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Responses of anthocyanin-producing and non-producing cells of *Glehnia littoralis* to radical generators

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Abstract

The responses of anthocyanin-producing (violet) and non-producing (white) cells of *Glehnia littoralis* to radical generators were compared. Cell growth, anthocyanin content, phenylalanine ammonia-lyase (PAL) activity and furanocoumarin production were determined after treatment with H_2O_2, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), X-ray and yeast extract, independently. AAPH and H_2O_2 repressed the growth of both violet and white cells, but violet cells grew better than white cells. On the other hand, the anthocyanin content in violet cells decreased. Neither X-ray nor yeast extract affected cell growth or pigment production. Treatment with H_2O_2, yeast extract, and X-ray, but not AAPH, induced PAL activity and furanocoumarin production in white cell cultures, whereas violet cell cultures did not produce furanocoumarin following any of the treatment employed. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Glehnia littoralis*; Apiaceae; Cell culture; Radical generator; H_2O_2; 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH); X-ray; Phenylalanine ammonia-lyase (PAL); Anthocyanin; Furanocoumarin

1. Introduction

During evolution, plants have developed various defense mechanisms. One of these is induction of the formation of plant-specific stress compounds, such as phytoalexins, in response to stimuli including attack by pathogens and treatment with various elicitors (Tal and Robeson, 1986; Pare et al., 1991). At the earliest stage of such responses, an oxidative burst that generates O_2^- and H_2O_2 occurs in various plant cells, where these radicals are expected to be involved in signaling mechanisms (Legendre et al., 1993; Degousee et al., 1994; Levine et al., 1994; Lamb and Dixon, 1997; Wojaszek, 1997). On the other hand, plants have also developed systems to eliminate such radicals as another type of defense mechanism. Low-molecular-weight compounds such as flavonoids and vitamins as well as high-molecular-weight compounds such as peroxidase and catalase counteract these radicals and oxidants (Culter et al., 1989; Wakamatsu and Takahama, 1993; Tsuda et al., 1994; Yamasaki et al., 1996).

Although an intact plant can produce these different kinds of defense compounds, it is not yet clear whether a plant cell can produce different kinds of defense compounds at the same time. In fact, there has been no previous report on attempts to induce a new defense compound in a plant cell in which a defense compound has already accumulated. As an approach to this question, we introduce here two suspension cell lines of *Glehnia littoralis* as simple systems. In a previous study, we found that furanocoumarins were newly induced in cell suspension cultures of *G. littoralis* (Apiaceae) after treatment with yeast extract (Kitamura et al., 1998). At the same time, we established a high anthocyanin-producing (violet) cell line from a non-producing (white) cell line of *G. littoralis* by selection (Miura et al., 1998). With these two cell lines, we can determine whether or not a radical generator, like yeast extract, can induce furanocoumarin formation in white cell cultures, and also what kind of radical might induce furanocoumarin. In addition, we can examine if it is possible to induce furanocoumarin in anthocyanin-producing cell cultures using the same treatment as in non-anthocyanin-producing cell cultures.

Using H_2O_2, 2,2′-azobis(2-amidinopropane) dihydrochloride [2997-92-4] (AAPH) and X-ray as radical generators, we found that H_2O_2 and X-ray irradiation,

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but not AAPH, induced furanocoumarin production in white cell cultures. On the other hand, no furanocoumarin was induced in violet cell cultures with any treatment.

2. Results

2.1. Changes in cell growth, PAL activity and anthocyanin content during culture without any treatment

Before examination with potent radical generators, we needed to determine when plant cells are most sensitive to detect their effects. Therefore, we determined the changes in cell growth, PAL activity, and anthocyanin and furanocoumarin contents of violet and white cell cultures over 3 weeks. PAL activities in the two cell lines were monitored because both anthocyanins and furanocoumarins are biosynthesized through the phenylpropanoid (acetate) pathways, the first common step of which is the conversion of phenylalanine to *trans*-cinnamic acid catalyzed by PAL.

