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Similarities of Tobacco Mosaic Virus-Induced Hypersensitive Cell Death and Copper-Induced Abiotic Cell Death in Tobacco

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Hypersensitive cell death of plants during incompatible plant-pathogen interactions is one of the efficient defense mechanisms of plants against pathogen infections. For better understanding of the molecular mechanisms involved in the plant hypersensitive response (HR), TMV-induced biotic plant cell death and $CuSO_4$-induced abiotic plant cell death were compared in terms of expression patterns of ten different defense-related genes as molecular markers. The genes include five pathogenesis-related protein genes, two plant secondary metabolite-associated genes, two oxidative stress-related genes and one wound-inducible gene isolated from tobacco. Northern blot analyses revealed that a same set of defense-related genes was induced during both biotic and abiotic cell death but with different time and magnitude. The expression of defense-related genes in tobacco plants was temporarily coincided with the time of cell death. However, when suspension cell cultures was used to monitor the expression of defense-related genes, different patterns of the gene expression were detected. This result implies that there are common and, in addition, also different branches of signaling pathways leading to the induced expression of defense-related genes in tobacco during the pathogen- and heavy metal-induced cell death.

Keywords : disease resistance, heavy metal, plant cell death, PR-genes, tobacco mosaic virus.

Infection of plants by a nonpathogen or an avirulent strain of a pathogen elicits rapid collapse of the challenged host cells, so-called hypersensitive response (HR), and deployment of a battery of inducible defenses in both the challenged and the surrounding cells (Lamb et al., 1989). In contrast, attack by a virulent strain does not elicit rapid localized hypersensitive cell death. Instead, the induction of defense responses is often substantially delayed, and thus disease ensues. In addition to the local plant defense response, systemic acquired resistance (SAR), which develops in noninfected parts of the plant, is considered as a subset of defense response triggered by localized HR (Ryals et al., 1996).

The HR-associated cell death is the most commonly activated response by which plants exert efficient disease resistance against invading pathogens. Activation of plant cell death after recognition of incompatible pathogen results in formation of a zone of dead cells localized around the site of pathogen penetration (Dorey et al., 1997). This zone of dead cells, also called a HR lesion, is thought to inhibit the proliferation and systemic infection of the invading pathogen (Goodman and Novacky, 1994).

Many studies have shown that the HR is accompanied by biochemical changes both at the site of infection and at distant sites on the plant (Malamy and Klessig, 1992). At the infection site, the HR is correlated with a transient burst of active oxygen species (AOS), activation of specific defense-related genes, accumulation of antimicrobial compounds, and alterations of plant cell wall morphology. Expression of a number of defense-related genes, in particular the pathogenesis-related (PR) genes, correlates with the establishment of the SAR (Kang et al., 1998). However, the molecular connections between incompatible pathogen-induced cell death and these gene expression are not fully understood. Plants produce AOS during interaction with various pathogens, and it is considered that AOS are involved in both disease resistance and symptom development in plant pathogenesis (Tzeng and De Vay, 1993). AOS, including H_2O_2, are very important components of plant defense, since they are known to be generated during biotic and abiotic stresses of plants (Baker and Orlandi, 1995). All of these responses are considered to contribute overall disease resistance in plants (Hammond-Kosack and Jones, 1996).

Heavy metal ions at high concentration also induce rapid plant cell death. In addition, it has been reported that the heavy metal ions also causes oxidative burst in plant cells. However, molecular biological characteristics of the metal-induced plant cell death and oxidative burst remains to be elucidated. Thus, it is still unclear whether molecular events involved in the metal-induced cell death is identical to or different from the pathogen-induced HR.

We have investigated the molecular response involved in either of biotic and abiotic plant cell death. In the present

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study, expression of a set of isolated plant defense-related gene has been monitored following TMV inoculation or exogenous application of $CuSO_4$ to tobacco plants. Although there were differences in magnitude and time of the gene expression, our results clearly indicated that there are similar patterns of activation of defense-related genes during the biotic and the abiotic cell death of tobacco. Molecular events in the pathogen-induced biotic and the copper-induced abiotic plant cell deaths in relation to defense-related gene expression are discussed.

