Involvement of Oxidative Burst in Isoflavonoid Metabolism in Elicited Cell Suspension Cultures of *Pueraria lobata*

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Hydrogenperoxide exogenously administerd to the suspension-cultured cells of *Pueraria lobata* stimulated a rapid disappearance of the constitutive isoflavonoids (malonylglucosides of daidzein and genistein) from the methanol soluble fraction of the cells, in a similar manner as observed in the cells treated with yeast extract or glycoprotein preparation of *Phytophthora megasperma* f. sp. *glycinea.* Rapid generation of H_2O_2 was detected within 10 min after elicitation at the cell surface, presumably preceded by the activation of plasma membrane NAD(P)H-oxidases. Pretreatment of some representative NAD(P)H-oxidase inhibitors such as SHAM, DEDTC and KCN (1 mM each) to the cells 1 h before elicitation resulted in almost complete inhibition of the elicitor-stimulated metabolism of isoflavonoids for 4 h, whereas treatment of the inhibitors at 1 h after elicitation showed no significant effect on the rapid isoflavonoid metabolism. These findings suggest that this type of response to elicitation could be regarded as a plausible initial defense strategy prior to the appearance of *de novo* synthesized defensive barriers such as phytoalexins and lignin in plant-pathogen interaction.

Introduction

The cell suspension cultures of *Pueraria lobata* (Leguminosae) and elicitors have been employed as a suitable model system for studies of isoflavonoid biosynthesis (Hakamatsuka *et al.,* 1990, 1991a, 1991b; Hashim *et al.,* 1990). Recently we have reported that major isoflavonoids exist constitutively as their 7-*O*-glucosides-6″-*O*-malonylesters in the cells (Park *et al.,* 1992), and elicitation of the cells with commercial yeast extract or glycoprotein preparation of *P. megasperma* f. sp. *glycinea (Pmg)* cell wall gave rise to a rapid disappearance of the pre-existing isoflavonoids within the first 4 h, followed by reaccumulation of the conjugates. No turnover product could be detected in the methanol soluble fraction during 4 h after elicitation, and the metabolic fate of the isoflavonoids lost by elicitation was shown to be associated closely with non-extractable lignocellulose fraction of the plant cell walls (Park *et al.,* 1995). On the other hand, elicitation of *P. lobata* cells with yeast extract also stimulated the formation of daidzein-dimers which are possible by-products of peroxidase(s) induced for lignin biosynthesis (Hakamatsuka *et al.,* 1992). Both of the isoflavone dimers and lignin can be synthesized *via* phenoxy radicals mediated by the action of peroxidases and H_2O_2. From the above observations, we could expect that certain oxidative reactions together with *de novo* synthesis of phenylpropanoids were induced or activated in elicited *P. lobata* cells. Elicitation led to a rapid stimulation of an oxidative burst in *Glycine max* cell suspension cultures (Apostol *et al.,* 1989). Rapid generation of H_2O_2 at cellular surface of elicited cells was postulated to be responsible for the production of pterocarpan phytoalexin glyceollins. In elicited *Trifolium repens* and *Nicotiana tabacum* cells, rapid oxidative burst consisting of H_2O_2 and superoxide anion has also been reported, but it may not be a necessary element of the signaling system for HR (hypersensitive reaction) and phytoalexin formation (Devlin and Gustine, 1992). These recent findings suggest the common involvement of oxidative burst in plant cells in response to elicitation. In *P. lobata* cells the elicitor-induced metabolism of

Abbreviations: SHAM, salicylhydroxamic acid; DEDTC, diethyldithiocarbamate; HRP, horseradish peroxidase; SOD, superoxide dismutase.

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isoflavonoids, in particular, rapid association of the constitutive isoflavone malonylglucosides with cell wall lignocellulose, may be a plausible defense response appearing at the early stage prior to *de novo* synthesis of lignin and pterocarpan phytoalexin tuberosin. But there was no information on a cause affecting the rapid metabolism of isoflavonoids, which prompted us to elucidate the cellular events started by elicitation. We report here that exogenously added H_2O_2 triggers a similar metabolism of the constitutive isoflavonoids as observed in the cells treated with yeast extract or *Pmg* glycoprotein, and also demonstrate that a rapid generation of H_2O_2 at the cell surface is closely involved in the subsequent disappearance of the isoflavonoids in elicited *P. lobata* cells.

