal to soluble activity. Incubation of microsomal HMGR preparations with soluble protein extracts, Mg^{2+} and ATP caused an apparent inhibition of HMGR, consistent with published reports of post-translational inactivation of HMGR by phosphorylation. The apparent inhibition was completely reversed, however, by 5 mM mevalonate and was found to be an artefact caused by the presence of mevalonate kinase, the next enzyme in the pathway, in the soluble fraction. HPLC assays for mevalonate kinase and mevalonate phosphate kinase were developed and used to measure the activities of these enzymes following wounding and elicitation. While HMGR levels increased 30-fold following arachidonic acid treatment and 15-fold following wounding, mevalonate kinase and mevalonate phosphate kinase only increased two- to four-fold following these treatments, and the levels in arachidonic acid treated tubers were only 20–40% higher than in wounded tubers. While HMGR levels are extremely low in untreated tissues, the activities of the two kinases are relatively high, suggesting that they do not serve as control points for the synthesis of terpenoids.

INTRODUCTION

Plants show a large number of biochemical and molecular responses following attacks by microbial pathogens and herbivores. Part of the complicated defence response against pathogens is the rapid accumulation of phytoalexins, low molecular weight metabolites with antimicrobial activity [1]. Phytoalexins are often synthesized in a narrow region surrounding the infection site, where they can accumulate to high levels [2, 3]. Plants also show a number of responses to wounding, such as cutting or chewing by insect and mammalian herbivores. In many cases, wounding induces the accumulation of metabolites which are highly toxic, suppress feeding or otherwise interfere with the maturation of herbivores [4].

The majority of the phytoalexins that have been described in Solanaceous plants are 15-carbon sesquiterpenoid derivatives [5]. Rishitin, lubimin, phytuberin and solvetivone, as well as several other structurally related sesquiterpenoid phytoalexins (SPs) have been extensively studied as antifungal compounds accumulating in potato (*Solanum tuberosum*) tuber tissue in response to attacks by fungal pathogens. Phytoalexin synthesis can also be triggered in potato tuber slices by compounds termed elicitors, including purified components of fungal cells [6]. Arachidonic acid (AA) is one of the natural lipid components of *Phytophthora infestans*, a potato pathogen which elicits the accumulation of phytoalexins in resistant cultivars

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and may serve as a signal to the plant that a pathogen is present. It has been shown that wounding of potato tubers induces the rapid accumulation of toxic and bitter steroid glycoalkaloids (SGAs), including α-solanine and α-chaconine [7]. The synthesis of SGAs is inhibited by AA treatment and fungal infection, while the synthesis of SPs is not induced by wounding [8–14]. Both SPs and SGAs are terpenoid products of the mevalonate pathway (Fig. 1). The increased synthesis of these compounds following elicitation or wounding should require increased flux through this pathway, but the regulation of the synthesis of these compounds is not fully understood.

The first committed step in the biosynthesis of terpenoids is carried out by 3-hydroxy-3-methylglutaryl CoA reductase (HMGR, EC 1.1.1.34), which catalyses the NADPH-dependent reduction of HMG-CoA to mevalonate (MVA) (Fig. 1). HMGR activity has been detected in a wide variety of plant species [15], and has been studied at the protein and gene level in several of these systems. The regulation of HMGR in plants is very complex, particularly in potato [16–20]. In potato there are at least three classes of HMGR genes which appear to be differentially regulated; one class is strongly induced by wounding, but suppressed by AA treatment, while the other two classes are only slightly induced by wounding and are strongly induced by AA and pathogen infection [14]. Post-translational modifications and compartmentation of isoforms may also play a role in regulating the biosynthesis of the many terenoid products in the cell [17, 20].

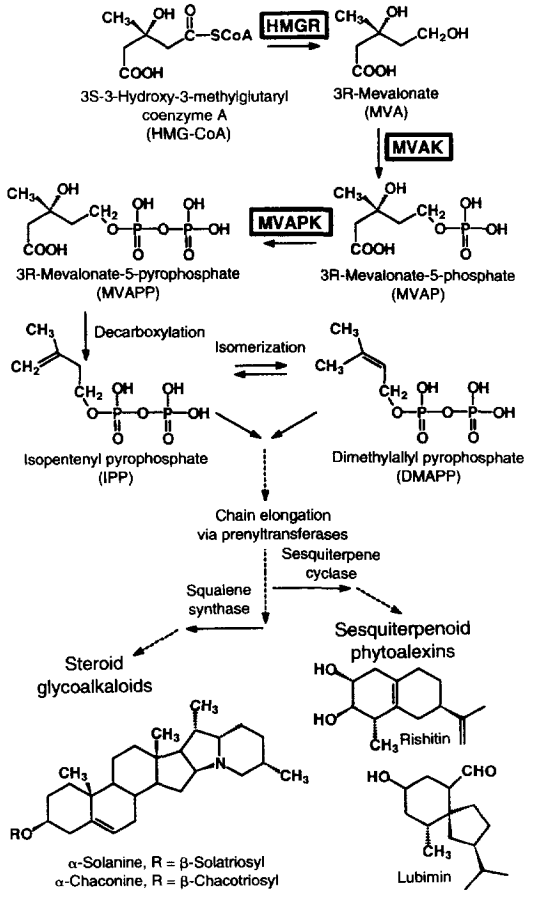

Fig. 1. Early MVA pathway enzymes, leading to the synthesis of SGAs and SPs. Enzymes assayed in this study are abbreviated as HMGR (HMG-CoA reductase), MVAK (mevalonate kinase) and MVAPK (mevalonate phosphate kinase).