Materials and Methods

Plant materials and treatments. *Nicotiana tobacum* cv. Xanthi nc grown in a greenhouse under a regime 16:8 hr light:dark at 25± 2°C were used as plant materials. Ten-week-old tobacco plants were sap-inoculated for induction of HR cell death. A mock inoculated plant leaf was used as a control. For heavy metal treatments, 10 mM $CuSO_4$ solution was prepared in distilled water for spraying onto tobacco leaf surface. Plant tissues were sampled and total RNA was isolated from leaf tissues at the indicated time after treatments. Expression of the selected defense-related genes was monitored by Northern blot hybridization.

Cell suspension culture and treatments. Cell suspension cultures were established from *N. tobacum* cv. Xanthi nc and maintained in a shaking incubator at 26°C, 80 rpm, with continuous light. Cells were transferred every 7 days to fresh Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1 mg/L 2,4-D. Treatments were performed 3~4 days after the transfer. Induction of plant cell death was performed in 250 ml Erlenmeyer flask with 25 ml aliquots of tobacco cell culture. $CuSO_4$ solution (100 μM final concentration by addition) was prepared in H_2O and added into the tobacco cell suspension cultures. Cells were harvested by vacuum filtration and frozen in liquid nitrogen for total RNA isolation.

Determination of cell death. Plant cell cultures were treated with copper solution (10 mM) and cell death was determined by Evans blue staining. Suspension cells (100 mg) harvested at indicated time after treatment was incubated for 15 min with 0.5 ml of 0.05% Evans blue solution followed by washing extensively to remove excess and unbound dye (Turner and Novacky, 1974). After washing, dyes bound to dead cells were solubilized in 50% methanol with 1% SDS at 50°C for 30 min. The solublized dye was quantified using spectrophotometer by measuring absorbance at 600 nm.

Preparation of defense-related gene probes. The probes used in the Northern blot analyses of PR-1 and PR-2 (acidic β-1,3-glucanase) genes were prepared as described peviously (Yun et al., 1996; 1998). PR-3 (acidic chitinase), SAR 8.2, phenylalanine ammonia lyase (PAL), hydroxymethylglutaryl coenzyme-A reductase (HMGR), ascorbate peroxidase (APX), glutathion-S-transferase (GST) and proteinase inhibitor genes (Table 1) were isolated by differential screening of TMV-induced cDNA library in our separate experiments for cloning of pathogen responsive tobacco genes (unpublished).

Table 1. Defense-related genes of tobacco used as probes in this study

Group	Gene name	Reference
Pathogenesis-related protein genes	Pathogenesis-related protein-1(acidic form)	Yun (1996)
	β-1,3-glucanase (acidic form)	Yun (1998)
	Chitinase (acidic form)	MIP*
	Osmotin-like protein	MIP
	SAR 8.2	MIP
Secondary metabolite-associated genes	Phenylalanine ammonia-lyase	MIP
	Hydroxymethylglutaryl coenzyme A reductase	Choi (1992)
Oxidative burst-related genes	Glutathion S-transferase	MIP
	Ascobate peroxidase	MIP
Wound-inducible gene	Proteinase inhibitor	MIP

*manuscripts in preparation

Total RNA isolation and Northern blot hybridization. RNA was isolated from treated leaf tissues according to the previously described methods (Choi et al., 1996). Total RNA (20 μg) was fractionated by formaldehyde-containing agarose gel electrophoresis and transferred onto Nytran membrane (Amersham). The loading of equal amount of RNA was checked by hybridization of the membrane with ^{32}P-labelled 25S rDNA as a probe. Hybridization of RNA blot with ^{32}P-labelled cDNA probe was carried out in 5X SSC containing 50% formamide at 42°C, and washed with 2X SSC under same conditions.

Results

Comparison of plant cell death induced by pathogen and heavy metal. When tobacco leaves were inoculated with common strains of TMV, typical HR lesions are usually appeared about 48 hr after infection. The lesions initiated as dark water soaked spots and eventually developed as brown necrotic lesions (Fig. 1A). In contrast, when the same stage of tobacco leaves were sprayed with 10 mM concentration of CuSO4, rapid necrotic cell death was observed (Fig. 1B). Copper-induced necrotic plant cell death is often observed within 3 hr after spraying and is very similar to the TMV-induced early water soaked lesions which appeared at least 40 hr after TMV infection. Even though the phenotypic symptoms were similar, the biological events undergoing in both plant cell death mechanisms have not been compared.

