Symbiosis, 25 (1998) 145–157
Balaban, Philadelphia/Rehovot

Glomus intraradices Colonization Regulates Gene Expression in Tobacco Roots

IDIT GINZBERG[1], RAKEFET DAVID[1], ORNA SHAUL[1], YIGAL ELAD[1], SMADAR WININGER[1], BRORIA BEN-DOR[1], HANNA BADANI[1], YIWEN FANG[2], PIETERNEL VAN RHIJN[2], YING LI[2], ANN M. HIRSCH[2], and YORAM KAPULNIK[1*]

[1]*Institute of Field and Garden Crops, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel, Tel. +972-3-9683461, Fax. +972-3-9669642, E-mail. kapulnik@agri.huji.ac.il; and*
[2]*Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, CA 90095-1606, USA*

Received July 30, 1997; Accepted November 30, 1997

Abstract

Mycorrhizae are the structural unit resulting from the symbiotic interaction between soil fungi and plant roots, and are accompanied by considerable alteration of the metabolism of both partners. In this study changes in the gene expression of *Glomus intraradices* inoculated tobacco roots were detected following the application of the plant defense chemical activators 2,6-dichloroisonicotinic acid (INA) or (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH). In response to mycorrhizal fungal colonization, mRNA steady state level of the pathogen-related protein 1a (PR-1a) and the protein accumulation of PR-1a and basic chitinase were reduced. The suppression of PR-1a and basic chitinase coincided with a significant increase in the level of Zeatin riboside (ZR)-like cytokinin compounds in mycorrhizal roots compared to non-mycorrhizal controls. Application of kinetin and 6-benzylaminopurine (BAP) mixture at 10^{-5} M each, to tobacco leaves resulted in a significant reduction in PR-1a and basic chitinase protein levels. The possible link between the altered expression of these specific plant genes and the elevated cytokinin level in AM fungus-colonized plants is discussed.

Keywords: Arbuscular mycorrhizae (AM), pathogen-related (PR) proteins, cytokinin

Presented at the Second International Congress of Symbiosis, April 13–18, 1997, Woods Hole, MA

*The author to whom correspondence should be sent.

0334-5114/98/$05.50

1. Introduction

Vesicular arbuscular mycorrhizal (AM) fungi have been recognized as obligate symbionts of about 80% of the land plants in most ecosystems. The symbiosis is biotrophic and normally mutualistic; the compatible interactions are based on nutritional transfer between the symbiotic partners. Although no morphological changes are observed in the colonized root, substantial modifications take place inside that tissue, with the development of elaborate intracellular fungal structures (arbuscules and vesicles) in the host cell (Bonfante-Fasolo, 1988). Moreover, changes in plant gene expression have been described during the early stages of the mycorrhizal symbiosis (van Rhijn et al., 1997), and low or transient defense responses seem to be activated in endomycorrhizae (Bonfante-Fasolo and Perotto, 1990; Harrison and Dixon, 1994; Gianinazzi-Pearson et al., 1995; Kapulnik et al., 1996). In general, few so-called "defense transcripts" or defense-related molecules have been found in mycorrhizal *Phaseolus vulgaris* L., *Medicago truncatula* and *M. sativa* roots (Lambais and Mehdy, 1993; Harrison and Dixon, 1993; Volpin et al., 1994; 1995). Recently, we showed down-regulation of the expression of a tobacco basic chitinase gene during the early stages of the establishment of the AM fungus symbiosis (David et al., 1998).

The mechanism governing the modulation of plant-defense-related gene expression during AM colonization is not understood. One possibility is that a symbiotic function(s), which is induced following mycorrhizal establishment, has the capacity to alter the plant's defense mechanisms. However, as yet, no such gene(s) or product(s) has been identified. Another possibility is that arbuscular fungi induce changes in the phytohormonal balance of the roots, leading to modulated gene expression (Lambais and Mehdy, 1993). Host-AM fungus associations result in elevated levels of ABA, cytokinin or gibberellin-like substances in various plant organs (Allen et al., 1980, 1982). Others (Smith and Gianinazzi-Pearson, 1988; Schwab et al., 1991) have noted that hormones are involved in establishing or regulating most of the growth changes observed following mycorrhization of the host plant (Danneberg et al., 1992; Beyrle, 1995), and plant hormones may influence other plant responses as well. In fact, significant reductions were noted in the plant pathogenesis-related (PR) enzyme, chitinase, and its mRNA level in cultured tobacco following elevation of cytokinin in the medium (Shinshi et al., 1987). However, this phenomenon has not yet been reported in whole-plant studies.