Few furanocoumarins were detected in either violet or white cell cultures during subculturing (data not shown). There was little difference in cell growth between the two cell lines: logarithmic growth started on day 9 (Fig. 1A). PAL activity between the two cell lines was very different and reflected whether or not anthocyanins were being biosynthesized (Fig. 1B). In white cell cultures, PAL activity was detected at a very early stage of culturing: maximum activity was found on day 3 and negligible activity was found after day 9. On the other hand, much higher PAL activity was detected throughout the 3 weeks in the violet cell cultures; the activity decreased until day 6, increased vigorously, and then again decreased from day 15 onwards. The anthocyanin content in violet cell cultures decreased from days 3 to 6, then reached a minimum, and increased from day 9 to reach around 4 μmol/g fresh weight (Fig. 1C).

Cell growth, anthocyanin content and PAL activity of violet cell cultures were logarithmic from days 9 to 12, which is the most sensitive time for detecting the effects of radical generators. Based on this result and our previous work (Kitamura et al., 1998), we decided to treat both white and violet cell cultures with potent radical generators on day 10, and then to determine their effects on cell growth, PAL activity, anthocyanin contents and furanocoumarin production 2 days later.

2.2. Effect of potent stress inducers on cell growth, PAL activity, anthocyanin content and furanocoumarin induction

2.2.1. H_2O_2

Hydrogen peroxide was added to the media of 10-day-old violet and white cell suspension cultures to give

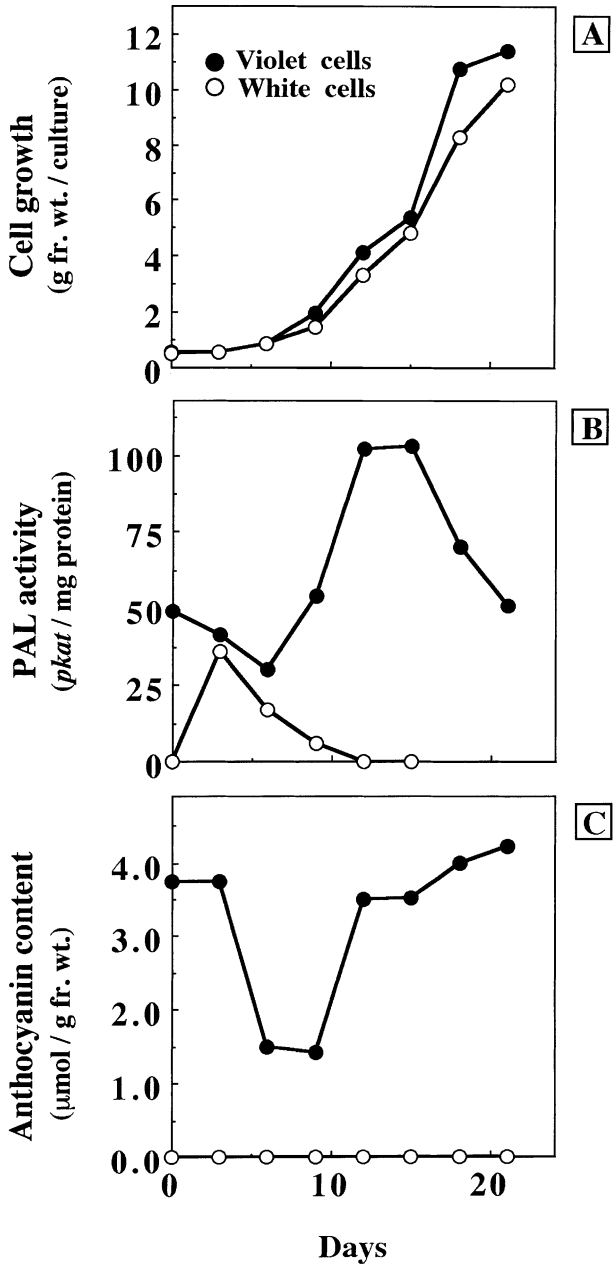

Fig. 1. Changes in (A) cell growth, (B) PAL activity and (C) anthocyanin contents in violet and white cell cultures during 3 weeks of culturing. Open circle, white cell culture; closed circle, violet cell culture.