Compared to HMGR, very little is known concerning the potential regulatory significance of the enzymes immediately downstream of HMGR in the mevalonate pathway. Mevalonate kinase (MVAK, mevalonate 5-phosphotransferase, EC 2.7.1.36) catalyses the ATP-dependent phosphorylation of *R*-mevalonate to *R*-mevalonate phosphate (MVAP) (Fig. 1). The third enzyme, mevalonate phosphate kinase (MVAPK, or phosphomevalonate kinase, EC 2.7.4.2) phosphorylates MVAP to mevalonate pyrophosphate (MVAPP). MVAK has been assayed in at least 12 plant species [21–25] while MVAPK has been assayed in only a few species [21–23, 26]. Neither kinase has been purified or thoroughly characterized. In three plant species examined, MVAK was inhibited by isoprenoid pyrophosphates such as farnesyl and phytyl pyrophosphate, suggesting that MVAK may be a control point for isoprenoid biosynthesis, regulated by feedback inhibition by late pathway intermediates [27]. Recently, MVAK has been cloned from *Arabidopsis thaliana* by genetic complementation of a yeast mutant [28], but no analysis of MVAK gene transcript levels was reported. Only two studies observing MVAK and/or MVAPK levels in response to external stimuli have been reported; these involved studies on the effects of light and abscisic acid in peanut and gibberellic acid in hazelnut [22, 23]. The response of MVAK and MVAPK to infection or mechanical injury in plants has not been previously reported.

In the process of investigating reports of post-translational modification/inactivation of HMGR by protein phosphorylation [29, 30], we determined that the apparent decrease in HMGR activity was actually due to MVAK activity present in the soluble fraction, which further metabolized the MVA produced by HMGR catalysis during the assay. In the light of these findings, and due to the lack of available information on the regulation of MVAK and MVAPK activity in plants, we examined the levels of HMGR, MVAK and MVAPK following wounding and elicitation, and correlated these results with the production of SGAs and SPs. To facilitate the analysis, we also developed an HPLC assay to quantitate MVAK and MVAPK levels quickly and easily in plant extracts. During the course of our studies, we also found that the published extraction conditions resulted in a severe underestimation of the microsomal HMGR activity in potato tubers; the addition of protease inhibitors greatly improved the quantitative recovery of HMGR activity.

RESULTS AND DISCUSSION

Improved recovery of HMGR using protease inhibitors

The buffers commonly used for extraction of HMGR activity contain no protease inhibitors other than low levels of EDTA and 2-mercaptoethanol. Five protease inhibitors active against different classes of proteases were tested for potential effects on the recovery of HMGR activity from 24 hr AA-treated tissues. Leupeptin and E-64 {*N*-[*N*-(L-3-*trans*-carboxyran-2-carbonyl)-L-leucyl]agmatine}, both cysteine protease inhibitors, produced four- and nine-fold increases, respectively, in the total amount of HMGR activity associated with the microsomal fraction, and greatly increased the ratio of microsomal to soluble HMGR activity (Fig. 2A). A combination of the two inhibitors was only as effective as E-64 alone. PMSF (serine protease inhibitor), aprotinin (serine protease inhibitor) or phosphoroamidon (metalloprotease inhibitor) had little or no effect on the recovery of HMGR activity and still allowed the majority of the HMGR activity to be recovered in the soluble fraction.

Although HMGR is known to be a membrane-associated enzyme [15], without the inclusion of cysteine protease inhibitors the largest portion of HMGR total activity is found in the post-105 000 *g* supernatant, or so-called 'soluble enzyme fraction'. While the specific activity of the supernatant is very low due to its high protein content, and the activity is very dilute, the relatively large volume of this fraction results in a considerable contribution to the total activity. Addition of cysteine protease inhibitors significantly decreased the amount of HMGR activity in the supernatant, while at the same time greatly increased the microsome-associated activity. In mammalian systems it has been shown that the degradation of HMGR *in vivo* can be decreased by the presence of cysteine protease inhibitors [31]. It is thought that proteolytic cleavage may be part of a regulatory mechanism for MVA synthesis and HMGR enzyme turnover [17, 31]. A long linker region is located between the hydrophobic domain, which contains several membrane-spanning domains, and the more hydrophilic catalytic domain at the C-terminal end of the protein in HMGR from both animals and plants. Treatment of mammalian microsomal-associated HMGR preparations with calpain II (a cysteine protease) cleaves this linker region, releasing a catalytically active protein fragment; subcloning and expression of the C-terminal region of the protein also results in a soluble protein with HMGR activity [31]. Evidently, a similar situation may exist in potato extracts, and in the absence of cysteine protease inhibitors, Some microsomal HMGR is solubilized by cleavage from the membrane and retains measurable activity, but the majority of HMGR activity is lost by protease degradation.