Materials and Methods

Cell cultures

Cell suspension cultures of *Pueraria lobata* Ohwi were maintained at 28 in the dark in MS liquid medium containing 2,4-D (2 ppm), kinetin (0.1 ppm) and sucrose (3%) as described earlier (Takeya and Itokawa, 1982). Cells were transferred to fresh medium for subculture with 14 to 15 days intervals.

Elicitation of cells

Commercial yeast extract (Difco) used as an elicitor was added to the suspension-cultured cells as previously described (Park *et al.,* 1995; Hakamatsuka *et al.,* 1992). Five day old cell suspensions of early growth phase (150 ml medium in 500 ml Erlenmeyer flask) were combined in an autoclaved 2 L flask just before elicitation and they were subdivided into 150 ml in 500 ml flask for increasing the cell homogeneity. Yeast extract (150 mg), dissolved in 2 ml sterile water, was aseptically added to the cells for elicitation. The control cells were prepared by applying an equal volume of sterile water without yeast extract.

Application of exogenous H_2O_2

H_2O_2 (0.5 mM or 1.0 mM, final concentration) diluted in sterile water was individually applied to the cell cultures (150 ml) on day 5 after subculture. The cells (1 g fr. wt) were harvested by filtration at appropriate time, extracted with methanol (10 ml) for 30 min under ultrasonication, and an aliquot (10–20 μl) was subjected to HPLC for quantitation of isoflavonoids. The detailed condition of HPLC analysis is described previously (Park *et al.,* 1992). To evaluate H_2O_2 dose-dependent effect on isoflavonoid metabolism, freshly harvested cells (1 g fr. wt) were aseptically transferred to an autoclaved test tube containing 10 ml of conditioned liquid medium. To this, 1–2 mM of H_2O_2 was individually applied and the cells were incubated at 28 °C for 30 min, with reciprocal shaking (200 rpm). The cells were harvested and extracted with methanol for quantitation of isoflavonoids by HPLC as mentioned above.

Calibration curve for H_2O_2 quantitation

Varied amounts of H_2O_2 (0–50 mM, final concentration) were individually applied to the assay solution containing 10 μg of N,N-dimethyl-*p*-phenylene-diamine and 2 μg horseradish peroxidase (HRP) (Sigma P-8000) in 5 ml of 0.1M Na-acetate buffer (pH 5.0). The mixture was vortexed, kept at r.t. for 5 min, and then its absorbance at 515 nm was measured on Hitachi U-2000 spectrophotometer.

Detection of extracellular H_2O_2

The assay solution was prepared by adding N,N-dimethyl-*p*-phenylenediamine and HRP to 0.1 M Na-acetate buffer (pH 5.0) as described above. The cells elicited with yeast extract, or control cells which were added with an equal volume of sterile water, were harvested by filtration at appropriate time, washed with distilled H_2O, and immediately used for detection of H_2O_2. To the cells (1 g fr. wt) was added 5 ml of the assay solution and the mixture was kept at r.t. for 5 min. The aliquot (3 ml) was passed through a membrane filter (Acrodisc, 0.22 μm) and the filtrate was directly measured for its absorbance at 515 nm, and H_2O_2 was quantitated using the calibration curve.

Experiments of NAD(P)H-oxidase inhibitors

Various concentrations of NAD(P)H-oxidase inhibitors, namely, salicylhydroxamic acid (SHAM), diethyldithiocarbamate (DEDTC), KCN and $K_4[Fe(CN)_6]$, dissolved in distilled H_2O and filter-sterilized, were added to the cultured cells 1 h be-

fore elicitation. Control cells were added with an equal volume of sterile water. After incubation for 4 h, changes in the cellular isoflavonoid contents were monitored by HPLC. To investigate the effect of timing of the inhibitor treatment, 1 mM of each inhibitor was applied to the cultures 1 h before or 1 h after elicitation. Catalase (1.0×10^5 units, Sigma C-10) and superoxide dismutase (1.5×10^4 units, Sigma S-2139) dissolved in 1 ml of 0.1 M K-phosphate buffer (pH 6.0) and filter-sterilized, were added to the cells (150 ml) 1 h before elicitation by yeast extract. In parallel experiments, the enzymes were individually added to the cell culture, and the cells were incubated for 4 h without elicitation. For control, the cells were administered with an equal volume of buffer or yeast extract (1 mg ml^{-1}) without pretreatment of the enzymes. Their inhibitory effects against elicitor-stimulated metabolism of the constitutive isoflavonoids were evaluated by monitoring changes of the cellular isoflavonoid contents after additional incubation for 4 h.