In plants, cytokinins are potent growth factors, necessary for cell growth and differentiation. In addition, it has been hypothesized that they activate specific genes and modify the composition of the mRNA pool in plant cells. However, little is known about cytokinin biosynthesis in the plant or its

mechanism of action. An interaction between cytokinin and auxin has been proposed but exactly how these two phytohormones mediate development, especially in the roots, is unclear.

In this study, the capacity of *Glomus intraradices* to alter plant gene expression was examined, and its ability to enhance cytokinin levels in colonized roots demonstrated. Earlier, we found transcripts for *MsENOD2* and *MsENOD40*, two early nodulin genes, in cytokinin-treated and mycorrhizal fungus colonized alfalfa-roots (van Rhijn et al., 1997). In this paper, we report that the PR proteins PR-1a and basic chitinase are down-regulated in AM-colonized tobacco roots, and that this down-regulation coincides with mycorrhizal-fungus colonization and enhanced cytokinin levels.

2. Materials and Methods

Plant culture

Surface-sterilized seeds of *Nicotiana tabacum* cv. Xanthi nc were grown under axenic conditions (Patterson et al., 1990) in 0.5-kg pots containing autoclaved sand, as specified by Volpin et al. (1994). The pots were watered to field capacity twice a week with a modified Johnson solution (Johnson et al., 1957) containing 8 mM NH_4NO_3 and 0.1 mM KH_2PO_4 which permitted similar growth patterns of AM and non-AM plants. Semi-synchronous infection by AM fungi was performed by applying 300–500 spores of *G. intraradices* NPI strain 26 in a layer 4 cm below the soil surface, prior to sowing. Control plants were treated in the same way except for the application of spores; no fungal colonization was detected in the controls during the experiments.

In experiments where the synthetic chemicals (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Friedrich et al., 1996) or 2,6-dichloroisonicotinic acid (INA) (Vernooij et al., 1995) were used to induce the expression of PR proteins, the treated plants received a single application of a solution containing 50 ppm of the chemical by watering or by foliar spraying, 4 or 2 days prior to the first sampling, respectively.

For cytokinin detection, Zeatin riboside (ZR) was extracted from a 1-cm root segment collected from the infected area (3–4 cm below the soil surface) and its level was quantified by radioimmunoassay (RIA) using polyclonal antibodies as described by van Rhijn et al. (1997). Results were expressed as the amount of ZR per gram fresh weight.

The cytokinins kinetin and 6-benzylaminopurine (BAP) (Sigma, MO, USA) were applied to leaves by infiltration at a concentration of 10^{-5} M following the method described by Klement (1963).

RNA extraction and Northern blot analysis

RNA was extracted from a 1-cm root segment collected from the infected area (3–4 cm below the soil surface) using Tri-Reagent (Molecular Research Center Inc., Ohio, USA). Samples of 10 µg total RNA were applied to a 1.1% formamide-agarose gel (Sambrook et al., 1989), followed by blotting to a nylon membrane (Hybond N, Amersham, UK). A PR-1a clone (Payne et al., 1988; kindly donated by J. Ryals, Novartis, USA), and tomato rDNA (kindly donated by E. Lifschitz, Technion, Israel) were used as probes. Hexamer-labeling of the probes and hybridization procedures were performed as described by Sambrook et al. (1989). Blots were exposed to either X-ray film with an intensifying screen at –70°C, or a Phosphor-Imager screen for quantification of radioactivity using the Phosphor-Imaging Program (Fujix BAS1500; Fuji, Japan).