In the light of the increased microsomal and total HMGR activity recovered, the cysteine protease inhibitors were included in all subsequent extractions of HMGR activity. Repeated analyses indicated that the inclusion of aprotinin caused only a slight decrease in the total HMGR values, and no negative effect on the specific activity of HMGR in the microsomal fraction (Fig. 2B), and was therefore also included in the enzyme extraction buffer.

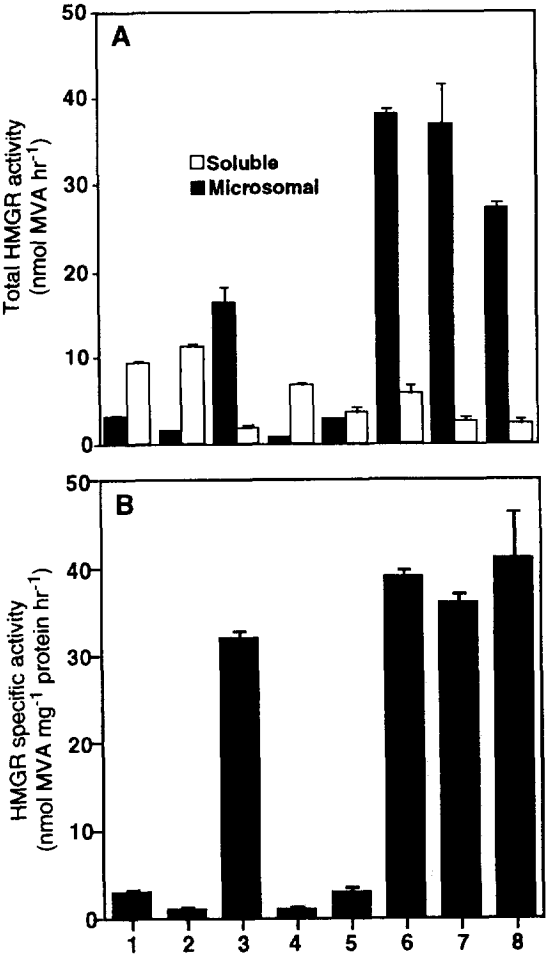

Fig. 2. Recovery of HMGR activity in the presence of protease inhibitors. (A) Total HMGR activity of the microsomal fraction and the post-microsomal supernatant were determined when the following protease inhibitors were included in the extraction and assay buffers: **1**, none; **2**, 1 mM PMSF; **3**, 5 μg ml^{-1} leupeptin; **4**, 5 μg ml^{-1} phosphoramidon; **5**, 2 μg ml^{-1} aprotinin; **6**, 5 μg ml^{-1} E-64; **7**, leupeptin and E-64; **8**, leupeptin, aprotinin and E-64. (B) HMGR specific activity of the microsomal fractions. The extraction of HMGR and activity assays were carried out on 24-hr AA-treated tissue as described in the Experimental. Values are means ± variation from the mean for two determinations.

Detection of MVAK and MVAPK activities

Several lines of evidence suggest that HMGR activity is controlled by post-translational modification of the

HMGR protein. Russell [29] indicated that pea HMGR may be rapidly inactivated by phosphorylation. The inactivation required a cytosolic (soluble) protein fraction, ATP and Mg^{2+}, and HMGR could be reactivated by treatment with bacterial phosphatase [29]. Recently, MacKintosh *et al.* [30] purified a protein kinase from califlower which phosphorylated a peptide sequence from HMGR and rapidly inactivated the enzyme.

We began an investigation to determine if protein phosphorylation played a role in the rapid decrease of HMGR activity several hours after elicitation [9] or in the suppression of HMGR accumulation in the presence of light [29]. Using assay conditions similar to those used in other protein kinase studies, we found that, for AA treated potato tuber microsomal fractions, the presence of Mg^{2+}, ATP and supernatant protein were all required to reduce the apparent activity of HMGR *in vitro* (Fig. 3). Addition of the supernatant protein alone, or addition of Mg^{2+} and ATP alone, had comparatively little effect on the observed HMGR activity. The apparent inhibitory activity of the supernatant protein extracts increased following wounding or AA treatment, although well in advance of the decrease in total HMGR activity (data not shown). In insect and mammalian systems, it has been proposed that MVA may directly or indirectly regulate HMGR levels; addition of MVA to cultured insect cells causes a rapid decrease in HMGR activity [32]. Therefore, we tested whether MVA could increase the inhibitory activity of the supernatants *in vitro* (Fig. 3). However, the addition of 5 mM unlabelled MVA during the incubation of HMGR with Mg^{2+}, ATP and the soluble protein fraction completely abolishes the observed inhibitory effect. The addition of MVA to the standard HMGR assay mixture or to the assays supplement with only soluble protein (no Mg^{2+} or ATP) has no effect on observed HMGR activity. As MVA would not be expected to inhibit a protein kinase, we concluded that HMGR kinase activity was not the responsible agent in the soluble protein (supernatant) fraction. Instead, we speculated that this fraction contained MVAK activity. The addition of Mg^{2+} and ATP could affect the *apparent* activity of HMGR by providing the cofactors for MVAK, the enzyme following HMGR in the biosynthesis of isoprenoids. MVAK would then convert a portion of the pool of radiolabelled MVA produced by HMGR into MVAP, which would most likely remain at the origin in the TLC system used for HMGR analysis. This speculation was supported in two ways. First, the soluble protein fraction was directly assayed for MVAK activity and found to have relatively high activity under the conditions of the HMGR assay (30.7 ± 4.7 nmol MVAP hr^{-1} mg^{-1} $protein^{-1}$), using an ion exchange method [33]; later experiments using highly responsive batches of tubers showed even higher values using an HPLC method (see below). Second, it was confirmed that radiolabelled MVAP standard did remain at the origin in the HMGR assay TLC system. The addition of relatively large amounts of unlabelled MVA protects the labelled HMGR-produced MVA from phosphorylation, allowing the labelled MVA to migrate at the correct R_f in the TLC system. Under these modified assay conditions, we found no evidence for true inhibition or inactivation of HMGR by a protein kinase using these extracts.

