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Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi

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ABSTRACT **Enhanced ethylene production is an early response of plants to pathogen attack and has been associated with both resistance and susceptibility to disease. Tobacco plants were transformed with the mutant *etr1–1* gene from *Arabidopsis*, conferring dominant ethylene insensitivity. Besides lacking known ethylene responses, these transformants (Tetr) did not slow growth when contacting neighboring plants, hardly expressed defense-related basic pathogenesis-related proteins, and developed spontaneous stem browning. Whereas hypersensitive resistance to tobacco mosaic virus was unimpaired, Tetr plants had lost nonhost resistance against normally nonpathogenic soil-borne fungi.**

The gaseous plant hormone ethylene is involved in the regulation of various developmental processes encompassing seedling emergence, leaf and flower senescence, fruit ripening and organ abscission, as well as in the reaction to abiotic and biotic stresses (1, 2). Upon wounding and pathogen attack its production is stimulated in the affected tissues, from which it diffuses into surrounding cell layers before escaping into the atmosphere. In the tissue it acts as a local signal, leading to the activation in neighboring cells of adaptive mechanisms that can alleviate the effects of the stress condition. Enhanced ethylene production is an early, active response of plants to the perception of a pathogen attack and appears to be involved in the induction of defense reactions (3). Strong stimulation of ethylene production is a common characteristic of hypersensitive reactions resulting from the incompatible combination of an avirulent pathogen and its resistant host, in which the pathogen is quickly restricted because of localized tissue necrosis near the site of tissue penetration (4). Hypersensitive resistance is associated with the accumulation of antimicrobial phytoalexins and pathogenesis-related proteins (PRs), and with the fortification of cell walls (5). Depending on the plant species, ethylene can induce or stimulate enzymes of aromatic biosynthesis necessary for isoflavonoid phytoalexin production and lignification, as well as promote synthesis of PRs. However, even though exogenous ethylene is sufficient to induce these biochemical and structural alterations, enhanced endogenous ethylene production may not be required for the induction of defense responses (3). In fact, treatment of plants with ethylene or its precursors can reduce or increase disease incidence, depending on the plant–pathogen interaction. One reason may be that stimulation of plant defenses may be offset by induced senescence processes. Thus, treatment of tobacco plants with the ethylene-releasing compound ethephon induces expression of PRs and stimulates the virus-localizing mechanism before infection with tobacco mosaic virus (TMV), whereas the increase in endogenous ethylene production accompanying lesion formation after virus infection accelerates leaf senescence and promotes lesion enlargement (6, 7). In other plant–pathogen combinations ethylene has likewise been associated with increased resistance or susceptibility (3), making its significance in plant–pathogen interactions far from clear. To elucidate the role of ethylene in resistance of tobacco to TMV, we have devised experiments to interfere with ethylene production or perception.

Previously, we were unable to reduce ethylene production in tobacco leaves to sufficiently low levels by transforming plants with antisense gene constructs of the ethylene biosynthetic enzymes 1-aminocyclopropane-1-carboxylic acid (ACC)-synthase and ACC oxidase (8). Because dominant genes conferring insensitivity to ethylene have been characterized in *Arabidopsis thaliana* (9, 10) and tomato (11), genetic modification of ethylene perception was attempted by using the mutant *etr1–1* gene from *Arabidopsis*. The *ETR1* gene appears to be an ethylene receptor (12) and, because of the dominant character of the mutation, *etr1–1* plants lack several ethylene responses present in wild-type plants, such as promotion of seed germination, inhibition of root and hypocotyl elongation, stimulation of peroxidase activity, acceleration of leaf senescence, and feedback suppression of ethylene production (13–15). By expressing the mutant *etr1–1* gene under the direction of viral 35S promoters in tomato and petunia, Wilkinson *et al.* (16) recently demonstrated significant delays in fruit ripening, flower senescence, and flower abscission, indicating that *etr1–1* can function in heterologous plants. By transforming tobacco plants with a gene conferring insensitivity to ethylene, we herewith demonstrate that ethylene perception is required for basic PR-gene expression and nonhost resistance against normally nonpathogenic fungi, whereas it is not required for hypersensitivity to TMV.