Expression of tobacco pathogenesis-related protein genes during TMV-induced HR and copper-induced necrotic cell death. To study the comparative effects of the biotic and abiotic cell death on the expressions of plant defense-related genes, 10 different genes (Table 1) and TMV-resistant tobacco cultivar Xanthi nc were used. When total RNA samples isolated from tobacco leaf tissues following inoculation with TMV or spraying with copper

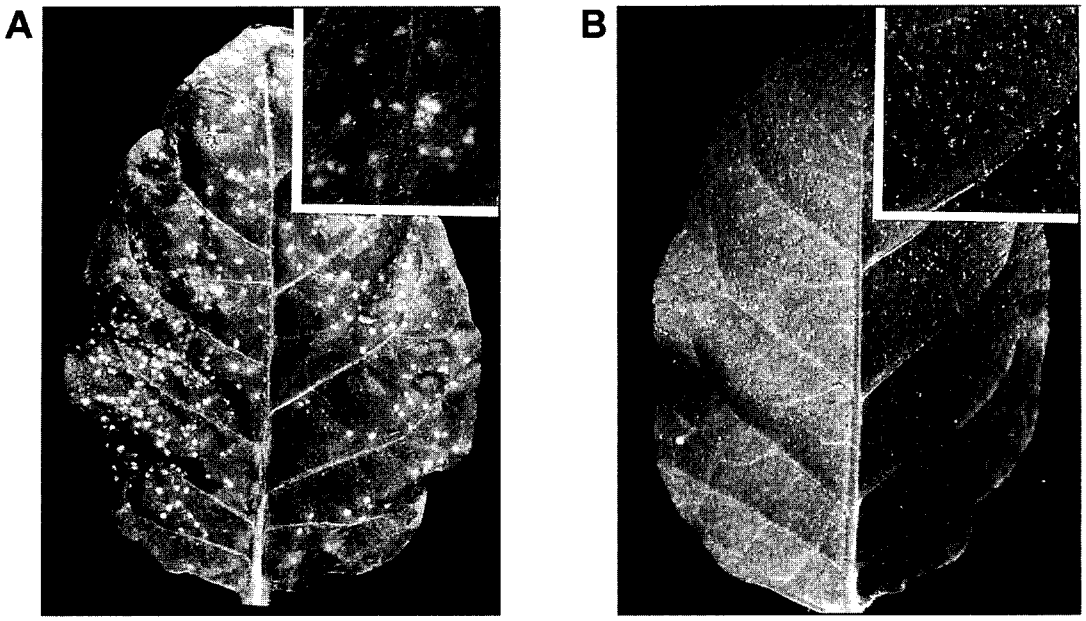

Fig. 1. Biotic and abiotic cell death induced by TMV infection or copper treatment. (A) HR-cell death symptom elicited 48 hr after infection of TMV. Ten-week-old tobacco plants were inoculated with sap prepared from TMV-infected tobacco. (B) HR-like cell death induced by heavy metal ion. 10 mM $CuSO_4$ solution was prepared in H_2O and sprayed onto tobacco leaf surface. Cell death occurred within 3 hr after treatment and pictures were taken 24 hr after treatment.

solution were subjected to Northern blot analyses with 5 different PR genes, differential expression was observed

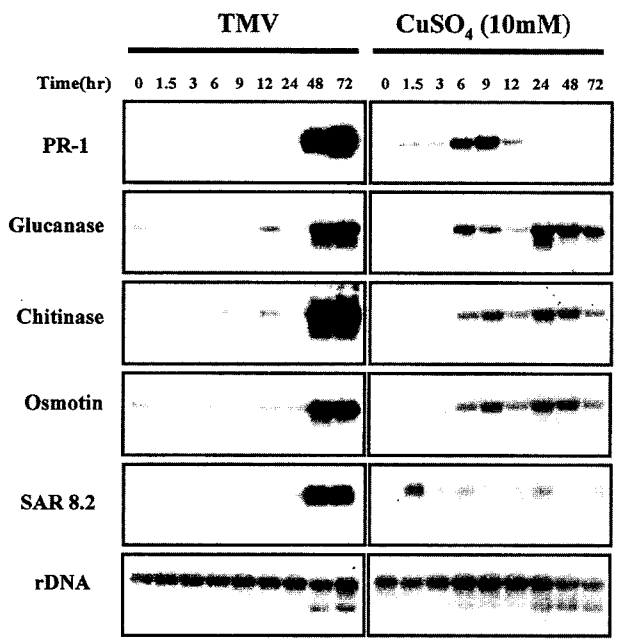

Fig. 2. Time course expression of PR-protein genes during HR-like cell death of tobacco plant by TMV-infection or $CuSO_4$ application. Ten-week-old tobacco plants (*N. tabacum* cv. Xanthi nc) were inoculated with TMV or treated with 10 mM $CuSO_4$ and then placed at 25±2°C culture room to develop cell death. Leaf tissues were harvested at the indicated time point after treatments for total RNA isolation. RNA blots were hybridized with ^{32}P-dCTP-labeled cDNA probes indicated in the figure.