Results

Effect of exogenously added H_2O_2 on isoflavonoid metabolism

We have attempted to investigate the effect of H_2O_2 on isoflavonoid metabolism in *P. lobata* cells. Direct administration of H_2O_2 (0.5–1 mM) to the cells led to a rapid decrease of the constitutive malonylglucosides of isoflavones within 4 h in a similar time course as observed in the cells treated with yeast extract (1 mg ml^{-1}) (Fig. 1). The cells applied with a glycoprotein preparation (40 μg ml^{-1}) of *Pmg* were also reported to show the similar response (Park *et al.*, 1995). As has been found in yeast extract- or *Pmg* glycoprotein-elicited cells, no alternative turnover product could be detected either in the medium or methanol soluble fraction of the cells during the same time period (data not shown). It suggests that the disappeared isoflavonoids are possibly associated with insoluble cell wall component(s) as reported previously (Park *et al.*, 1995).

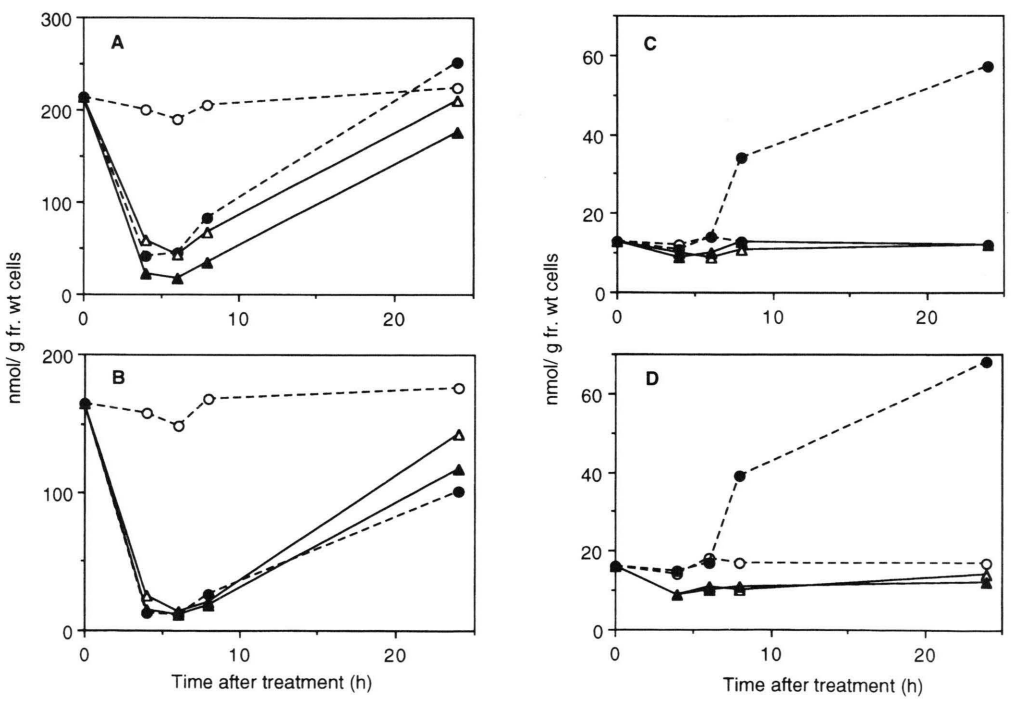

Fig. 1. Effect of exogenous H_2O_2 on metabolism of isoflavanoids. A, daidzein-malonylglucoside; B, genistein-malonylglucoside, C, daidzein; D, genistein: ○, control cells; ●, cells treated with yeast extract (1 mg ml^{-1}); cells treated with H_2O_2 (△, 0.5 mM and ▲, 1.0 mM).