Protein extraction and Western blot analysis

Proteins were extracted from a 1-cm root segment from the infected area of the same plants that were analyzed for mRNA levels. Root tissue (0.1 g) was ground in phosphate-buffered saline (PBS) pH 7.0, containing 'protease-inhibitor-cocktail tablets' (Complete; Boehringer Mannheim, Germany) according to the manufacturer's protocol. Protein samples, of 10 µg, as determined by Bradford assay (Bradford, 1976), were loaded onto a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose filter (Gelman, USA) using BIO-RAD equipment, according to their manual. Western assays were performed with polyclonal antibodies (kindly provided by R. Fluhr, Weizmann Institute of Science, Rehovot, Israel) and secondary peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Lab Inc., PA, USA), using the ECL Kit (Amersham) for detection according to the manufacturer's protocol.

3. Results

Plant growth and fungal colonization

To study changes in the expression of PR-1a and basic chitinase genes during establishment of AM symbiosis, tobacco plants were grown in the presence of *G. intraradices* (NPI, isolate 26). Seventy two percent of the inoculated tobacco roots were colonized 5 weeks after sowing. The root cortex contained numerous arbuscules and vesicles but no new spores (data not shown). Mycorrhizal and non-mycorrhizal plants showed the same growth rate pattern as reflected by roots and shoots biomasses (Table 1).

Table 1. The effect of AM fungal inoculation on plant dry weight accumulation. Tobacco plants were grown for 5 weeks after sowing, in dune sand watered with low P nutrient solution. Numbers are means of five replicates ± SE.

Treatment	Shoot (dry weight) mg/plant	Root (dry weight) mg/plant
–AM	60 ± 8	20 ± 3
+AM	62 ± 5	22 ± 4

Expression of PR-1a in mycorrhizal and non-mycorrhizal tobacco roots

Western blot analysis was used to investigate the expression of the PR-1a protein in mycorrhizal and non-mycorrhizal roots of tobacco plants. PR-1a was not expressed constitutively in tobacco roots (Fig. 1A, B, –BTH). Thus, in order to examine the effect of AM fungus on PR-1a suppression, plants were treated with BTH to induce PR-1a expression. BTH was applied to 21-day-old mycorrhizal and non-mycorrhizal plants, and the level of PR-1a protein was monitored at different times after application. PR-1a protein accumulated to high levels in BTH-treated, non-mycorrhizal roots 24 days after application (45 days after sowing), but no accumulation of this protein was detected in BTH-treated mycorrhizal roots (Fig. 1A, +BTH). Northern analysis showed a similar pattern of PR-1a expression (Fig. 1B, C). Taken together, these results suggest that *G. intraradices* colonization of the plant roots is involved with suppression of PR-1a gene expression.

Expression of basic chitinase in mycorrhizal and non-mycorrhizal tobacco roots

In earlier work we demonstrated that AM colonization suppressed basic chitinase gene expression in tobacco roots following BTH application (David et al., 1998). In order to examine the effect of AM fungal infection on basic chitinase accumulation, the chemical INA was used (Friedrich et al., 1996) as an alternative inducer of PR proteins. When plants were watered with 50 ppm INA, significant accumulation of the basic chitinase protein was observed in the control plants (Fig. 2, +INA, –AM), but the level of this protein in INA-treated mycorrhizal plants was significantly lower (Fig. 2, +INA, +AM). These data are consistent with the detection of basic chitinase mRNA and protein levels observed earlier by David et al. (1998), using the BTH as the inducer of PR proteins.