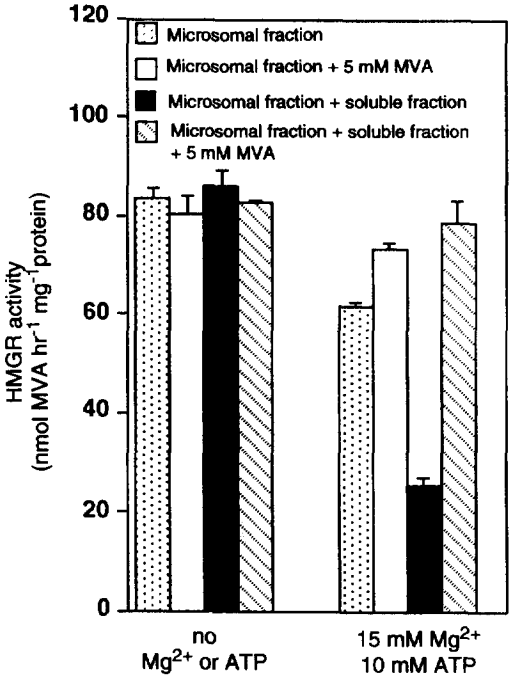

Fig. 3. The effect on apparent HMGR activity of the addition of soluble protein fraction and MVA in combination with Mg^{2+} and ATP. To 50 μl of the standard HMGR 2X assay cocktail were added 25 μl of the microsomal fraction (*ca* 11 μg of protein) from 21-hr treated tuber tissue and 25 μl containing the following combinations of additives: water only, 5 mM MVA (final assay concentration), soluble protein fraction (105 000 *g* supernatant, 10 μl containing 11 μg of protein), or 5 mM MVA plus soluble fraction. The same four combinations were also assayed in the presence of 15 mM Mg^{2+} and 10 mM ATP. The soluble fraction was obtained from tissues 21-hr post-elicitation, the time at which HMGR activity was beginning to decline in this particular time-course. Specific HMGR activity was measured and expressed as described in the Experimental.

Our future investigations of HMGR kinase activity in potato will include tests for possible assay artefacts caused by the presence of MVAK in the samples. Studies in rat liver clearly showed that MVAK could cause interference in HMGR inactivation studies [33], and MVAK might also be present in the pea tissue extracts used in the HMGR phosphorylation studies of Russell [29] and others. However, regulation of HMGR activity by phosphorylation was demonstrated by MacKintosh *et al.* [30] via purification from cauliflower of a protein kinase which phosphorylates and inactivates HMGR from potato and other species. Purification was monitored using a peptide-phosphorylation assay, which is not subject to interference by MVAK. Use of this assay system, in combination with cold MVA-supplemented HMGR assays as described above, may

indicate if phosphorylation of HMGR is even significant in our system. While an HMGR kinase may be an important regulatory element under certain conditions in some species, the rapid decrease in HMGR activity after 24 hr of elicitation in potato may not be due to protein phosphorylation, but rather to protease attack and protein turnover.

There are no previously published reports of possible changes in activities of MVAK or MVAPK (the next enzyme in terpenoid biosynthesis) in response to wounding or fungal infection. We therefore investigated the levels of these two enzyme activities in a wounded and elicited potato tuber system, and compared their levels with HMGR and two classes of end products of mevalonate metabolism. Published methods for assaying MVAK include spectrophotometric determination of ADP produced, monitoring the disappearance of lactonizable MVA, and detection of radioactively labelled MVAP following paper chromatography or ion-exchange column chromatography to separate MVA from MVAP [25]. We chose to use the last strategy, monitoring the incorporation of [^{3}H]-MVA into MVAP in the MVAK assay, and [^{14}C]-MVAP into MVA pyrophosphate in the MVAPK assay. Previous studies have used a wide range of buffers, substrate concentrations and reducing agents for the enzyme reaction. We found that the same levels of DTT and MOPS buffer used for the HMGR extraction were compatible with MVAK and MVAPK assays. The MVAK and MVAPK activities observed were fairly constant in the range 5–15 mM ATP and 10–20 mM $MgCl_2$ (data not shown) and we therefore selected the intermediate concentrations of 10 mM ATP and 15 mM $MgCl_2$ for routine assays. Our assay conditions most closely resemble those described for peanut [23]. For the MVAPK assay, the same level of activity was obtained when either the dibrucine salt or the relatively expensive ammonium salt of unlabelled MVAP was used to dilute the radioactive substrate, and therefore the dibrucine salt was used routinely (data not shown).

