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ACCUMULATION OF THE PHYTOALEXIN LETTUCENIN A AND CHANGES IN 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE ACTIVITY IN LETTUCE SEEDLINGS WITH THE RED SPOT DISORDER

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Abstract—The sesquiterpenoid lactone phytoalexin lettucenin A was isolated from seedlings of lettuce with the red spot physiological disorder. Accumulation of lettucenin A was preceded by increased activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase. The red spot syndrome may provide a tractable mimic of the hypersensitive reaction of lettuce to microbial challenge.

INTRODUCTION

Red spot is a physiological disorder of lettuce (*Lactuca sativa* L). which develops in seedlings produced from aged seed. Shoots grow in a distorted manner and cotyledons develop a characteristic reddening (red spot) close to their petioles [1]. The development of red spot has been associated with increases in lipid peroxidation in the cotyledon and embryo during prolonged storage of seed [2]. The characteristic red spot symptom is visually similar to the appearance of restricted lesions produced in cotyledons undergoing localized resistant reactions to attempted colonization by the fungal pathogens *Botrytis cinerea* or *Bremia lactucae* [3]. Lesion mimics such as red spot have recently received increasing attention as model systems for the examination of signal transduction leading to the hypersensitive reaction [4, 5].

In a search for phytoalexins in lettuce, TLC plate bioassays of extracts of cotyledons with red spot symptoms or challenged by *B. cinerea*, revealed the similar accumulation of one predominant band of inhibition of the test fungus *Cladosporium herbarum* [6]. Seedlings with the red spot disorder were used as a source of the inhibitor which was identified as the sesquiterpenoid lactone lettucenin A initially characterized by Takasugi *et al.* [7] from lettuce infected with *Pseudomonas cichorii*. In this paper we describe the analysis of changes in concentrations of the phytoalexin in seedlings with red spot symptoms. Accumulation of the sesquiterpenoid lactone was found to be preceded by increased activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC.1.1.1.34) a key enzyme regulating the flux of intermediates through the isoprenoid pathway [8].

RESULTS AND DISCUSSION

Changes in lettucenin A concentrations during development of the red spot syndrome are given in Fig. 1. Accumulation of lettucenin A was associated with the appearance of discoloration in affected cotyledons. The localization of lettucenin A within seedlings was examined 7 days after sowing. The major accumulation of the phytoalexin was within distorted cotyledons with the red spot symptom (6.2 $\mu g\,g^{-1}$ fr. wt). Lettucenin A was found in trace amounts ($< 0.01\ \mu g\,g^{-1}$ fr. wt) in the roots but was not detected in hypocotyls.

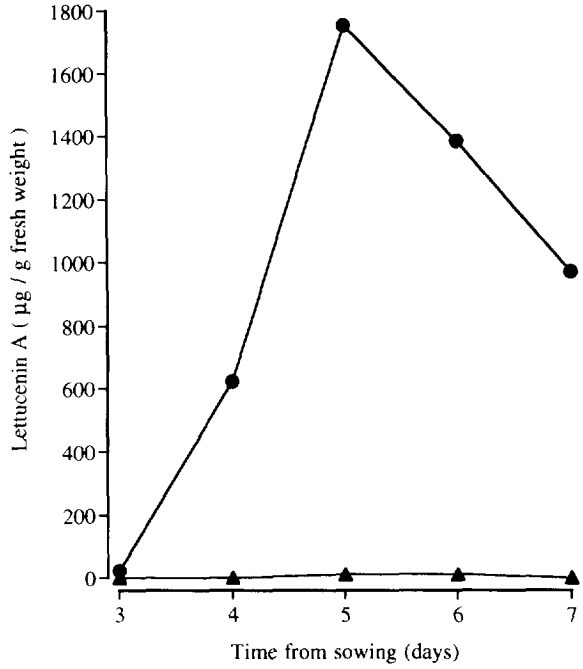

Fig. 1. Accumulation of lettucenin A in lettuce seedlings with red spot (●) or in healthy controls (▲). Data are the means from two samples.

The enzyme HMGR catalyses conversion of 3-hydroxy-3-methylglutaryl co-enzyme A to mevalonic acid — the first committed step in the pathway leading to isoprenoids such as lettucenin A [8, 9]. Seedlings with red spot provided a potential source for the characterization of HMGR in lettuce. Enzyme activity was detected in microsomal (P120) and plastidic (P20) preparations with higher levels in the P120 than P20 fractions. Seedlings developing red spot symptoms showed elevated HMGR activity compared with healthy controls during the early stages of growth following germination. The high activity recorded in Fig. 2 preceded the accumulation of lettucenin A.

Defence related induction of HMGR activity has been demonstrated in members of the Convolvulaceae and Solanaceae producing sesquiterpenoid phytoalexins, e.g. in sweet potato roots [10], potato tubers [11, 12] and tomato cell cultures [11, 13]. In each of these studies either fungal infection or an abiotic elicitor was used to induce both phytoalexin accumulation and HMGR synthesis. In lettuce (a member of the Compositae) it is clear that an exogenous elicitor is not required to induce lettucenin A accumulation. The link between the metabolic lesion causing red spot and phytoalexin biosynthesis remains unknown. The association of lipid peroxidation with the physiological disorder suggests analogy with the hypersensitive reaction (HR) [2, 6, 14, 15]. Possibly, lipid peroxidation leading to irreversible membrane damage may occur in isolated cells during the development of red spot. Isoprenoid biosynthesis may then be activated in surrounding undamaged cells by endogenous elicitors released following decompartmentation [6, 17]. Analysis of mechanisms of disease resistance in lettuce has largely been confined to physiological studies [3, 6, 14]. Characterization of the phytoalexin lettucenin A and HMGR reported here provide biochemical markers for the molecular dissection of the HR and other stress responses in lettuce [18].