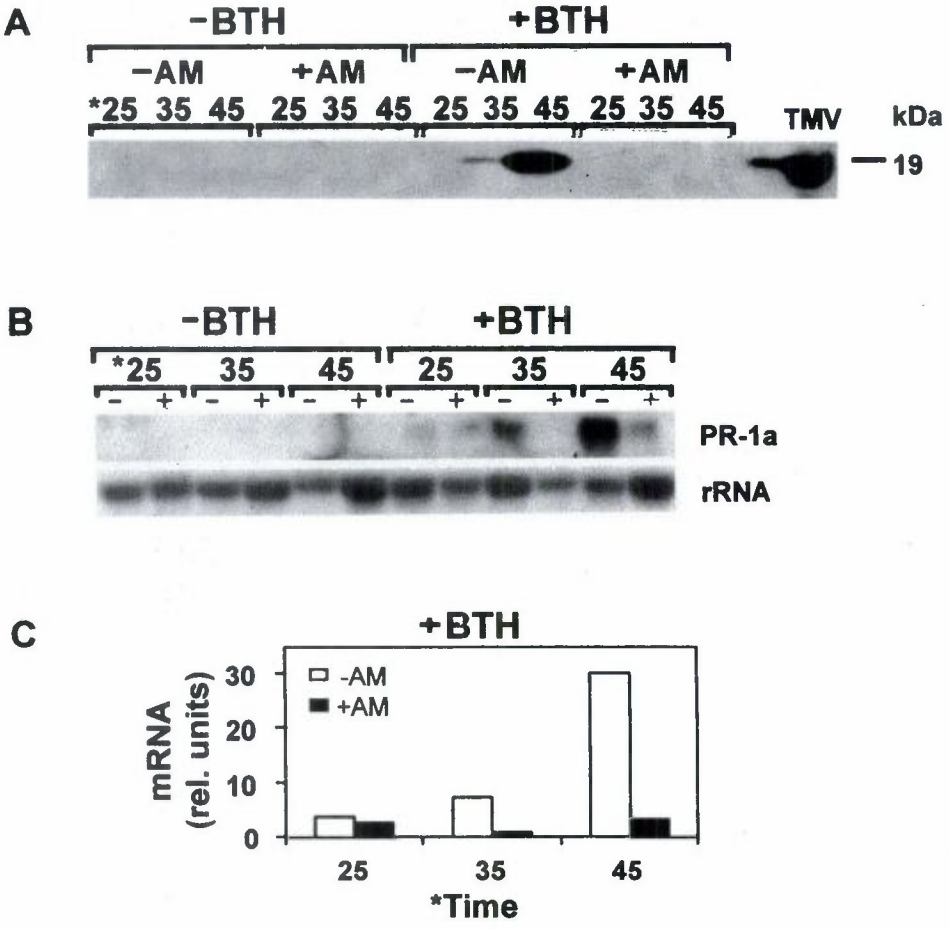
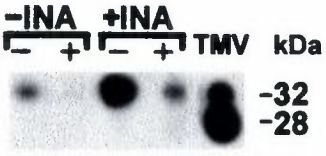


Figure 1. Down-regulation of PR-1a expression. Proteins and total RNA were extracted from BTH-treated or non-treated roots of mycorrhizal (+AM) and non-mycorrhizal (–AM) tobacco plants 25, 35, and 45 days after sowing. Protein samples (10 μg) were analyzed by Western blotting probed with polyclonal antibodies against tobacco PR-1a, using an extract from TMV-infected leaves as the marker (A). Northern blot analysis with 10 μg of total RNA probed with PR-1a, and with tomato rDNA (B). Radioactivity values were analyzed with the Phosphor-Image Program, and normalized according to the rRNA content of each sample. The PR-1a mRNA steady-state level in mycorrhizal roots (black boxes) was compared with its level in non-mycorrhizal roots (white boxes) (C).



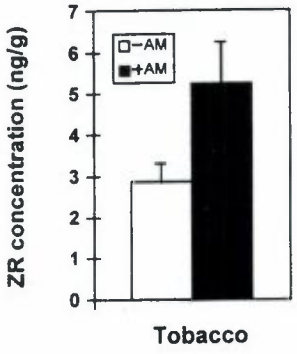


Fig. 2 Fig. 3.

Figure 2. Reduced levels of tobacco basic chitinase. Proteins were extracted from INA-treated or non-treated roots of mycorrhizal (+) and non-mycorrhizal (–) tobacco plants 35 days after sowing. Protein samples (10 μg) were analyzed by Western blot probed with polyclonal antibodies against tobacco chitinase, using an extract from TMV-infected leaves as the 32-kDa basic chitinase and 28-kDa acidic chitinase markers.

Figure 3. The effect of AM fungus colonization on the concentration of ZR-like cytokinins in tobacco roots. Analysis was carried out in 5-weeks-old plants. Vertical bars represent SE.

Effect of AM fungi on cytokinin production in tobacco roots

The effect of AM fungus colonization on cytokinin level was tested in tobacco roots. Extracts of 5-week-old tobacco plants contained 1.6 times more ZR-like cytokinin compounds in the mycorrhizal roots than in the non-mycorrhizal control roots (Fig. 3).