initial concentrations of 0.01, 0.1, 1, and 10 mM, respectively. Cell growth in both cell lines decreased in a dose-dependent manner by H_2O_2 treatment, but violet cells were less damaged than white cells (Fig. 2A). Both violet and white cells nearly doubled from days 10 to 12 without stress treatment (Fig. 1A). At 10 mM, cell growth in the white cells was about 30% of that in the control (without H_2O_2 treatment), while the violet cells kept growing at more than 50% of that of the control despite a drastic color loss (Fig. 2B). Anthocyanin contents at 1 and 10 mM were less than half and less than a

quarter of that of the control, respectively, and the PAL activity decreased as well.

In the white cell cultures, the PAL activities were detected for the first time after addition of hydrogen peroxide (Fig. 2C). With an increase in PAL activity, the furanocoumarin bergapten was detected in the medium of cell suspension cultures. Bergapten production and PAL activity tended to increase in a dose-dependent manner.

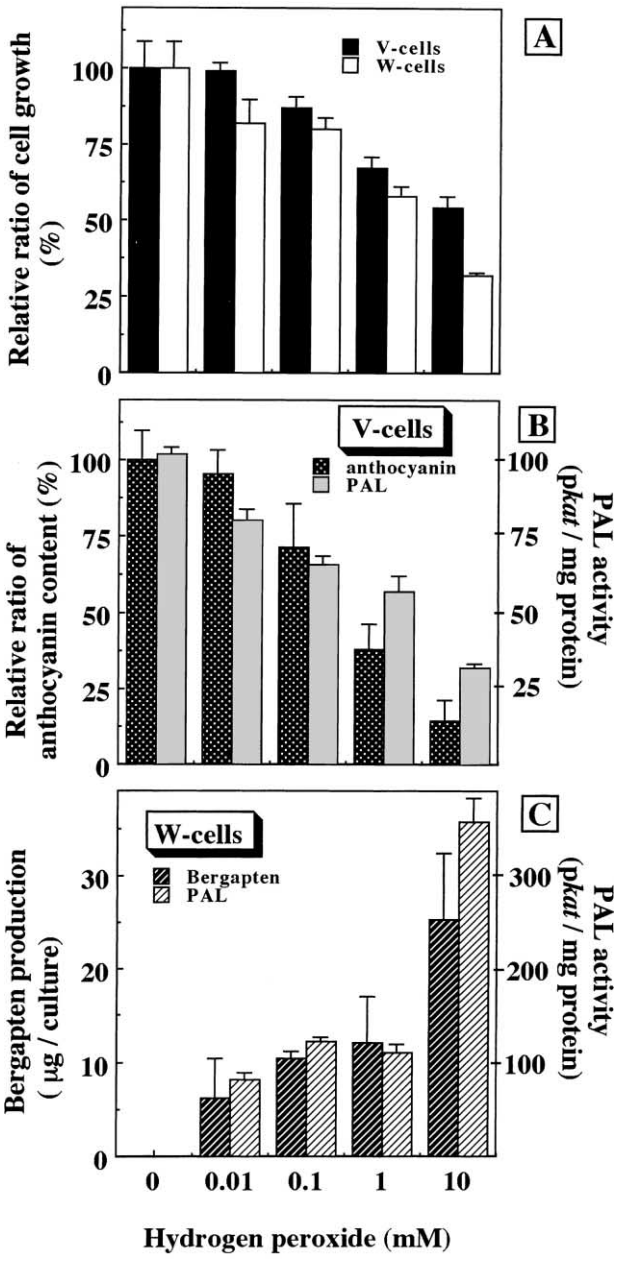

Fig. 2. Effects of various concentrations of hydrogen peroxide on (A) cell growth of violet and white cell cultures, (B) anthocyanin content and PAL activities in violet cell cultures, and (C) bergapten production and PAL activity in white cell cultures. Data are the means of triplicate determinations and bars represent S.D.