To separate and quantitative the radioactive substrates and products, we used an HPLC system utilizing a polymeric reversed phase column and tetrabutylammonium phosphate as an ion-pair reagent. A similar system has been used to separate several mevalonate pathway intermediates in extracts of radiolabelled fruit fly cells [32]. We found it to be less labour-intensive and more reproducible than an open-column ion-exchange method [33], and obtained similar MVAK values with either method. Recently, a similar HPLC method was described for the separation of the pathway intermediates in peppermint leaf extracts; in this method a C-18 silica column was used with tetrabutylammonium sulphate as the ion pair reagent [34].

By reducing the initial acetonitrile concentration and the flow rate through the column relative to previously published conditions [32], we were able to resolve radiolabelled MVA, MVAP, MVAPP and isopentenyl pyrophosphate (IPP) in under 40 min. In MVAK assays, [^{3}H]-MVA was converted into not only [^{3}H]-MVAP but also [^{3}H]-MVAPP and [^{3}H]-IPP. Similar multi-step conversions were reported for extracts of *Nepeta cataria* leaves, where the levels of labelled MVAP, MVAPP and IPP all increased with incubation time [24]. As the synthesis of all three phosphorylated compounds requires the action of MVAK, the total radioactivity from all three product peaks was used to calculate MVAK activity. The duration of the gradient was shortened to decrease the assay time per sample; however, while the resolution between the [^{3}H]-MVA substrate and the products was maintained, MVAPP and IPP were no longer fully resolved from each other. In MVAPK assays, the slope of the gradient was decreased to improve the resolution between the high levels of the radioactive substrate [^{14}C]-MVAP and the products [^{14}C]-MVAPP and [^{14}C]-IPP. The total radioactivity in both the MVAPP and IPP peaks was used to calculate MVAPK activity.

Increases in HMGR, MVAK, MVAPK and related metabolites following wounding and elicitation

The regulation of HMGR activity and gene expression in potato in response to wounding, elicitation and other environmental stimuli has been previously reported [9, 14, 15, 18]. We chose to use sliced tuber discs as a model system for studying wound-induced changes, and to simulate fungal pathogen infection (in combination with wounding) by elicitation with AA.

The activities of HMGR in the wounded and elicited samples were almost identical until 24 hr incubation (Fig. 4A). From 12 to 18 hr, there was a very rapid increase in HMGR activity. After 18 hr, the HMGR activity in elicited tissues continued to increase and remained high until 60 hr, while the activity in wounded tissues decreased and later increased again. The elicited HMGR activity values were two to four times higher than the wounded values from 24 to 48 hr incubation. The general patterns of HMGR activity (including the two peaks of activity in the wounded tissues) were reproducible in a number of independent trials. The overall patterns of increases in HMGR are roughly similar to a previously published time course with much fewer time points [9]. However, the maximal activity observed in our study was 10-fold higher than in the previous study, most likely due to our inclusion of protease inhibitors in the extraction buffer. Also, our study indicates that even at 72 hr post-treatment, the HMGR activity had not yet returned to basal levels, while the previous study had shown a return to pretreatment levels by 48 hr. Again, this may be due to the increased sensitivity of our assay in the presence of added protease inhibitors, or to natural variation between batches of potato tubers.

The activities of both MVAK and MVAPK increased over time following both wounding and elicitation (Fig. 4B and C). However, the differences between kinase levels following the two treatments were much less pronounced than in the case of HMGR. In replicate experiments, the elicited tissues had significantly higher

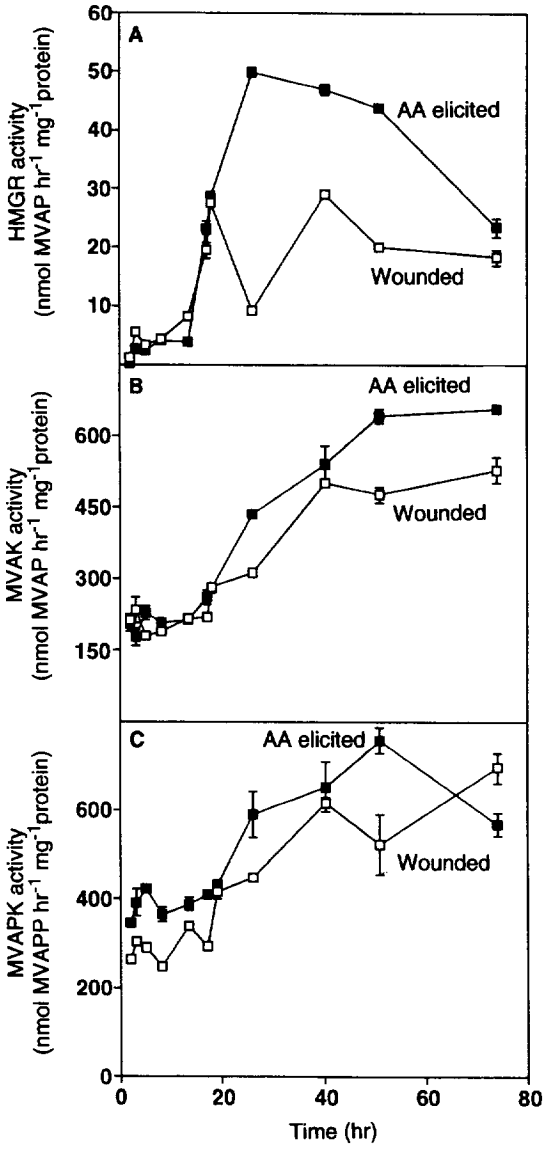

Fig. 4. Increases in (A) HMGR, (B) MVAK and (C) MVAPK specific activity following wounding or wounding plus AA elicitation of potato tubers. Values are the means ± variation from the mean for two determinations.