Cytokinin affects basic chitinase and PR-1a accumulation

Western blots were used to investigate the PR-1a, acidic chitinase and basic chitinase levels in tobacco leaves following cytokinin application. Infiltration of water or a mixture of cytokinins (at 10^{-5} M) into the first expanded tobacco leaf was followed 48 h later by INA application (foliar spray) to induce PR proteins. Two and four days after the INA application, a significant reduction in PR-1a and basic chitinase accumulation was observed in cytokinin (CK)-treated leaves as compared to the non treated (C) or water infiltrated (H_2O) controls (Fig. 4). The level of the acidic chitinase accumulation was affected to a lesser extent by the cytokinin application at both sampling dates (Fig. 4).

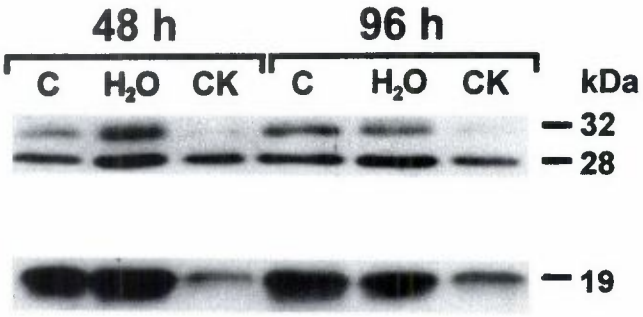


Figure 4. PR-1a and chitinase protein levels followed cytokinin mixture application. Tobacco leaves were infiltrated with a mixture of cytokinins consisted of kinetin and BAP at 10^{-5} M each (CK), with water (H_2O), or left untreated (C), and two days later sprayed with 50 ppm INA. After 48- and 96 h following the INA treatment, protein extracts (10 μg) were analyzed by Western blots probed with polyclonal antibodies against 32 kDa basic chitinase, 28 kDa acidic chitinase or 19 kDa acidic PR-1a.

4. Discussion

Mycorrhizal fungal colonization in tobacco roots resulted in significant suppression of PR-1a gene expression and protein accumulation relative to non-mycorrhizal roots. Likewise, a reduction in basic chitinase accumulation was observed in the mycorrhizal roots. Coordinated regulation of gene transcription and protein level has also been observed in mycorrhizal bean (Lambais and Mehdy, 1993) and alfalfa (van Rhijn et al., 1997) roots, however, this report considers the suppression of not only a constitutively expressed gene (basic chitinase), but also of a chemically induced gene (PR-1a) in tobacco roots (see Figs. 1 and 2). Although the exact function of PR-1a is unknown, it is considered to have anti-pathogenic activity: constitutive high-level expression of PR-1a in transgenic tobacco resulted in tolerance to infection by two oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* (Alexander et al., 1993).

Mycorrhizal fungus colonization in tobacco and alfalfa roots resulted in significantly elevated levels of ZR-type cytokinins relative to non-mycorrhizal control roots (van Rhijn et al., 1997; this work). The enhancement in ZR-type cytokinin levels was evident 5 weeks after germination of tobacco, while any morphological changes could have been observed in the inoculated plant. An increase in the number of cytokinin-like molecules has been previously observed in some monocotyledonous (Allen et al., 1980) and dicotyledonous (Dixon et al., 1988) plants colonized by AM fungi. The

enhancement of cytokinin production in mycorrhizal Citrus trees was not related to the ability of AM fungi to improve plant uptake of mineral nutrients, mainly phosphorus (Dixon et al., 1988). Moreover, several studies have shown that the endomycorrhizal fungus, *G. mosseae* (Barea and Azcon-Aguilar, 1982) and some ectomycorrhizal fungi are very good producers of cytokinin *in vitro* (Crafts and Miller, 1974). Other ectomycorrhizal fungi, which failed to release cytokinin-like substances in culture media, increased cytokinin activity in needles of Pinus (Beyrle, 1995), leading to the hypothesis that this activity may be internally regulated in the plant. It is also difficult to extrapolate data from *in vitro* studies to intact roots where complex combinations of metabolites may interact. At present, we cannot rule out the possibility that the elevated cytokinin levels obtained in tobacco roots were related to the ability of *G. intraradices* to produce the compound(s) in the plant roots. If so, the mechanisms by which these changes occur need to be elucidated.