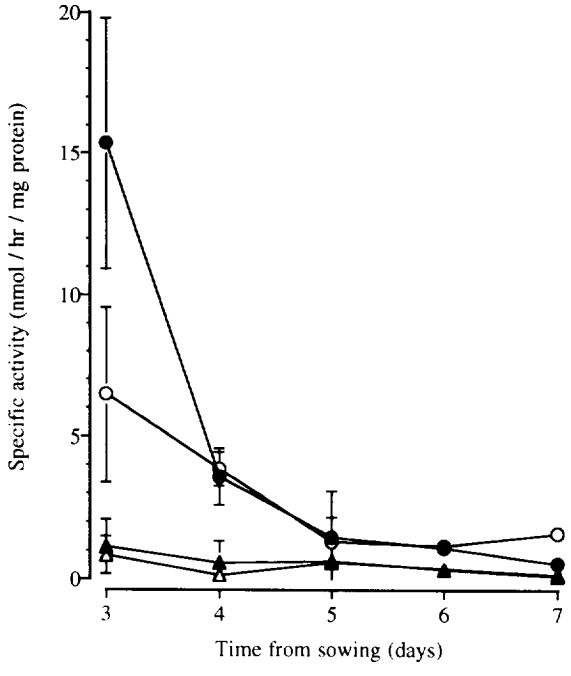

Fig. 2. Activity of HMGR in lettuce with red spot (circles) or in healthy controls (triangles); closed and open symbols represent P120 and P20 fractions respectively. Data are means ± s.e.m. from four samples.

EXPERIMENTAL

Plant material. Lettuce seeds (*Lactuca sativa* cv. *Diana*) which had been stored at 4° for more than 1 year, were sown in deep seed trays on 3MM filter paper moistened with tap water. They were grown in a 16 hr day length and a constant temperature of 24°. The seedlings were harvested daily after the first signs of germination 3 days after sowing. The sample of stored seed used in these experiments produced more than 80% of seedlings with the red spot disorder.

HMGR assay. The following method was adapted from ref. [19]. Whole seedlings (*ca* 15 g at each harvest) were ground in a pre-cooled pestle and mortar containing sand and 3 vols of homogenizing buffer (0.33 M sorbitol; 100 mM Tris–HCl, pH 7.6; 30 mM EDTA; 1% PVPP and 15 mM β-mercaptoethanol, added freshly). The homogenate was squeezed through two layers of Miracloth (Calbiochem) and the filtrate spun at 2500 *g* for 5 min. The pellet was discarded and the supernatant centrifuged at 20 000 *g* for 25 min to pellet the plastids (P20). The supernatant was spun again at 120 000 *g* for 75 min and the resultant pellet containing microsomes (P120) was retained. Both pellets were resuspended in a 100 mM phosphate buffer (pH 7.2) with 50 mM dithiotreitol (DTT) and 2% polyoxyethylene ether (Brij) W-1. Protein levels in the final extracts were determined using a detergent compatible protein assay kit obtained from Bio-Rad.

Enzyme extract (containing 100 μg protein, always in less than 15 μl) was incubated in 25 μl buffer containing 200 mM phosphate (pH 7.2), 20 mM DTT and 80 μM HMG-CoA (Sigma), 2.5 μl [3-^{14}C]-HMG-CoA (1.85 kBq, 1.8–2.2 TBq mol^{-1}; New England Nuclear). The volume was made up to 40 μl with H_2O and the mixture allowed to equilibriate at 37° for 2 min before addition of 10 μl of 10 mM $NADPH_2$ (Sigma) to initiate the reaction. The mixture was incubated in a shaking water bath at 37° for 30 min and the reaction stopped by addition of 10 μl 6 M HCl, followed by 10 μl of [^{3}H]-mevalonate (0.37 kBq, 96.3 MBq mol^{-1}, Amersham) and the mixture allowed to lactonize for 30 min. Following lactonization the solution was streaked on to silica gel TLC plates (250 μm thick 20 × 20 cm; Merck, BDH), the plates were air dried and developed in Me_2CO-toluene (1:1). The plates were visualized using iodine and the band corresponding to mevalonate ($R_f \approx 0.5$) scraped into scintillation vials and counted using Ecolite™ (ICN Flow) as scintillant. Activity was expressed as nmol mevalonate produced hr^{-1} mg^{-1} protein.

Phytoalexin accumulation. Lettucenin A concentrations were determined after HPLC using fluorescence detection as previously described [6]. Samples of 20 seedlings were homogenized in 70% MeOH (3 μl mg^{-1} fr. wt). The homogenate was microfuged for 10 min (14 000 *g*) and the pellet re-extracted with 70% MeOH (2 μl mg^{-1} fr. wt). Supernatants were combined, filtered (0.45 μm) and analysed by reversed phase HPLC [6]. Duplicate samples were taken at each time point. Seedlings with severe symptoms 7 days after germination were dissected to localize lettucenin A accumulation; at least 100 μg each of cotyledon, root and hypocotyl tissue were collected for analysis.

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