After 6 h of H_2O_2 addition, the isoflavone malonylglucosides began to reaccumulate in the cells, however, no accumulation of isoflavone aglycones such as daidzein and genistein was observed during 24 h. Neither daidzein-dimers nor pterocarpan tuberosin was produced for 24 h after addition of H_2O_2 (data not shown), in contrast to those observed in the cells treated with yeast extract or *Pmg* elicitor. The fact that H_2O_2 could not stimulate the production of either isoflavones nor pterocarpan tuberosin indicates the active oxygen may not be directly linked to a signal transduction for phytoalexin synthesis in *P. lobata* cells.

To evaluate dose-dependent effect of H_2O_2 on the rapid disappearance of isoflavone malonylglucosides, various concentrations of H_2O_2 were added to the cells and the changes in the amount of isoflavonoids were monitored by HPLC. Table I shows that the constitutive isoflavone malonylglucosides disappeared from the methanol extract in a dose-dependent manner. Treatment with excess (≧ 2 mM) amount of H_2O_2 resulted in almost complete disappearance of the isoflavonoids.

Detection of H_2O_2 in elicited cells

Since exogenously added H_2O_2 stimulated a rapid decline in the amount of the constitutive isoflavonoids in *P. lobata* cells, we investigated whether the generation of H_2O_2 in elicited cells is indeed involved in the elicitor-induced metabolism of isoflavonoids. N,N-Dimethyl-*p*-phenylenediamine was used for detection of H_2O_2. The compound is oxidized by H_2O_2 under the catalysis of HRP to give a stable red radical (λ_{max} 515 nm), which enabled the precise quantitation of extracellular H_2O_2 in *Solanum tuberosum* (Murai *et al.*, 1995). The suspension-cultured cells of *P. lobata* on day 5 of early growth phase were elicited with yeast extract, harvested at appropriate time, and the cells (1 g fr. wt) were immediately used for quantitation of H_2O_2. It revealed that elicitation prompted a rapid generation of H_2O_2 at the cell surface within the first 10 min, and after 1 h of elicitation its amount reached a maximum level (approx. 20 μmol g^{-1} fr. wt) (Fig. 2). Its level lowered to about 50% of the maximum level 8 h after elicitation, though it showed no significant decline furthermore during the experiment period of 36 h.

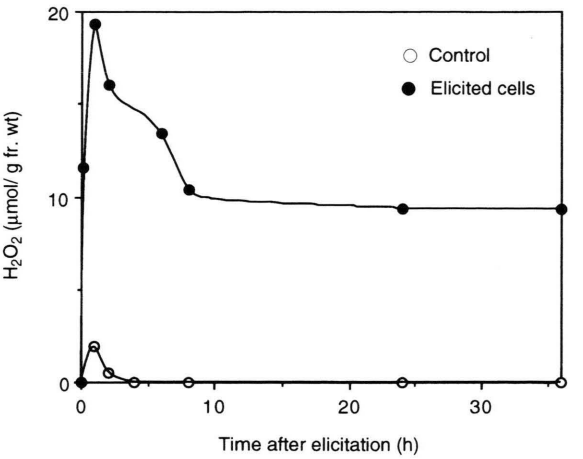

Fig. 2. Rapid generation of H_2O_2 in *P. lobata* cells elicited with yeast extract.

Inhibition of elicitor-induced metabolism of isoflavonoids

Hydrogenperoxide has been reported to be hypothetically generated by so called NAD(P)H-oxi-

Table I. Dose dependent effect of exogenous H_2O_2 on rapid loss of constitutive isoflavonoids from methanol-soluble fraction. After 1 g (fr. wt) cells were treated for 1 h with each concentration of H_2O_2, the isoflavonoids were quantitated by HPLC as described in "Materials and Methods". Values in parentheses represent the relative amounts compared to that of control cells (n.d.: not detected).