An elevated level of cytokinin in mycorrhizal roots may elicit additional biochemical activity in the infected roots, some of which could be related to plant growth and development. For example, high cytokinin levels can enhance both photosynthesis and transpiration rates by opening stomata (Incoll and Whitelam, 1977), influencing transport (van Steveninck, 1976), and regulating chlorophyll levels (Richmond and Lang, 1957). Moreover, cytokinins have been shown to facilitate phosphorus utilization in tomato plants (Menary and van Staden, 1976). High levels of cytokinin reduce plant resistance to fungal invasion and delay the induction of the hypersensitive reaction (HR) of tobacco callus to fungal zoospores (Haberlach et al., 1978) or potato tuber to *Phytophthora infestans* (Beckman and Ingram, 1994). The mechanism by which plant cytokinin contributes to the suppression of both the HR and disease resistance is not yet understood; however, chitinase and glucanase activities and their transcript levels have been found to be significantly reduced in tobacco tissues, following alterations in the auxin/cytokinin balance (Felix and Meins, 1986). Furthermore, endochitinase and β-1-3-endoglucanase enzyme activities and their mRNA levels were reduced in mycorrhizal bean roots at high and low P levels, as compared with non-mycorrhizal controls (Lambais and Mehdy, 1993). Nevertheless, it is still possible that mycorrhizal fungi have the capacity to produce a non-hormone suppressor of the plant-defense mechanism, similar to the hypersensitive inhibiting factor isolated from *Phytophthora infestans* (Doke et al., 1979).

The fact that PR-1a and chitinase are induced in plants infected by pathogens has led to the hypothesis that they may play some role in the plant-defense reaction (Bell, 1981). In this study, we demonstrated down-regulation of PR-1a and basic chitinase gene products following AM fungus colonization. Shinshi et al. (1987) demonstrated blocked accumulation of

tobacco basic chitinase mRNA due to alteration in auxin and cytokinin levels in tobacco tissue. It is tempting to speculate that the elevated level of cytokinin found in mycorrhizal roots suppresses the induction of some PR-protein genes. A direct application of cytokinins to tobacco leaves resulted in reduced accumulation of chitinase and PR-1a proteins (Fig. 4 and Shaul et al., personal communication). However, the influence of cytokinin on PR gene induction needs to be directly confirmed in tobacco roots at physiological concentrations of the hormone.

In conclusion, changes in hormonal levels of mycorrhizal roots appear to present a new class of secondary plant responses to AM fungus colonization. Whether the alteration in gene expression during the plant-fungus colonization has a functional role in the AM fungus symbiosis or whether it is elicited by a non specific signal transduction pathway, remains to be clarified.

Acknowledgments

We thank Dr. J. Ryals for the PR-1a clone, Prof. E. Lifschitz for the tomato rDNA clone and Prof. R. Fluhr for polyclonal antibodies. This work was partially supported by GIF project No. G-0388-199.12 to YK and by NSF 96-30842 to AMH.

REFERENCES

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., and Ryals, J.A. 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proceeding of the National Academy of Science USA* **90**: 7327–7331.

Allen, M.F., Moore, T.S. Jr., and Christensen, M. 1980. Phytohormone changes in *Bouteloua gracilis* infected by vesicular-arbuscular mycorrhizae. I. Cytokinin increases in the host plant. *Canadian Journal of Botany* **58**: 371–374.

Allen, M.F., Moore, T.S. Jr., and Christensen, M. 1982. Phytohormone changes in *Bouteloua gracilis* infected by vesicular-arbuscular mycorrhizae. II. Altered levels of gibberellin-like substances and abcisic-acid in the host plant. *Canadian Journal of Botany* **60**: 486–471.

Barea, J.M. and Azcon-Aguilar, C. 1982. Production of plant growth-regulating substances by vesicular-arbuscular mycorrhizal fungus *Glomos mosseae*. *Applied and Environmental Microbiology* **43**: 810–813.