MATERIALS AND METHODS

Plant Material and Inoculation. Plants of *Nicotiana tabacum* cv. Samsun NN, resistant to TMV, were grown in the greenhouse with a 16-h period of light. The light intensity was 5,000–6,000 lux and the humidity was maintained at 65%. The temperature was 24°C during the day and 21°C at night. For all experiments, 6- to 9-week-old plants were used. For inoculation with TMV, carborundum-dusted leaves were rubbed with water or virus solution (2.5 μg/ml) and rinsed with water. For fungal inoculation, plants grown in twice-autoclaved soil were

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Abbreviations: PR, pathogenesis-related protein; TMV, tobacco mosaic virus; ACC, 1-aminocyclopropane-1-carboxylic acid.
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inoculated at the stem base by placing four 1-cm diameter plugs taken from a culture of *Pythium sylvaticum* grown for 3 days on potato dextrose agar (PDA).

Transformation of Plants. pBluescript containing the 4.25-kB *Eco*RI fragment encompassing the *etr1–1* promoter and gene (9) was digested with *Pvu*II, and the 7.8-kb product was ligated into the *Sma*I-digested transformation vector pMOG800. pMOG800 contains the NPTII gene for selection on kanamycin and the left and right border for transfer by *Agrobacterium tumefaciens*. This construct was transformed into *Agrobacterium tumefaciens* strain LBA4404 by using electroporation. *Agrobacterium*-mediated leaf disc transformation was carried out as described previously (17). Primary transformants were allowed to self-pollinate. T1 seed was germinated on Murashige and Skoog medium containing 100 μg/ml kanamycin, after which surviving plantlets were transferred to soil. P12 tobacco plants, transformed with the P1 and P2 genes of alfalfa mosaic virus, were used as transgenic control plants. These plants were in all respects phenotypically similar to untransformed Samsun NN tobacco plants, and no differences between untransformed or P12 plants were observed in any of the characteristics investigated.

Ethylene Analysis. In 30-ml vials, four leaf discs of 1 cm diameter were floated on either 10 ml water, 0.01% Tween-20, or 10 ml 1 mM α-aminobutyric acid (AB), 0.01% Tween-20. After incubation for 3 days in the light, ethylene levels were measured by GC as described previously (18). Measurements were performed in triplicate. Ethylene production was expressed relative to the levels obtained from control plants incubated on water.

Triple-Response Assay. For the triple-response assay, surface-sterilized seeds were germinated for 8 days on Murashige and Skoog medium, pH 5.8, with 100 μg/ml kanamycin, 0.7% bactoagar, with or without 20 μM ACC (Sigma) and grown in the dark for 8 days at 25°C.

RNA Analysis. Total RNA was isolated from leaves as described previously (19). For Northern blots, 10 or 20 μg RNA was separated on a 1% agarose gel in 15 mM sodium phosphate, pH 6.5, and transferred to Hybond N (Amersham) filters. Hybridization was performed at 65°C in 250 mM sodium phosphate, pH 7/1 mM EDTA/7% SDS/1% BSA with one of the following randomly labeled probes: (*i*) 544 bp *etr1–1 Eco*RI/*Sst*I DNA (9); (*ii*) 450 bp acidic PR-1a cDNA (20); (*iii*) 793 bp basic PR-1 g cDNA (21) .

Isolation of *Pythium sylvaticum*. Diseased and nondiseased stem parts from Tetr and control plants were surface-sterilized for 1 min in 10% H_2O_2, plated on 1.5% water agar and incubated at 23°C. After 4 to 5 days mycelium from the growing zone was replated on 1.5% PDA. Pure cultures were identified at the Central Bureau for Fungal Cultures (CBS), Baarn, The Netherlands. Additional fungi present in the diseased stem parts were identified at the Dutch Plant Protection Service (PD), Wageningen, The Netherlands.