No bergapten was detected in the violet cell cultures by treatment with H_2O_2 at any concentration. Other treatments (AAPH, X-ray, and yeast extract) described below also did not induce furanocoumarin production/accumulation in the violet cell cultures.

2.2.2. AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) [2997-92-4]

According to Ohlsson et al. (1995), AAPH was added to the medium of the cell suspension cultures at final concentrations of 1, 2, 4, 8, and 16 mM, respectively. Cell growth with the addition of AAPH at any concentration was less than that without AAPH, although there was little difference in cell growth among the AAPH concentrations examined (Fig. 3A). As with H_2O_2, anthocyanin content decreased with AAPH treatment; the content decreased in a dose-dependent manner from 1 to 8 mM and then reached a steady state, where the relative content was 50% of that of the control (Fig. 3B).

PAL activity in cell suspension cultures treated with AAPH was extremely different from that in the cells treated with H_2O_2. In white cells, no PAL activity was

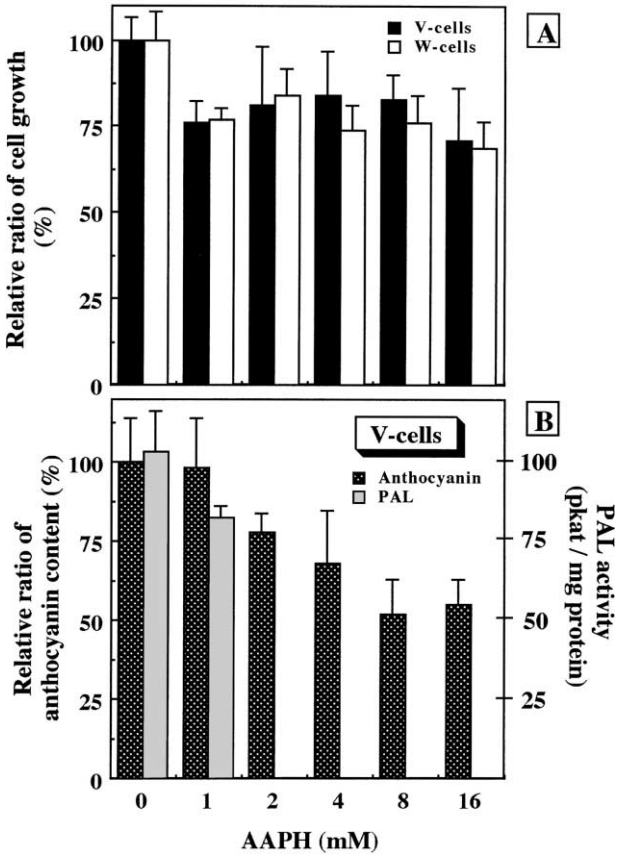

Fig. 3. Effects of various concentrations of AAPH on (A) cell growth of violet and white cell cultures at various concentrations and (B) anthocyanin contents and PAL activities in the violet cell cultures. Data are the means of triplicate determinations and bars represent S.D.

detected with any AAPH concentration. Bergapten was also not detected in the white cells, which is consistent with the lack of PAL activity. Even in the violet cells, PAL activity was detected only at 1 mM.

2.2.3. X-ray and yeast extract

X-ray irradiation did not affect either cell growth or anthocyanin content in the white and violet cell cultures at 1 and 10 Gy (data not shown). However, bergapten production, together with increased PAL activity, were detected in the white cell cultures (Fig. 4). Similarly, the yeast extract did not affect either cell growth or anthocyanin content. Yeast extract induced PAL activity and bergapten production only in the white cell cultures. Interestingly, PAL activity induced by X-ray irradiation was much higher than that induced by yeast extract despite the lower production of furanocoumarin.