Beckman, K.B. and Ingram, D.S. 1994. The inhibition of the hypersensitive response of potato tuber tissues by cytokinins: similarities between senescence and plant defence responses. *Physiological and Molecular Plant Pathology* **44**: 33–50.

Bell, A.A. 1981. Biochemical mechanisms of disease resistance. *Annual Review of Plant Physiology* **32**: 21–81.

Beyrle, H. 1995. The role of phytohormones in the function and biology of mycorrhizas. In: *Mycorrhiza Structure, Function, Molecular Biology and Biotechnology*. A. Varma and B. Hock, eds. Springer-Verlag, Berlin, Heidelberg, pp. 365–390.

Bonfante-Fasolo, P. 1988. The role of the cell-wall as a signal in mycorrhizal associations. In: *Cell to Cell Signals in Plant, Animal and Microbial Symbiosis*, NATO ASI Ser., Vol. 17. S. Scannerini, D.C. Smith, P. Bonfante-Fasolo, and V. Gianinazzi-Pearson, eds. Springer-Verlag, Berlin, pp. 219.

Bonfante-Fasolo, P. and Perotto, S. 1990. Mycorrhizal and pathogenic fungi: do they share any features? In: *Electron Microscopy Applied in Plant Pathology*. K. Mendgen and E.D. Lesemann, eds. Springer, Berlin, Heidelberg, New York ,Tokyo, pp. 265–275.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248–254.

Crafts, C.B. and Miller, C.O. 1974. Detection and identification of cytokinins produced by Mycorrhizal fungi. *Plant Physiology* **54**: 586–588.

Dannenberg, G., Latus, C., Zimmer, W., Hundeshagen, B., Schneider-Poetsch, H.J., and Bothe, H. 1992. Influence of vesicular-arbuscular mycorrhiza on phytohormone balances in maize (*Zea mays* L). *Journal of Plant Physiology* **141**: 33–39.

David, R., Itzhaki, H., Ginzberg, I., Gafni, Y., Galili, Y., and Kapulnik, Y. 1998. Suppression of tobacco basic chitinase gene expression in response to colonization by the arbuscular mycorrhizal fungus *Glomus intraradices*. *Molecular Plant-Microbe Interactions*, in press.

Dixon, R.K., Garrett, H.E., and Cox, G.S. 1988. Cytokinins in the root pressure exudate of *Citrus jambbiri* Lush colonized by vesicular-arbuscular mycorrhizae. *Tree Physiology* **4**: 9–18.

Doke, N., Garas, N.A., and Ku'c, J. 1979. Partial characterization and aspects of mode of action of hypersensitivity-inhibiting factor (HIF) isolated from *Phytophthora infestans*. *Physiological Plant Pathology* **15**: 127–140.

Felix, G. and Meins, F. Jr. 1986. Developmental and hormonal regulation of (β-1,3-glucanase in tobacco. *Planta* **167**: 206–211.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Gut-Rella, M., Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, J.P., Kessmann, H., and Ryals, J. 1996. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant Journal* **10**: 61–70.

Gianinazzi-Pearson, V., Gollotte, A., Lherminier, J., Tisserant, B., Franken, P., Dumas-Gaudot, E., Lemoine, M.C., van Tuinen, D., and Gianinazzi, S. 1995. Cellular and molecular approaches in the characterization of symbiotic events in functional arbuscular mycorrhizal associations. *Canadian Journal of Botany* **73**: S526–S532.

Haberlach, G.T., Budde, A.D., Sequeira, L., and Helgeson, J.P. 1978. Modification of disease resistance of tobacco callus tissues by cytokinins. *Plant Physiology* **62**: 522–525.

Harrison, M.J. and Dixon, R.A. 1993. Isoflavonoid accumulation and expression of defence gene transcripts during the establishment of vesicular-arbuscular mycorrhizal associations in roots of *Medicago truncatula*. *Molecular Plant-Microbe Interactions* **6**: 543–654.

Harrison, M.J. and Dixon, R.A. 1994. Spatial patterns of expression of flavonoid/isoflavonoid pathway genes during interaction between roots of *Medicago truncatula* and the mycorrhizal fungus *Glomus versiforme*. *Plant Journal* **6**: 9–20.