Concentration (mM)	Daidzein malonylglucoside (nmol/g fr. wt)	Genistein malonylglucoside (nmol/g fr. wt)
None	210 (100)	170 (100)
0.05	195 (93)	163 (96)
0.10	167 (79)	135 (79)
0.50	112 (53)	88 (52)
1.00	43 (20)	34 (20)
2.00	n.d.	n.d.

dases bound to plasma membrane in elicited plant cells (Apostol *et al.*, 1989; Vianello and Marci, 1991). From the above observations, it is assumed that the generation of H_2O_2 in elicited *P. lobata* cells also involves plasma membrane NAD(P)H-oxidases because of its rapid appearance. We tested a series of known inhibitors of plant NAD(P)H-oxidases for their abilities to interfere with the elicitor-induced disappearance of the isoflavonoids. Their inhibitory effects were evaluated by monitoring changes in isoflavonoids in the cells elicited with yeast extract for 4 h. Table II displays the results of the experiments using several representative inhibitors. Pretreatment of SHAM, DEDTC and KCN to the suspension-cultured cells of *P. lobata* 1 h before elicitation was capable of suppressing the elicitor-stimulated disappearance of malonylglucosides in dose-dependent manner and almost complete inhibition was observed with 1 mM of SHAM and DEDTC. Whereas slightly weak inhibition was noted at the same concentration of KCN, a known inhibitor of IAA oxidase and other enzymes involved in O_2 metabolism. It suggests that a KCN-sensitive enzyme may be a component of the H_2O_2 generating system, but KCN-independent oxidation reactions also contribute to the elicitor-induced generation of H_2O_2. In contrast, more than 10 mM of $Fe(CN)_6^{-4}$ was necessary for complete inhibition against the elicitor's action to isoflavonoid metabolism.

To confirm the importance of rapid H_2O_2 generation leading to metabolism of the constitutive isoflavonoids, we applied the NAD(P)H-oxidase inhibitors (1 mM each) such as SHAM, DEDTC and KCN to the cultured cells 1 h after elicitation. But they could not block the action of elicitor on isoflavonoid metabolism, in contrast to that observed in the cells pretreated with the inhibitors 1 h prior to elicitation (Table III). These results strongly support that H_2O_2 generated by rapid activation of NAD(P)H-oxidases is responsible for subsequent rapid disappearance of the constitutive isoflavonoids in elicited cells of *P. lobata*. The inhibitory effect of two antioxidant enzymes, catalase and SOD, was also tested to further characterize the nature of this oxidative burst. Unexpectedly, no inhibition against the elicitor's effect on isoflavonoid metabolism could be observed even though these enzymes were added to the culture 1 h before elicitation. It is likely to result from an elicitor-like effect of the proteins themselves,

Table II. Effect of NADH-oxidase inhibitors on elicitor-stimulated disappearance of constitutive isoflavone malonylglucosides in cultured *P. lobata* cells.

Inhibitors	Concentration	Inhibition of decrease in amount[a]	
		Daidzein-malonylglucoside	Genistein-malonylglucoside
	[mM]	%	
None[b]	0.0	0	0
SHAM	0.1	<10	<10
	0.5	89	95
	1.0	100	100
DEDTC	0.1	58	46
	0.5	84	83
	1.0	100	100
KCN	0.1	21	21
	0.5	57	60
	1.0	85	94
$K_4[Fe(CN)_6]$	1.0	<10	<10
	5.0	39	35
	10.0	88	81

[a] Inhibitory effects were determined 4 h after addition of yeast extract (1 mg ml^{-1} medium) to the cells which had been treated with each inhibitor for 1 h as described in "Materials and Methods"; [b] the cells were stimulated only with yeast extract in the absence of inhibitors.

Table III. Treatment-timing effect of some inhibitors on elicitor stimulated disappearance of isoflavone malonylglucosides.

Treatment	Amount (nmol/g fr. wt)	
	Daidzein-malonylglucoside	Genistein-malonylglucoside
Control[a]	215	162
Elicitor[b]	62	40
SHAM (1 mM) + elicitor[c]	217	149
DEDTC (1 mM) + elicitor[c]	214	158
Elicitor + SHAM (1 mM)[d]	71	48
Elicitor + DEDTC (1 mM)[d]	69	45
Catalase + elicitor[e]	58	41
Catalase[f]	123	72

[a] Content of isoflavonoids in the cells was determined 0 h after elicitation; [b] one mg yeast extract was added per ml of cells. Cellular isoflavonoids were quantitated 4 h after addition of yeast extract as described in "Materials and Methods"; [c] Elicitor was added 1 h after introduction of inhibitors; [d] inhibitors were added 1 h after elicitation; [e] catalase was administered to the cell culture for 1 h prior to elicitation; [f] catalase was added to the cell culture for 4 h in the absence of elicitor. Similar results were obtained with SOD.

because the cells treated only with enzymes showed similar rapid disappearance of the constitutive isoflavonoids.