RESULTS

Construction and Characterization of Tobacco Expressing the *Arabidopsis etr1–1* Mutant Gene. The mutant *etr1–1* gene from *Arabidopsis* containing its own promoter and flanked by sequences of 2.7 kb upstream of the putative transcription initiation site and 1 kb downstream of the polyadenylation site (9) was cloned into the transformation vector pMOG800 containing a kanamycin resistance gene. *Agrobacterium*-mediated transformation of tobacco leaf discs resulted in 21 primary transformants, designated Tetr1 to Tetr21. Expression of the *Arabidopsis etr1–1* gene was examined on Northern blots (Fig. 1). In four randomly selected Tetr transformants, an *etr1–1* transcript of the correct size was present, whereas no cross-hybridizing bands were detectable in non-*etr1–1*-containing control plants. The *Arabidopsis etr1* promoter thus

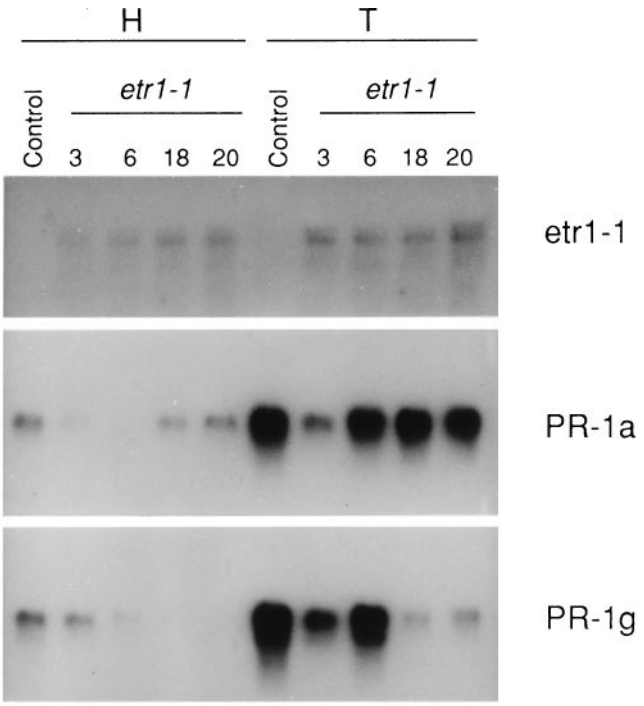

FIG. 1. Northern blot analysis of Samsun NN tobacco and four primary transformants of *etr1–1*. Total RNA was isolated from healthy plants (H) and plants inoculated with TMV 3 days earlier (T). Total RNA was electrophoresed, blotted, and hybridized with *etr1–1*, PR-1a, and PR-1g probes.

is functioning in tobacco. Measurements of ethylene production indicated enhanced basal ethylene production in three of four transformants (Fig. 2). Moreover, application of the chemical inducer AB, which mimics the effect of virus infection (8), stimulated ethylene production to 5- to 10-fold higher levels in the Tetr plants than in the control plants. This higher stimulation conforms to the lack in ethylene feedback control caused by the loss of ethylene perception associated with the *etr1–1* mutation in *Arabidopsis* (13).

The Tetr transformants and their selfed progeny were tested further for absence of typical ethylene effects and other phenotypic characteristics. Germinating seeds of control plants in the dark in the presence of ACC induced the "triple response": the reaction of etiolated seedlings to ethylene consisting of inhibition of both hypocotyl and root elongation, radial swelling of the hypocotyl, and exaggeration of the apical hook (15). In the Tetr seedlings the triple response was completely absent (data not shown), indicating that the *Arabidopsis* mutant *etr1–1* gene is blocking ethylene functioning in tobacco.

When transformed and control plants were grown individually in pots, little difference in the morphology of the plants was noticeable. Tetr plants tended to be somewhat greener than control plants, reflecting a lesser rate of leaf senescence. However, when seedlings were grown together, growth of control plants slowed before leaves of neighboring plants overlapped. These plants remained relatively small and exhibited accelerated leaf senescence (Fig. 3). In contrast, Tetr plants did not appear to perceive their neighbors. They did not show a reduction in growth, resulting in a "crowding effect" of interdigitating leaves, which, moreover, remained fully green. These observations indicate that impaired ethylene responsiveness leads to an impaired perception of neighboring plants.