Incoll, L.D. and Whitelam, G.C. 1977. The effect of kinetin on stomata of the grass *Anthephera pubescesns Nees*. *Planta* **137**: 243–245.

Johnson, C.M., Stout, P.R., Beyer, J.C., and Carlson, A.B. 1957. Comparative chlorine requirements of different species. *Plant Soil* **8**: 337.

Kapulnik., Y., Volpin, H., Itzhaki, H., Ganon, D., Galili, S., David., R., Shaul, O., Elad, Y., Chet, I., and Okon, Y. 1996. Suppression of defense response in mycorrhizal alfalfa and tobacco roots. *New Phytology* **133**: 59–64.

Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. *Nature* **199**: 299–300.

Lambais, M.R. and Mehdy, M.C. 1993. Suppression of endochitinase, β-1-3-endoglucanase, and chalcone isomerase expression in bean vesicular-arbuscular mycorrhizal roots under different soil phosphate conditions. *Molecular Plant-Microbe Interactions* **6**: 75–83.

Menary, R.C. and van Staeden, J. 1976. Effect of phosphorus nutrition and cytokinins of flowering in the tomato, *Lycopersicon esculentum* Mill. *Australian Journal of Plant Physiology* **3**: 201–205.

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J., and Dennis, E.S. 1990. Chitinase, β-1-3-glucanase, osmotin and extensin are expressed in tobacco explants during flower formation. *Plant Cell* **2**: 673–684.

Patterson, N.A., Chet, I., and Kapulnik, Y. 1990. Effect of mycorrhizal inoculation on nodule initiation, activity and contribution to legume productivity. *Symbiosis* **8**: 9–20.

Payne, G., Parks, T.D., Burkhart, W., Dincher, D., Ahl, P., Metraux, J.P., and Ryals, J. 1988. Isolation of the genomic clone for pathogenesis-related protein 1a from *Nicotiana tabacum* cv. Xanthi-nc. *Plant Molecular Biology* **11**: 89–94.

Richmond, A. and Lang, A. 1957. Effect of kinetin on protein content and survival of detached *Xanthium* leaves. *Science* **125**: 650–651.

Sambrook, J., Maniatis, T., and Fritsch, E.F. 1989. *Molecular Cloning. A Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schwab, S.M., Menge, J.A., and Tinker, P.B. 1991. Regulation of nutrient transfer between host and fungus in vesicular-arbuscular mycorrhizas. *New Phytologist* **117**: 387–398.

Shinshi, H., Mohnen, D., and Meins, F. Jr. 1987. Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. *Proceeding of the National Academy of Science USA* **84**: 89–93.

Smith, S.E. and Gianinazzi-Pearson, V. 1988. Physiological interactions between symbionts in vesicular arbuscular mycorrhizal plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **39**: 221–244.

van Rhijn, P., Fang, Y., Galili, S., Shaul, O., Atzmon, N., Wininger, S., Eshed, Y., Lum, M., Li, Y., To, V., Fujishige, N., Kapulnik, Y., and Hirsch, A.M. 1997. Expression of early nodulin genes in alfalfa mycorrhizae indicates that signal transduction pathways used in forming arbuscular mycorrhizae and *Rhizobium*-induced nodules may be conserved. *Proceeding of the National Academy of Science USA* **94**: 5467–5472.

van Steveninck, R.F.M. 1976. Effect of hormones and related substances on ion transport. In: *Transport in Plants II*, Part B. Luttge, U., and Pittman, M.G., eds. Springer-Verlag, New York, NY. pp. 307–342.

Vernooij, B., Friedrich, L., Ahl-Goy, P., Staub, T., Kessmann, H., and Ryals, J. 1995. 2,6-Dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. *Molecular Plant-Microbe Interactions* **8**: 228–234.

Volpin, H., Elkind, Y., Okon, Y., and Kapulnik, Y. 1994. A vesicular arbuscular mycorrhizal fungus (*Glomus intraradices*) induces a defense response in alfalfa roots. *Plant Physiology* **104**: 683–689.

Volpin, H., Phillips, D.A. Okon, Y., and Kapulnik, Y. 1995. Suppression of an isoflavonoid phytoalexin defense response in mycorrhizal alfalfa roots. *Plant Physiology* **108**: 1449–1454.