(Fig. 2). In case of TMV-induced HR of tobacco plants, expression of 5 classes of PR genes was highly induced in 48 and 72 hr following inoculation. This PR-gene expression was correlated with the HR lesion development on tobacco leaves. In contrast, much rapid induction of PR-gene expression was observed in copper-induced plant cell death but it was still correlated with appearance of cell death. The magnitude of PR gene expression in copper treated tobacco tissues was lower than that of TMV-inoculated leaf tissues. Among PR-protein genes, expression of PR-1 and SAR 8.2 was different from that of other PR-genes by copper treatment, suggesting that there are at least two different signaling pathways leading to PR-gene expression during copper induced plant cell death. These results indicated that a same set of PR-gene is induced by both TMV- and copper-induced plant cell death.

Expression of secondary metabolite-associated and oxidative stress-related genes by TMV-induced HR and copper-induced plant cell death. We also examined the expressions of several defense related genes other than PR-genes during the biotic HR and copper treated cell death of tobacco plants. Total RNA were isolated from leaves harvested at 0 to 72 hr after TMV infection and 10 mM $CuSO_4$ treatments. RNA blots were prepared and hybridized with several cDNA probes including PAL, HMGR, GST, APX, and proteinase inhibitor. As shown in Fig. 3, expression of all of the genes we used as probes was affected by TMV-infection as well as by $CuSO_4$ treatments. The time course of induced expression of each gene was different between

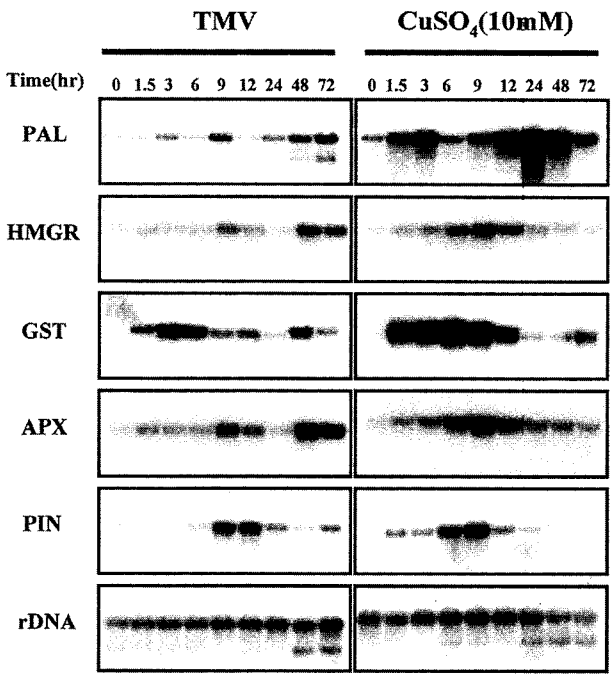

Fig. 3. Time course expression of defense-related gene during cell death of tobacco after TMV infection or copper treatment. Twenty μg of total RNA samples were blotted on Nytran membrane and hybridized with ^{32}P-labeled cDNA probes. A probe for 25S rRNA was used as a control for equal RNA loading. PAL, phenylalanine ammonia lyase; HMGR, 3-hydroxy-3-methylglutaryl Co-A reductase; GST, glutathion-S-transferase; APX, ascorbate peroxidase; PIN, proteinase inhibitor.