3. Discussion

In a previous study, we induced formation of the stress-related compound furanocoumarin in *G. littoralis* cell suspension cultures by treatment with a yeast extract (Kitamura et al., 1998). Active oxygen has been reported to be involved in signal transmission in inducible defense responses (Schwacke and Hager, 1992). Therefore, we determined whether or not a radical generator could be used instead of yeast extract to induce stress compounds, and if so, what kinds of radical generator could be used. We used three kinds of radical generators: (1) H_2O_2, a representative inorganic radical, is a well-known product of oxidative burst and is involved in intra- and inter-cell signal transmission (Alvarez et al., 1998); (2) AAPH, an organic radical generator. Two carbon-centered radicals and nitrogen gas are produced by decomposition of the symmetrical molecule in water (Dooley et al., 1990); (3) X- ray irradiation, both an organic and inorganic radical inducer. It has been reported that γ- and X-radiation induce long-lived organic radicals as well as short-lived inorganic radicals in hamster and human embryonic cells (Yoshimura et al., 1993; Koyama et al., 1998).

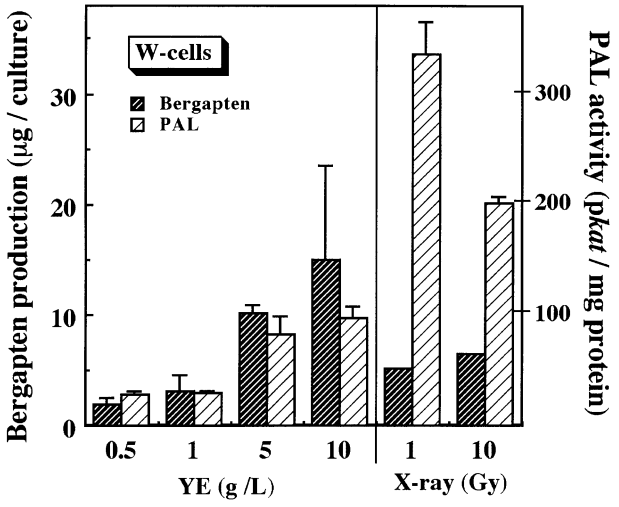

Fig. 4. Effects of X-ray irradiation and YE treatment on bergapten production and PAL activity in white cell cultures. Data are the means of triplicate determinations and bars represent S.D.

Our results showed that H_2O_2 and X-ray irradiation, but not AAPH, increased PAL activity and induced stress compound accumulation in non-anthocyanin-producing cell cultures. This means that an inorganic radical such as H_2O_2 can induce formation of stress compounds, though the participation of H_2O_2 was not determined in the case of X-ray irradiation. AAPH impaired cell growth, but did not induce formation of the stress compound. AAPH was first used for studying the defense system in *Catharanthus roseus*, *Catharanthus tricophyllus*, and *Pisum sativum* cell cultures by Ohlsson et al. (1995). They reported that PAL activity in *C. roseus* cell cultures increased more than 8-fold with the addition of 1 mM AAPH. However, no induction of PAL activity was observed in our white cell cultures under the conditions used. They also reported that the culture medium turned yellow together with the production of phenolic substances, while we observed a loss of color in the culture medium as the AAPH concentration increased.

X-ray irradiation induced PAL activity and production of the stress compound furanocoumarin in the white cell cultures. However, the PAL activity induced by X-ray irradiation was much higher than that induced by the yeast extract or hydrogen peroxide despite the lower production of furanocoumarin (Figs. 2C and 4). Similarly, an increase in PAL activity with induction of little furanocoumarin was observed in the white cell cultures when osmotically stressed by sucrose, irradiated with UV, and treated with heavy metals such as CrO3 and VOSO4 (data not shown). These results suggest that not only the biosynthetic pathway leading to furanocoumarin but also another phenylpropanoid pathway may be stimulated by X-ray irradiation.