Discussion

It has been postulated that plants possess a number of pre-formed and inducible defense mechanism conferring disease resistance against potential pathogens (Ebel and Grisebach, 1988). One of the most important factors affecting on the successful defense strategy is the expression timing of defense mechanism against pathogens. Although pre-formed mechanism (lignin, constitutively existing low molecular weight antimicrobial substances, *etc.*) may function as initial defensive barriers upon infection of pathogenic microorganisms, their sole action is not likely to be sufficient for rendering plants resistant. Therefore, rapid expression of the inducible defense mechanisms may be essential for protecting plants from continuous inıvasion of pathogens. Among them, phytoalexin accumulation is believed to be an important early defense response in several plant-pathogen interactions. However, the inducible defense mechanism needs at least several hours to appear and function as essential defense factors because the induction of phytoalexin synthesis comprises a whole complement of enzymes of the biosynthetic pathway, as demonstrated in a number of plant cells (Dixon, 1986; Dixon and Harrison, 1990). It leads to an assumption that there must exist more rapid defense mechanisms appearing in plants at very early stages of pathogen-invasion. For example, pre-formed glycosides such as tuliposide have been regarded as a candidate for the first defense line, because they are instantaneously split by endogenous glycosidases to yield their respective aglycones with remarkable antimicrobial activitiy when plant cells are damaged by pathogen invasion or mechanical stresses (Schoenbeck and Schroeder, 1972; Mansfield, 1983). Another example for rapid defense response is callose which was reported to be deposited in the cell wall within 20 min of treatment with chitosan elicitor to soybean cell suspension cultures (Kohle *et al.*, 1985). The oxidative burst, which derives predominantly from the activation of membrane-bound NAD(P)H-oxidases, may also serve as a first line of defense in soybean cells because of its speed of appearance (Apostol *et al.*, 1989). Thus, it is conceivable that there must commonly occur certain defensive response which precedes *de novo* synthesis of inducible defense mechanisms in plant cells.

In this study, we showed that exogenous addition of H_2O_2 to the suspension-cultured cells of *P. lobata* triggered a rapid metabolism of the constitutive isoflavonoids in a similar manner as observed in the cells treated with yeast extract or *Pmg* glycoprotein, and also demonstrated that elicitation stimulated a rapid generation of H_2O_2 at the cell surface leading to a subsequent disap-

pearance of the constitutive isoflavonoids. Involvement of H_2O_2 in this elicitor induced metabolism was further confirmed by the experiments using NAD(P)H-oxidase inhibitors (SHAM, DEDTC, and KCN). Application of the inhibitors to the cultured cells 1 h before elicitation resulted in almost complete inhibition of elicitor-induced disappearance of the isoflavonoids, whereas treatment at 1 h after elicitation could not. The result indicates that H_2O_2 production by rapid activation of H_2O_2 generating enzymes, presumably NAD(P)H-oxidases, must be involved in the elicitor-induced disappearance of isoflavonoids in elicited *P. lobata* cells. SHAM and KCN could inhibit iron containing peroxidases, possible components of subsequent metabolism leading to the binding of isoflavone aglycones to cell walls. However inhibition by a copper chelating agent, DEDTC, indicates that the primary target of these inhibitors in the isoflavonoid metabolism under discussion must be NAD(P)H-oxidases and not peroxidases. Our failure to detect the effect of the impermeable enzymes, namely catalase and SOD, on isoflavonoid metabolism was due to the unexpected elicitor-like activity of the enzymes themselves. This result is contrary to that reported in soybean cells, where the pretreatment with catalase 1 h before elicitation supressed the function of H_2O_2 as a signal transducer for phytoalexin production (Apostol *et al.*, 1989).