Ethylene is known to accelerate flower fading, and interference with ethylene perception prolongs vase life of, e.g., carnation (22). Tobacco flowers are short-lived and wilt within 3 days from opening in control plants. However, flowers of

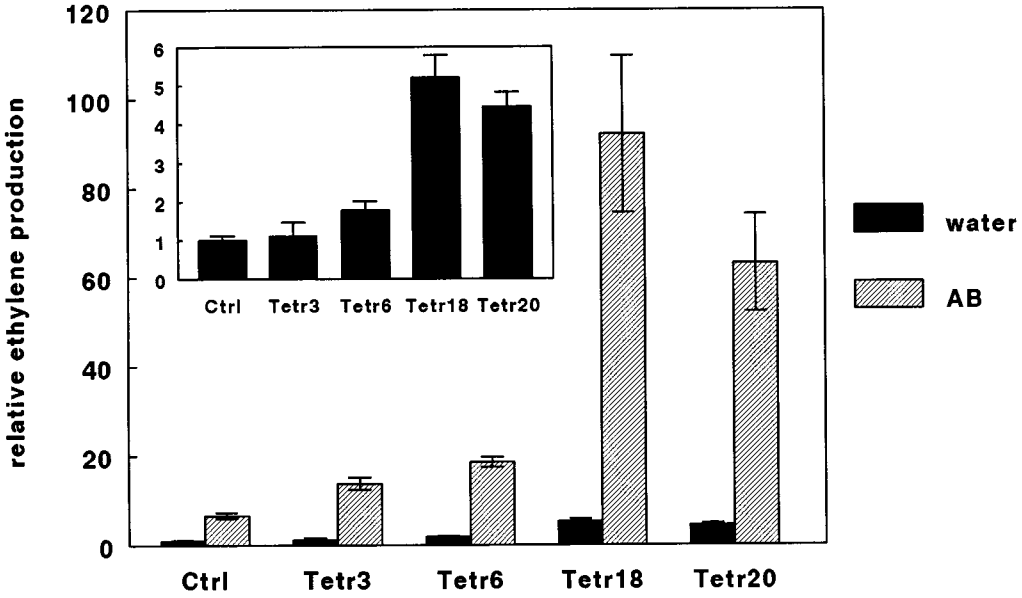

FIG. 2. Endogenous and AB-induced ethylene production of Samsun NN tobacco and four primary *etr1–1* transformants. Relative ethylene levels are expressed as a percentage of endogenous ethylene production of Samsun NN plants. *Insert* shows an expanded view of the endogenous ethylene levels. A relative ethylene production rate of 1 corresponds to 0.12 nmol ethylene/g fresh weight per hr.

Tetr18 plants had a prolonged lifespan, resulting in many flowers blooming simultaneously (Fig. 4). The stage up to flower opening was unaffected (phase a), but flowers of Tetr plants remained turgid for a prolonged period (phase b). Wilting was also slowed, and the corolla did not abscise during seed set, in contrast to control plants.

Ethylene-Insensitive Tobacco Reacts to TMV with Reduced Expression of Basic PR Genes. Because of the presence of the resistance gene *N*, Samsun NN tobacco reacts hypersensitively to TMV. This reaction was fully maintained in the Tetr transformants, indicating that lack of ethylene perception does not affect *N* gene-mediated resistance to virus. Because defense-related gene expression in TMV-infected tobacco involves production of PRs and exogenous ethylene induces PRs in tobacco (23–26), the effect of the mutant *etr1–1* gene on

FIG. 3. Transgenic P12 plants and Tetr18 plants of the same age grown at high density. Control plants develop a "crowding effect," which is absent in the Tetr18 plants.