In fact, responses of tobacco suspension cells to methyl jasmonate and pathogenic elicitor are distinct, though PAL activity increased in both cases; scopoletin and scopolin were found only from methyl jasmonate-treated cell cultures (Sharan et al., 1998). In the case of parsley plants, the cross-induction of defense pathways leading to furanocoumarins and flavone glycosides was found after exposure to ozone (Eckey-Kaltenback et al., 1994). In birch seedlings, UV-B radiation significantly increased PAL activity and the accumulation of flavonoids and phenolic acids such as chlorogenic acid (Lavola et al, 2000). In addition, lignin, an important structural component of cell wall, biosynthesized through phenolics, participated in the response of

cucumber roots exposed to allelochemicals (Polytycka, 1999). Similarly, soybean leaves exposed to ozone responded with increased PAL activity followed by increase in cell-wall-bound phenolics and acid-insoluble lignin (Booker and Miller, 1998). Correlations between induction of PAL activity and production of soluble and insoluble phenolics as reported above have not yet been determined in *G. littoralis* cell cultures.

Interestingly, furanocoumarin biosynthesis was not induced in anthocyanin-producing cells of *G. littoralis* by any radical generators. There are at least two possible explanations for this results: (1) the cells produce anthocyanin at sufficiently high concentrations to capture radicals that participate in signaling mechanisms and to terminate further responses of inducible defense; and (2) cells that express one metabolic pathway such as anthocyanin biosynthesis may be inhibited in the expression of a competing pathway such as furanocoumarin biosynthesis, since both pathways branch off from phenylpropanoid metabolism. The available evidence does not allow us to conclude which of these hypotheses is correct.

In *C. roseus* cell cultures (CR19, PINK), AAPH reportedly reduced anthocyanin accumulation (Ohlsson et al., 1995). Similarly, anthocyanin accumulation in the violet cells of *G. littoralis* decreased following treatment with H_2O_2 and AAPH, and was accompanied by a reduced PAL activity (Figs. 2B and 3B). No PAL activity was detected upon treatment with more than 1 mM of AAPH, but the treated cells still accumulated at least 50% of the anthocyanin in non-treated cells. This means that anthocyanin in AAPH-treated cells was not newly accumulated, but remained without being degraded, since the amounts of anthocyanin in the violet cells nearly doubled from days 10 to 12 without stress treatment (Fig. 1C). In particular, AAPH gave the prolonged inhibition of PAL activity at the concentration of more than 1 mM, presumably due to its long half life. According to Koyama et al.(1998), the half life of a long-lived organic radicals induced by X-irradiation in human embryonic cells was over 20 h at room temperature, and was much longer than that of the inorganic OH radicals (70–200 ns). On the other hand, with H_2O_2, less anthocyanin production/accumulation was detected with higher PAL activity. Color bleaching and/or decomposition must occur in H_2O_2-treated cells. Since anthocyanin is usually stored in vacuoles via tonoplasts, H_2O_2 but not AAPH might more readily pass through tonoplasts and access the anthocyanins. In addition, anthocyanin-producing cells showed better growth than non-producing cells after H_2O_2 treatment. These results indicate that the cells may be damaged less by H_2O_2 treatment in exchange for color deterioration, probably because of the capture of radicals.

Treatment with H_2O_2 and AAPH reduced PAL activity as well as anthocyanin content in the violet cells in vivo, but it remained unknown whether these radical generators affected the enzyme directly or at the regulatory level. Therefore, effect of AAPH and H_2O_2 on PAL activity was tested in vitro at final concentrations of 0.2, 1, 2, 4, and 8 mM, and at final concentrations of 0.01, 0.1, 1, and 10mM, respectively. Neither AAPH nor H_2O_2 inhibited PAL activity in vitro at any concentration examined in the 10- to 12-day-old violet cell suspension cultures (data not shown). This suggests that AAPH as well as H_2O_2 may suppress PAL transcription and/or translation in vivo, but not directly enzyme activity. Induction of PAL transcript accumulation by exogenous H_2O_2 was observed in *Arabidopsis* and soybean suspension cells (Desikan et al., 1998, Levine et al,. 1994), but there has been few reports on suppression of PAL transcript accumulation by either H_2O_2 or AAPH. Time course experiments after stress treatment on PAL transcript accumulation and PAL activity in the violet as well as the white cell cultures of *G. littoralis* are now under investigation.