TMV-infected and copper-treated plants. In TMV-infected tobacco plant, expression of secondary metabolite-associated genes including PAL and HMGR, was coordinately induced in 48 hr after TMV infection, whereas expression of oxidative stress-related genes such as APX and GST was observed at an 1.5 hr post inoculation and detected until 72 hr (Fig. 3). The proteinase inhibitor gene was also induced after 9 hr and then reduced gradually until 24 hr after TMV infection (Fig. 3). In contrast to TMV inoculation, expression of PAL and HMGR gene in copper-treated plant tissues was detected earlier. Expression of PAL gene in tobacco by copper treatments was at an early stage with high magnitude and mRNA level of PAL gene was increased until 24 hr. However, the level of HMGR mRNA was reached to maximum in 9 hr after treatment. Expression of APX and GST genes was also induced by copper. GST transcripts were strongly detected at early stages and then reached a maximum level in 6 hr after treatment. APX gene expression was also induced in 9 hr after copper treatment. Induced expressions of both genes by copper treatment was decreased 24 hr after treatment (Fig. 3). Maximum level of proteinase inhibitor mRNA was detected in 9 hr after copper treatment and the pattern of this gene expression was similar to that of TMV inoculation

All together, these results imply that expression of all the selected defense-related genes in this study are affected by both TMV and copper-induced cell death of plants. Differential expression of the selected genes also imply that there may be present multiple signalling pathways leading to defense-related gene expression following the pathogen or metal-induced plant cell death.

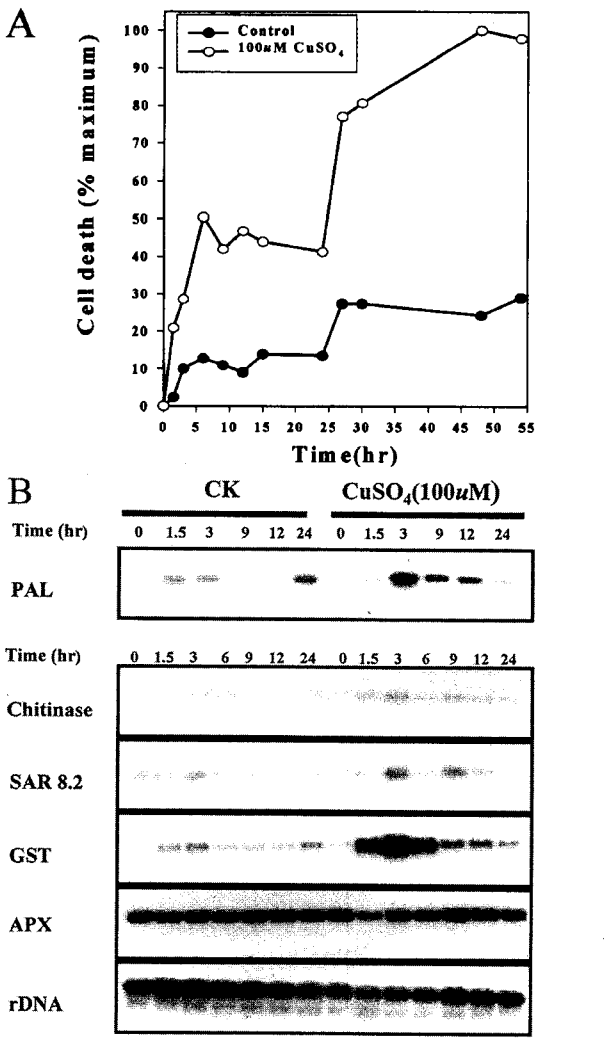

Fig. 4. Copper induced cell death and expression of defense-related genes in cultured tobacco cell. (A) Copper induced cell death. Cell death was assayed by using the Evans blue staining mothod. Cells were harvested and stained for 15 min with 0.05% Evans blue and then washed extensively with water to remove excess unbound dye. Bound dye was solubilized in 50% methanol with 1% SDS for 30 min at 50°C and quantified by measuring the absorbance at 600 nm. (B) Expression of defense-related genes. Cell suspension cultures of tobacco (*Nicotiana tabacum* cv. Xanthi nc) were grown in a shaking incubator at 26°C, 80 rpm under continuous light. 100 μM $CuSO_4$ were treated after 4 days of transfer to fresh medium supplemented with 1 mg/l 2,4-D. Cells were harvested by vacuum filtration and Northern blot analyses were performed as described in Materials and Methods. CK is water control.

Expression of defense-related genes in copper-induced cell death of tobacco suspension cultured cells. Cell suspension cultures were developed from TMV-resistant tobacco cultivar Xanthi nc to maintain homogenous conditions for this experiment. Using tobacco cell suspension culture, expression of defense-related genes following copper-induced cell death was examined.