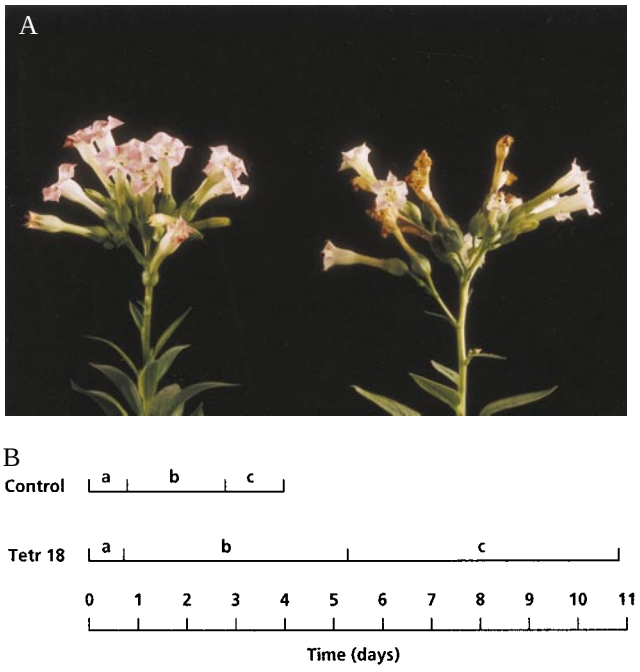

FIG. 4. (*A*) Flowers of P12 plants (*Right*) and Tetr18 plants (*Left*) of comparable age. (*B*) Different stadia of flowering of control and Tetr18 plants. a, unripe but colored flower; b, open mature flower with ripe pollen; c, wilted flower. Data represent the average of 50 flowers.

TMV-induced PR-gene expression was analyzed by using members of the PR-1 and PR-5 families as representatives. Expression of the hardly ethylene-responsive acidic PR-1 genes was little affected in Tetr plants. However, ethylene-inducible basic PR-1g gene expression was strongly reduced in lines 18 and 20 as compared with control plants (Fig. 1). Similar results were obtained for the basic PR-5c (data not shown). This demonstrates that in tobacco, basic PR-gene expression is regulated by ethylene.

Ethylene-Insensitive Tobacco Develops Spontaneous Stem Necrosis During Growth in Soil. Whereas lack of ethylene perception did not affect hypersensitivity to TMV, 12 of the 21 primary transformants spontaneously developed symptoms of disease at arbitrary stages up to flowering. Symptoms started with a browning of the stem base, associated with loss of turgor and wilting of the leaves, progressing into degeneration and necrosis of the basal part of the stem (Fig. 5*A*). These plants finally died, but young shoots were rescued by cutting or grafting to nontransgenic root stock. Eventually, seed was obtained from 15 selfed transformants. Because these symptoms had never before been observed in our plant growth facilities, either with nontransgenic tobacco or with tobacco transformed with other constructs, it was concluded that they correlated with the presence of the *etr1–1* gene.

On average, half of the individual plants ($n = 25$) of the T_1 offspring of several lines tested developed disease symptoms between 3 and 9 weeks after germination. This was irrespective of whether the primary transformed "mother" plant had been diseased or not. This phenomenon was observed at locations in Leiden and Utrecht, either in a growth chamber with fluorescent light or in a greenhouse with natural daylight.

Microscopic examination of transverse sections through the affected stem parts revealed degeneration of cortex tissue and browning of the vascular cylinder (data not shown), reminiscent of the effects of soil-borne fungal pathogens causing damping-off and stem lesions. To avoid any effect of microorganisms that might give rise to such symptoms, the potting soil used for growing the plants was autoclaved two times. Planted in this soil, the mutant *etr1–1*-containing plants grew and developed normally and no disease symptoms became apparent.

To determine which microorganism(s) was responsible for initiating disease, symptom-bearing and nondiseased stem parts of affected plants were surface-sterilized and plated on water agar. From diseased parts, abundant fungal growth ensued, whereas virtually none resulted from nondiseased stem parts. A fungus prevalent on diseased parts and absent from nondiseased parts was identified as *Pythium sylvaticum* Campbell and Hendrix. Additional fungi isolated from diseased Tetr18 plants were *Pythium splendens*, two other *Pythium* spp., *Rhizopus* spp., and *Chalara elegans*.