4. Experimental

4.1. Plant materials

White callus was induced from a petiole segment of *G. littoralis* (Apiaceae) and cultured on a solid Murashige and Skoog (1962) (MS) medium containing 1 mg/l 2,4-D and 0.01 mg/l kinetin. Anthocyanin-producing violet callus was established from white callus by mechanical selection as reported previously (Miura et al., 1998), and sustained on Gamborg B5 (Gamborg et al., 1968) medium containing 1 mg/l NAA and 0.01 mg/l kinetin. From both calli, cell suspension cultures were obtained and subcultured every 3 weeks by transferring 5 ml of suspension cells into 25 ml of fresh medium in a 100 ml Erlenmeyer flask. All cultures were incubated at 25 °C in the dark. For stress induction, 10-day-old cultures were treated with various radical generators. After treatment, cultures were harvested by vacuum filtration, separated into cells and media, and stored at −70 and −20 °C, respectively, until analysis. Before storage, the fr. wt of cells was determined.

4.2. Radical treatment

As potent radical generators, H_2O_2 (Wako), 2,2′-azobis(2-amidinopropane) dihydrochloride [2997-92-4] (AAPH) (Wako) and X-ray as well as yeast extract (Difco Laboratories) were used. H_2O_2 and AAPH were prepared just before use and then subjected to filter-sterilization, while yeast extract was autoclaved at 121 °C for 15 min before addition. X-ray was applied at 0.44 Gy/min (150 kV, 5 mA), using a soft X-ray generator (SOFTEX M-150WE).

4.3. Analysis

Anthocyanin content was determined as previously described (Miura et al., 1998). Furanocoumarin analysis and assay of phenylalanine ammonia-lyase [EC 4.1.3.5] (PAL) activity were performed as previously reported (Kitamura et al., 1998).

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References

Alvarez, M.E., Pennell, R.I., Meijer, P.-J., Ishikawa, A., Dixon, R.A., Lamb, C., 1998. Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92, 773–784.

Booker, F.L., Miller, J.E., 1998. Phenylpropanoid metabolism and phenolic composition of soybean (*Glycine max* Merr.) leaves following exposure to ozone. J. Exp. Bot. 49, 1191–1202.

Culter, A.J., Saleem, M., Coffey, M.A., Loewen, M.K., 1989. Role of oxidative stress in cereal protoplast recalcitrance. Plant Tiss.Org.-Cult. 18, 113–127.

Degousee, N., Triantaphylides, C., Monntillet, J.-L., 1994. Involvement of oxidative processes in the signaling mechanisms leading to the activation of glycerol synthesis in soybean (*Glycine max*). Plant Physiol. 104, 945–952.

Deskin, R., Reynolds, A., Hancock, J.T., Neill, S.J., 1998. Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defense gene expression in *Arabidopsis* suspension cultures. Biochem. J. 330, 115–120.

Dooley, M.M., Sana, N., Kawashima, H., Nakamura, T., 1990. Effects of 2,2′-azobis(2- amidinopropane)hydrochloride in vivo and protection by vitamin E. Free Radic. Biol. Med. 9, 199–204.

Eckey-Kaltenback, H., Ernst, D., Heller, W., Sandermann, J.-H., 1994. Biochemical plant responses to ozone. IV. Cross-induction of defensive pathways in parsley (*Petroselinum crispum* L.). Plant Physiol. 104, 67–74.

Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell. Res. 50, 151–158.

Kitamura, Y., Ikenaga, T., Ooe, Y., Hiraoka, N., Mizukami, H., 1998. Induction of furanocoumarin biosynthesis in *Glehnia littoralis* cell suspension cultures by elicitor treatment. Phytochemistry 48, 113–117.