Plant plasma membranes and cell walls have some NAD(P)H oxidation activities capable of generating superoxide anion and hydrogen peroxide (Vianello and Macri, 1991; De Luca *et al.*, 1984; Valenti *et al.*, 1990; Neufeld and Bown, 1987). They generate hydrogen peroxide through a reaction chain involving superoxide anion utilizing NAD(P)H as a reducing substrate. Although several kinds of H_2O_2 generating enzymes has been characterized in plants, their physiological role remains unclear (Harkin and Obst, 1973; Maeder and Fuessl, 1982). Their involvement in resistance mechanism against pathogens is also less understood. Frequently, as a consequence of an infection, a rapid and localized hypersensitive response occurs near to the region parasitized, which leads to death of cells surrounding the site of pathogen invasion. This reaction is mediated by multiple biochemical components which act together: production of phytoalexins, secretion of hydrolytic enzymes, deposition of extracellular molecular barriers and rapid formation of oxygen free radicals (Vianello and Macri, 1991). As described above, the rapid production of H_2O_2 does not only evoke abrupt destruction of some fluorescent dyes in combination with extracellular peroxidases but also function as a signal transducer for phytoalexin formation in soybean cells treated with elicitors, suggesting H_2O_2 plays a crucial role in defense response to pathogens (Apostol *et al.*, 1989; Chandra and Low, 1995; Tenhaken *et al.*, 1995).

In addition to our previous findings (Park *et al.*, 1995), our present results show that H_2O_2 is responsible for the elicitor-induced metabolism of constitutive isoflavonoids which consequently bind to insoluble lignocellulose fraction in *P. lobata* cells. The isoflavone skeleton with *O*-glycosidic linkage was shown to be essential for their binding to insoluble lignocellulose with the experiments using ^{14}C-labeled isoflavonoids (Park *et al.*, 1995). The association process can be explained as a phenol oxidative coupling reaction which is well established in lignin biosynthesis *via* polymerization of monolignols (Hakamatsuka *et al.*, 1992). Isoflavones have been shown to inhibit the peroxidase-catalyzed oxidation of the lignin precursor coniferyl alcohol, and their possible role in controlling cell wall bound peroxidase activity involved in lignification of hypocotyls of *Lupinus albus* has been suggested (Barcelo and Munos, 1989; Ferrer *et al.*, 1990). This may imply that isoflavones might be able to serve as excellent substrates of cell wall peroxidases competing with monolignols for lignification.

Participation of H_2O_2 is essential for lignin biosynthesis catalyzed by peroxidases. We have also reported that *in vitro* reaction of daidzein with HRP/H_2O_2 brings about the formation of its dimers together with some unidentified polymers, and that daidzein-dimers accumulate as possible by-products of lignification in *P. lobata* cells treated with yeast extract (Hakamatsuka *et al.*, 1992). Large increase in lignin contents (estimated by derivatization with thioglycolic acid) was also observed after 4 h of elicitation with the same time course response as production of daidzein-dimers in *P. lobata* cells (data not shown), indicating that the formation of daidzein-dimers may be accompanied by lignification in elicited *P. lobata* cells.

Hydrogenperoxide is not only a powerful antimicrobial agent in animal phagocyte cells (Lachman, 1986; Prince and Gunson, 1987) but also a defense-related signal transducer in plants (Apostol *et al.*, 1989; Murai *et al.*, 1995). Even though there is at present no evidence that rapid generation of H_2O_2 by elicitation can be directly involved in defense responses in *P. lobata* cells, it is clear that the active oxygen evokes a rapid flow of preexisting isoflavonoids from their soluble pool to non-extractable cell wall fraction, which may facilitate an increase in mechanical rigidity of plant cell wall. This process does not neccessarily involve gene transcriptions since enzymes required for binding of isoflavones to cell wall such as malonylesterase, β-glucosidase and cell wall bound peroxidase are highly expressed in cells irrespective of elicitor treatment (to be reported elsewhere) and disruption of compartmentation of relevant substrates and enzymes caused by rapidly generated H_2O_2 seems to be a key event leading to the observed metabolism. This type of rapid response (rapid generation of H_2O_2 and subsequent binding of pre-existing isoflavonoids to cell wall lignocellulose) stimulated by elicitation could be regarded as an early defense system of *P. lobata* cells prior to the expression of *de novo* synthesized defense mechanism such as phytoalexins and lignin in plant-pathogen interaction. To obtain more informations and evidences for our assumption, the studies are underway at cellular and enzymatic levels.

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