Copper induced cell death at the concentration of 100 μM, and several defense-related genes were expressed (Fig. 4). The copper-induced cell death was biphasic. The first phase of rapid cell death was triggered within 6 hr of copper treatment, and the second phase of cell death occurred within next 24 hr after copper application (Fig. 4A). Maximum cell death was detected 48 hr after addition of copper to the cell suspension.

The above data indicated that $CuSO_4$ treatment induced similar set of defense-related gene transcripts accumulation compared to TMV inoculation, but differed in extents and patterns. To invesigate the expression patterns of defense-related genes in tobacco cell suspension culture during copper-induced cell death, the same set of defense-related gene expression was monitored. Northern blot analyses following treatment of copper to tobacco suspension cell culture revealed that cultured cells showed different patterns of defense-related gene expression from those of whole plants. Expression of GST gene was strongly induced within 1.5 hr of copper treatments, but low level expression of chitinase and SAR 8.2 was detected. But in most of the cases, expression of defense-related genes which were detectable when whole plants were used as experimental materials were barely detectable (data not shown). This result indicates that cultured cells may respond to heavy metal ion in differnt ways from those of whole plants.

Discussion

HR is the most common feature of plant defense reactions triggered by all classes of pathogens such as viruses, bacteria, and fungi. In addition to such biotic challenges, some abiotic stress including heavy metal ion treatment also induces HR-like symptoms on the plant tissues. We have studied molecular mechanisms involved in the HR-associated cell deaths of tobacco plants. In the present study, we have observed that several defense-related genes are coordinately expressed during the TMV-triggered HR reaction and heavy metal-induced cell death in tobacco plants.

Both TMV-infected and copper-treated tobacco plant exhibited significant induction of PR-protein genes, secondary metabolite producing genes, oxidative bust-related genes, and a wound-inducible protease inhibitor. PR proteins examined in this study include PR-1 (acidic form), β-1,3-glucanase (acidic form), chitinase (acidic form), osmotin-like protein (PR-5), and SAR 8.2. Among the PR-protein genes, PR-1 gene expression is one of the unique features of disease resistance in infected plants (Ward et al., 1991). Our results showed that expression of PR-1 gene was induced by copper-treatment at an early stage, which was observed at 48 hr after inoculation in the pathogen-induced HR-cell death in tobacco. Expression of glucanase, chitinase, and osmotin genes is known to be unique in defense responses. Yun et al. (1996) previously reported that glucanase was also induced by copper treatment. Transcripts of these genes were also strongly detected at 48 to 72 hr in the TMV-infectced plant, exhibiting similar expression patterns as observed for PR-1. Expression pattern of the three genes induced by copper treatment was also similar to that of PR-1 in terms of time course and intensity of accumulated mRNA.

Genes encoding enzymes involved in secondary metabolism such as PAL and HMGR were induced at a late stage of defense response in TMV-induced HR. Expression of the genes was earlier upon copper treament than upon TMV infection. Expression of the genes were significantly induced by copper treatment, but did not reach at the highest level when PR-genes were fully expressed.

It has been reported that hypersensitive cell death is accompanied by generation of AOS including H_2O_2 (Tenhaken, et al., 1995). Recently, accumulated evidences have indicated that TMV-infection induces oxidative burst in the hypersensitively reacting cells. The AOS could serve as important protectants against invading pathogens, and could also be the signal activating further plant defence reactions including the HR of infected cells (Tenhaken et al., 1995).

H_2O_2 is stable and less reactive than O_2^-. However, in the presence of reduced transition metals, H_2O_2-dependent formation of $\cdot OH$ and O_2^- can occur and the O_2^- can act as the initial reducing agent for the metal. Under conditions normally found in plant cells, this reaction, however, proceeds rather slowly, and is ineffective in producing substantial amounts of $\cdot OH$. On the other hand, significant level of $\cdot OH$ could be formed through the cycle of reactions involving oxidation of transition metals such as Fe^{2+} or Cu^+ via Fenton reaction (Hammond-Kosack and Jones, 1996). In addition, highly reactive hydroxyl radicals are formed upon direct reaction of H_2O_2 and $\cdot O_2^-$.

Our results indicated that the HR-cell death of tobacco plants infected with TMV is accompanied by an increased level of transcripts of oxidative burst-related genes including APX and GST. Copper-treatment also induced and increased expression of the genes at the region of HR-like cell death of tobacco leaf without wounding effect. The gene expression was induced more rapidly upon the copper-treatment than upon the TMV-infection.