activity than the wounded tissues at later time points (24–48 hr), but the relative increases were small; the elicited values were only *ca* 20–40% higher than the wounded values. Also, while HMGR levels had increased more than 30-fold in 24 hr following elicitation, MVAK and MVAPK levels only increased approximately four- and two-fold, respectively, in 48 hr. While the absolute specific activity values for HMGR, MVAK and MVAPK varied among batches of tubers, the percentage increase and the timing of the activity changes were very reproducible. The kinases do have much higher apparent basal activities prior to treatment, and therefore a larger increase in activity may not be required to ensure sufficient flux through the MVA pathway.

To compare changes in metabolite levels with enzyme levels, samples were analysed for two classes of terpenoid end products. The accumulation of SGAs has been reported to be increased following wounding, but severely decreased following elicitation [7, 8]. TLC analysis indicated that in our wounded tissues the SGAs α-solanine, solanidine and α-chaconine increased from undetectable levels to easily detectable levels in wounded tissues, while no SGAs were detected in elicited tissues (Fig. 5A). Conversely, the accumulation of SPs has been reported to follow elicitation by fungi, fungal components or treatment with certain abiotic elicitors, but not wounding alone [9, 12]. TLC analysis confirmed that the SPs rishitin and lubimin rose from undetectable to high levels in our arachidonic acid-elicited tuber slices, but were not detected in the wounded tissues (Fig. 5B). The increases in both SPs and SGAs occurred after the first significant activity increases in HMGR and the kinases were observed (Fig. 4).

Although the secondary metabolite profiles of the wounded and elicited tissues are very different, in both cases the metabolites are terpenoid-derived products, and their syntheses require the presence of the early MVA pathway enzymes. Basal levels of these enzymes are required in unstressed plant cells for the synthesis of primary metabolites (e.g. ubiquinone, carotenoids, hormones, prenyl sidechains of proteins and tRNAs) [17, 20]. The rapid increased synthesis of high levels of

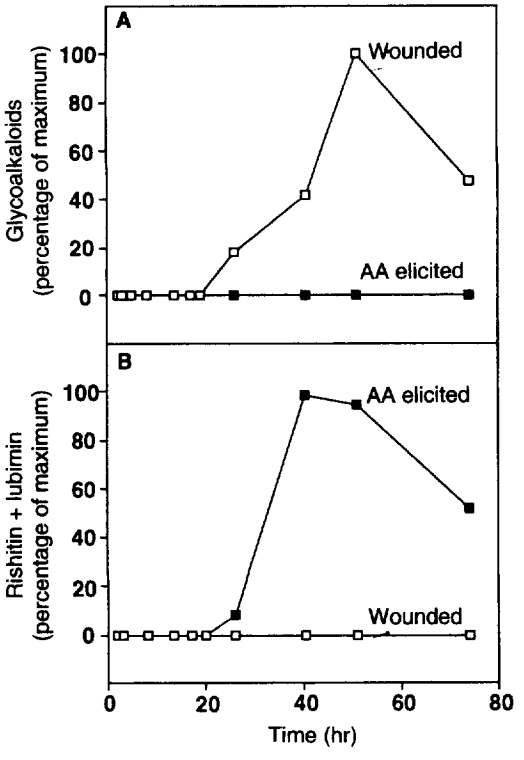

Fig. 5. Relative levels of total (A) SGAs and (B) SPs rishitin and lubimin following wounding or wounding plus AA elicitation of potato tubers. Levels are expressed as a percentage of the maximum observed for either glycoalkaloids or phytoalexins during the experiment.

stress-induced terpenoids such as SGAs and SPs apparently require the increased levels of HMGR, MVAK and MVAPK over these basal levels.

The higher fold induction of HMGR is consistent with its proposed role as the rate-limiting enzyme in terpenoid biosynthesis [12, 17]; once HMG-CoA is reduced, the carbon and energy invested in MVA is committed to terpenoid biosynthesis, and there is no need for another control step until the pathway reaches another branch point. Obviously, the total extractable levels of HMGR, MVAK and MVAPK do not determine the final class of terpenoids accumulating; this is determined by the relative activities of later branch-point enzymes such as sesquiterpene cyclase and squalene synthase [10–12].