Koyama, A., Kodama, S., Suzuki, K., Matsumoto, T., Miyazaki, T., Watanabe, M., 1998. Radiation-induced long-lived radicals which cause mutation and transformation. Mutat. Res. 421, 45–54.

Lamb, C., Dixon, R.A., 1997. The oxidative burst in plant disease resistance. Ann.Rev. Plant Physiol. Plant Mol. Biol. 48, 251–275.

Lavola, A., Julkunen-Tiitto, R., De la Rosa, T.M., Lehto, T., Aphalo, P.J., 2000. Allocation of carbon to growth and secondary metabolites in birch seedlings under UV-B radiation and CO_2 exposure. Plant Physiol. 109, 260–267.

Legendre, L., Rueter, S., Heinstein, P.F., Low, P.S., 1993. Characterization of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glycine max*) cells. Plant Physiol. 102, 233–240.

Levine, A., Tenhaken, R., Dixon, R., Lamb, C., 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79, 583–593.

Miura, H., Kitamura, Y., Ikenaga, T., Mizobe, K., Shimizu, T., Nakamura, M., Kato, Y., Yamada, T., Maitani, T., Goda, Y., 1998. Anthocyanin production of *Glehnia littoralis* callus cultures. Phytochemistry 48, 279–283.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Ohlsson, A.B., Berglund, T., Komlos, P., Rydstrom, J., 1995. Plant defense metabolism is increased by the free radical-generating compound AAPH. Free Radic. Biol. Med. 19, 319–327.

Pare, P.W., Dmitrieva, N., Mabry, T.J., 1991. Phytoalexin aurone induced in *Cephalocereus senilis* liquid suspension cultures. Phytochemistry 30, 1133–1135.

Politycka, B., 1999. Ethylene-dependent activity of phenylalanine ammonia-lyase and lignin formation in cucumber roots exposed to phenolic allelochemicals. Acta Soc. Bot. Pol. 68, 123–127.

Schwacke, R., Hager, A., 1992. Fungal elicitors induce a transient release of Active oxygen species from cultured spruce cells that is dependent on Ca^{++} and protein-kinase activity. Planta 187, 136–141.

Sharan, M., Taguchi, G., Gonda, K., Jouke, T., Shimosaka, M., Hayashida, N., Okazaki, M., 1998. Effect of methyl jasmonate and elicitor on the activation of phenylalanine ammonia-lyase and the accumulation of scopoletin and scopolin in tobacco cell cultures. Plant Sci. 132, 13–19.

Tal, B., Robeson, D.J., 1986. The induction, by fungal inoculation, of ayapin and scopoletin biosynthesis in *Helianthus annuus*. Phytochemistry 25, 77–79.

Tsuda, T., Watanabe, M., Ohshima, K., Norinobu, S., Choi, S.-W., Kawakishi, S., Osawa, T., 1994. Antioxidative activity of the anthocyanin pigments cyaniding 3-*O*-β-D-glucoside and cyaniding. J. Agric. Food Chem. 42, 2407–2410.

Wakamatsu, K., Takahama, U., 1993. Changes in peroxidase activity and in peroxidase isozymes in carrot callus. Physiol. Plant 88, 167–171.

Wojaszek, P., 1997. Oxidative burst: an early plant response to pathogen infection. Biochem. J. 233, 249–257.

Yamasaki, H., Uefuji, H., Sakihama, Y., 1996. Bleaching of red anthocyanin induced by superoxide radical. Arch. Biochem. Biophys. 332, 183–186.

Yoshimura, T., Matsuno, K., Miyazaki, T., Suzuki, K., Watanabe, M., 1993. Electron spin resonance studies of free radicals in gamma-irradiated golden hamster embryo cells: radical formation at 77 and 295 K, and radioprotective effects of vitamin C at 295 K. Radiat. Res. 136, 361–365.