Furthermore, we observed more clearly in experiments using tobacco suspension culture cells that copper actually

induced both cell death and expression of several genes related to the HR-cell death. The suspension cultures are physiologically more homogeneous than differentiated leaves, and thus show a more uniform biochemical response to stress than the heterogeneous cell populations in intact plants. Significant increase of mRNA level of the defense-related genes was detected at an early stage and then reached to a maximum level at 6 to 12 hr in the suspension culture. In particular, expression of GST gene was detected at 1.5 hr, and reached at the highest level at 3 hr, and then gradually reduced 9 hr after the copper-treatment.

All together, these results suggest that molecular mechanisms involved in cell death triggered either by pathogens or heavy metal ions are similar to each other. Possibly, AOS generated by either stress may cause the cell death. In addition, similarity of expression patterns of various defense-related genes upon the two different stresses might be a consequence of the similarity of the cell deaths.

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References

Atkinson, M. M., Keppler, L. D., Orlandi, E. W., Baker, C. J. and Mischke, C.F. 1990. Involvement of plasma membrane calcium influx in bacterial induction or the K+/H+ exchange and hypersensitivce responses in tobacco. *Plant Physiol.* 92:215-221.

Baker, C. J. and Orlandi, E. W. 1995. Active oxygen in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:299-321.

Choi, D., Kim, H. M., Yun, H. K., Park, J. A., Kim, W. T. and Bok, S. H. 1996. Molecular cloning of a metallothionein-like gene from *Nicotiana glutinosa* L. and its induction by wounding and tobacco mosaic virus infection. *Plant Physiol.* 112: 353-359.

Choi, D., Ward, B. L. and Bostock, R. M. 1992. Differential induction and suppression of potato 3-hydrxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. *Plant Cell* 4:1333-1344.

Dorey, S., Baillieul, F., Pierrel, M.-A., Saindrenan, P., Fritig, B. and Kauffmann, S. 1997. Spatial and temporal induction of cell death, defense genes, and accumulation of salicylic acid in tobacco leaves reaction hypersensitively to a fungal glycoprotein elicitor. *Mol. Plant-Microbe Interact.* 10:646-655.

Goodman, R. N. and Novacky, A. J. 1994. The hypersensitive reaction in plants to pathogens: A resistance phenomenon. American Phytopathological Society, St. Paul, MN.

Hammond-Kosack, K. E. and Jones, J. D. G. 1996. Resistance gene-dependent plant defense responses. *Plant Cell* 8:1773-1791

Kang, M.-K., Park, K.-S. and Choi, D. 1998. Coordinated expression of defense-related genes by TMV infection or salicylic acid treatment in tobacco. *Mol. Cells* 8:388-392.

Lamb, C. J. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* 76:419-422.

Malamy, J. and Klessig, D. 1992. Salicylic acid and plant disease resistance. *Plant J.* 2: 643-654.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tisssue cultures. *Physiol. Plant* 15:473-497.

Ryals, J., Uknes, S. and Ward, E. 1996. Systemic acquired resistance. *Plant Cell* 8:1809-1819.

Tenhaken, R., Levine, A., Brisson, L. F., Dixon, R. A. and Lamb, C. 1995. Function of the oxidative burst in hypersensitive disease resistance. *Proc. Natl. Acad. Sci.* USA 92:4158-4163

Turner, J. G. and Novacky, A. 1974. The quantitative relationship between plant and bacterial cells involved in the hypersensitive reaction. *Phytopathology* 64:885-890.

Tzeng, D. D. and De Vay, J. E. 1993. Role of oxygen radicals in plant disease development. *Adv. Plant Pathol.* 10:1-34.

Ward, E. R., Uknes, S. J., Williams, S .C., Dincher, S.S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Metraux, J.-P. and Ryals, J. A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3:1085-1094.

Yun, H. K., Yi, S. Y., Kim, S. U., Son, K. H., Yu, S. H., Bok, S. H. and Choi, D. 1996. Molecular cloning and induction of β-1,3-glucanase gene from *Nicotiana glutinosa* L. *Mol. Cells* 6:422-428.

Yun, H.-K., Yi, S.-Y., Yu, S.-H. and Choi, D. 1998. Cloning of a pathogenesis-related protein-1 gene from *Nicotiana glutinosa* L. and its salicylic acid-independent induction by copper and β-aminobutyric acid. *J. Plant Physiol.* (in press).