EXPERIMENTAL

Chemicals. *R,S*-3-[Glutaryl-3-^{14}C]-hydroxy-3-methylglutaryl CoA and *R*-[5-^{3}H]-MVA (NH_4 salt) were purchased from New England Nuclear (Boston, MA). Unlabelled *RS*-MVA-5-phosphate (NH_4 salt), *RS*-[2-^{14}C]-MVA-5-phosphate and *RS*-[5-^{3}H]-MVA pyrophosphate were purchased from American Radiolabeled Chemicals, St. Louis, MO. Unlabelled *RS*-MVA-5-phosphate (dibrucine salt) was purchased from Research Plus, Bayonne, NJ. Silica gel (Si250-19C) TLC plates were obtained from Baker (Phillipsburg, NJ). All other chemicals were from Sigma.

Preparation of plant material. Certified seed potatoes (*S. tuberosum* L. cv. Kennebec) were stored at 4° for 1 month or more until 24 hr prior to use when they were allowed to warm to room temp. Manipulation of tubers and prepn of tuber discs were as previously described [9]. In summary, tubers were washed and surface sterilized, and discs (22 × 5–7 mm) were cut from the central parenchymatous tissue with a sterile cork borer. The discs were washed with sterile distilled H_2O and placed in a petri dish. The prepn of tuber tissue was done with only a green safelight for illumination to prevent inhibition of HMGR activity by white light [9]. The tuber discs were placed in a single layer in petri dishes. To the upper surface of the discs was applied either 100 μl of sterile distilled H_2O or 100 μl 1.64 mM AA. After a given period of incubation in a dark humidified chamber, 1 mm slices of the upper surface of several discs were harvested, pooled, frozen in liquid N_2 and stored at −70°. One portion of the pooled sample was used for prepn of enzymes extracts (HMGR, MVAK and MVAPK) and a second portion was used for quantitation of SGAs and SPs.

Preparation of enzyme extracts. HMGR, an integral membrane protein, was assayed in the microsomal fr. prepd from treated tuber tissue [9], except where indicated. Tissue from 5 discs (*ca* 1.7 g frozen tissue) was powdered in a prechilled mortar and mixed with 8 ml ice-cold extraction buffer (0.2 M MOPS, pH 7.5, 10 mM EDTA, 0.35 M sorbitol, 20 mM 2-mercaptoethanol, 5% PVPP). Protease inhibitors were routinely added to the extraction and resuspension buffers at the following concns: 5 μg ml^{-1} leupeptin, 5 μg ml^{-1} E-64 and 2 μg ml^{-1} aprotinin, unless stated otherwise. The homogenate was filtered through miracloth (CalBiochem, La Jolla, CA). The filtrate was centrifuged first at 1200 *g* for 5 min to remove insoluble PVPP and cell debris. The supernatant was then further clarified by centrifugation at 16 000 *g* for 40 min. This supernatant was then centrifuged at 105 000 *g* for 2 hr. The 105 000 *g* pellet (microsomal fr.) was resuspended in 400 μl freshly prepd resuspension buffer containing 50 mM MOPS (pH 7.5), 30 mM DTT and the protease inhibitors. The 105 000 *g* supernatant (soluble enzyme fr.) was used for MVAK and MVAPK assays following concn and spin dialysis to dilute EDTA and other components of the extraction buffer. To achieve this, supernatant (1.5 ml) was diluted 10-fold with resuspension buffer (13.5 ml) and centrifuged in a Centriprep-10 (10 000 MW cut-off; Amicon, Beverly, MA) at 2000 *g*, 4°, for 1–2 hr to reduce vol. to *ca* 0.5 ml. After addition of resuspension buffer, enzyme prepns were allowed exposure to white light. The resuspended frs were stored at −70°. Protein content was estimated by the Bio-Rad dye-binding assay following the manufacturer's directions with BSA as standard.

Assay of HMGR. HMGR activity was determined by a radiochemical assay modified from ref. [29]. The assay reaction was started by adding 50 μl assay cocktail to 50 μl microsomal enzyme prepn (20–50 μg protein). The combined mixt. contained 50 mM MOPS (pH 7.5), 1 mg ml^{-1} BSA, 25 mM DTT, 12.7 nmol *R,S*-3-[glutaryl-3-^{14}C]-hydroxy-3-methylglutaryl CoA (62.5 nCi) and 2.5 mM NADPH. [Previously published assay conditions used 0.3 mM NADP and a NADPH regenerating system. We found that 2.5 mM NADPH alone gave maximal enzyme activity and prevented inhibition by ATP addition (data not shown).] Compounds to be added to the HMGR reactions were also made up in 50 mM MOPS (pH 7.5). After 40 min incubation at 30° the reaction was stopped by adding 10 μl 6 M HCl. The reaction mixt. was incubated at room temp. for at least 30 additional min to ensure complete lactonization of the MVA. The mixt. was then centrifuged for 3 min at full speed in a microfuge, and a 30 μl aliquot of the supernatant was applied to a thin-layer plate of activated silica gel. The plate was developed with $CHCl_3$–Me_2CO (2:1). The region of plate corresponding to a mevalonolactone standard was removed and the amount of ^{14}C was determined by liquid scintillation counting.

Assay of MVAK. A stock of radioactive MVA was prepd by mixing a soln of based-treated (delactonized) *R,S*-MVA lactone (2 μmol in 20 μl) with a soln of *R*-[5-^{3}H]-MVA (0.01 mCi in 0.3 nmol in 20 μl); only the *R*-isomer is known to be utilized in the reaction. The assay reaction was started by adding 50 μl assay cocktail to 50 μl soluble enzyme fr. (100–200 μg protein). The final reaction mixt. contained 50 mM MOPS (pH 7.5), 10 mM ATP, 15 mM $MgCl_2$, 15 mM DTT, 0.5 mM *R*-[5-^{3}H]-MVA (0.125 μCi). The mixt. was incubated at 37° for 15 min, and the reaction was

terminated by addition of 1 vol. of a soln of 0.28 M NH_4OH and 60% EtOH in H_2O. Samples were kept frozen at −70° until analysed by HPLC.

The reaction mixt. was fractionated using reversed phase ion pair HPLC, using conditions similar to those used for fractionating phosphorylated isoprenoid intermediates from insect cells [32]. The samples were thawed, vortexed and centrifuged (12 500 *g* for 15 min). An aliquot (100 μl) was injected on to a Hamilton PRP-1 column (5 μm poly(styrene–divinylbenzene) packing, 4.1 × 150 mm bed) equilibrated with 90% 10 mM tetrabutylammonium phosphate (pH 7.5) and 10% CH_3CN at a flow rate of 1 ml min^{-1}. Upon injection, a 23 min linear gradient from 10–45% CH_3CN was initiated. Frs (1 ml) were collected for 25 min and radioactive compounds were detected by liquid scintillation counting. The column was calibrated with the appropriate radiolabelled standards. MVA generally eluted in frs 2–5, MVAP in frs 9–13, and MVAPP (and IPP) in frs 16–19. The column was washed with 95% CH_3CN for 5 min after each sample and re-equilibrated for 10 min prior to next injection.

Assay of MVA-5-phosphate kinase. A stock of radioactive MVA was prepd by mixing a soln of *R,S*-MVA-5-phosphate (dibrucine salt) (2 μmol in 400 μl) with a soln of *R*-[5-^{3}H]-MVA-5-phosphate (1 μCi in 18 nmol in 20 μl); only the *R*-isomer is known to be utilized in the reaction. The assay reaction was started by adding 50 μl assay cocktail to 50 μl soluble enzyme fr. (100–200 μg protein). The final reaction mixt. contained 50 mM MOPS (pH 7.5), 10 mM ATP, 15 mM $MgCl_2$, 15 mM DTT, 0.5 mM *R*-[2-^{14}C]-MVA-5-phosphate (0.0125 μCi). The reaction mixt. was incubated at 37° for 15 min, and the reaction was terminated by addition of 1 vol. of 0.28 M NH_4OH in 60% EtOH. The reaction mixts were processed as described above except that a longer HPLC gradient was necessary to resolve fully the high levels of radioactive substrate (MVAP) from the product (MVAPP). The duration of gradient was changed to 35 min, and 1 ml frs were collected from 7 to 30 min after injection and analysed by scintillation counting. MVAP generally eluted in frs 10–15 min post-injection, and MVAPP together with IPP eluted in frs 19–25 min post-injection.

Estimation of potato phytoalexins and glycoalkaloids. Tissue from AA- or H_2O-treated potato discs was homogenized with $CHCl_3$–HOAc–MeOH (10:1:9) at a ratio of 2 g frozen tissue to 10 ml solvent mixt. After extracting overnight the homogenate was filtered and the filtrate evapd to dryness with a stream of N_2. The residue was resuspended in a mixt. of $CHCl_3$ and 0.2 M HOAc (1 ml each). The 2 layers were sepd and the $CHCl_3$ layer, which contained the SPs [7], was evapd to dryness under flowing N_2. Care was taken to remove tubes from the dryer as soon as solvent was evapd to avoid possible loss of the semi-volatile SPs. The extract was resuspended in 60 μl MeOH. A 40 μl aliquot was applied to a silica gel TLC plate. The plate was developed with EtOH–cyclohexane (1:1). The sepd compounds were located by spraying the plate with vanillin–H_2SO_4. The R_fs of rishitin and lubimin are 0.20 and 0.30, producing blue and blue–violet spots, respectively [35]. The identity of rishitin was also further indicated by spotting the authentic compound on the TLC plates. The HOAc phase (containing SGAs) was concd to 1/10 of original vol. and brought to pH 10 with conc. NH_4OH. The soln was heated at 80° in a water bath for 30 min and cooled at 4° for at least 3 hr. The soln was centrifuged at 15 000 *g* for 30 min and the pptd SGAs were washed with 2% NH_4OH, dried and dissolved in 50 μl MeOH–HOAc (19:1). An aliquot was applied to a silica gel TLC plate which was developed in the organic layer of $CHCl_3$–95% EtOH–1% NH_4OH (2:2:1) [36]. Commercial standards of α-solanine, solanidine and α-chaconine (min. detectable quantity <5 μg) were run for comparison. The glycoalkaloids were localized by spraying the plates with Carr–Price reagent (a mixt. of Sb in $CHCl_3$) [37].

For both SPs and SGAs, relative quantitations metabolites were performed by capturing a digitized image of the freshly stained TLC plate using an Elmo EV-308 visual presenter (Elmo Co., Japan) and integrating the area and intensity of the appropriate spots using JAVA Jandel Video Analysis Software (Jandel Scientific, Corte Madera, CA).

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