To analyze whether *P. sylvaticum* was responsible for the disease, 6-week-old Tetr18 and control plants grown in autoclaved soil were inoculated by placing agar plugs from a fungal culture at the stem base. Four to 7 days later symptoms started to develop in the Tetr18 plants, consisting of loss of turgor in the leaves and stem necrosis. Control plants were totally unaffected by *P. sylvaticum* (Fig. 5*B*). From the infected Tetr18 plants a fungus was isolated, which was identified as *P. sylvaticum* Campbell and Hendrix. This demonstrates that *P. sylvaticum* alone was sufficient to cause all symptoms and, thus, was at least one of the biotic factors involved in the disease. The isolation of additional fungi from the diseased plants indicates that Tetr plants had lost nonhost resistance against these soil-borne organisms.

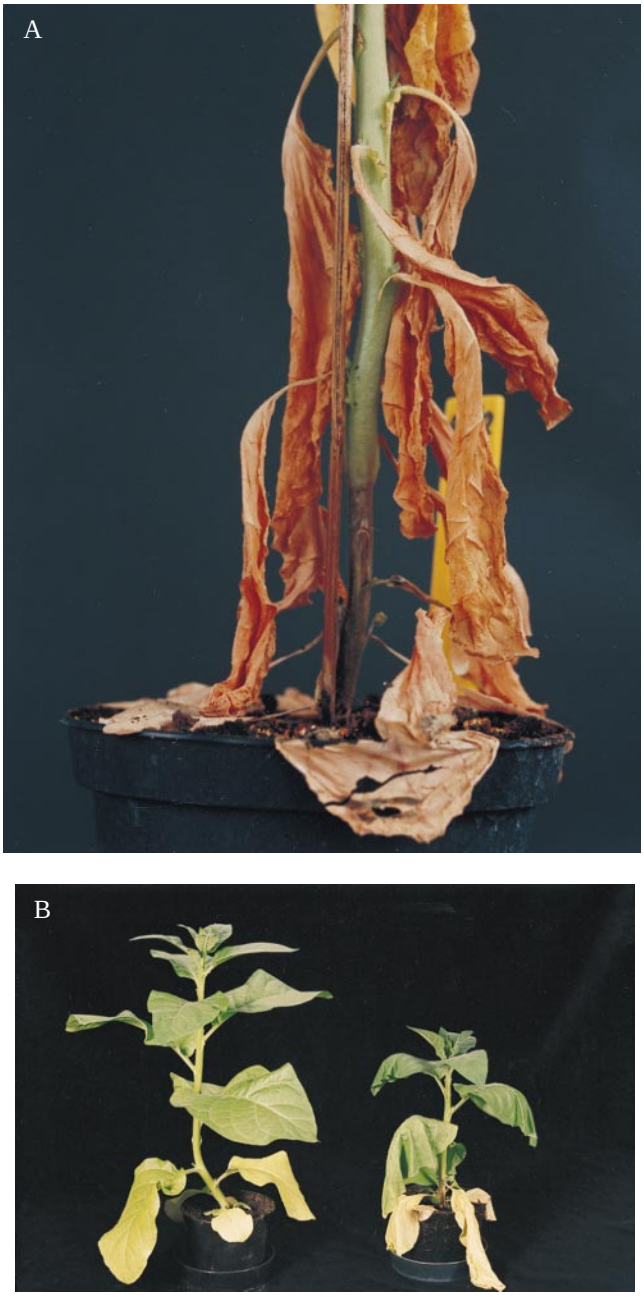

FIG. 5. (*A*) Primary *etr1–1* transformant showing spontaneously developed disease symptoms. (*B*) Control P12 plant (*Left*) and Tetr18 plant (*Right*) 11 days after inoculation with *Pythium sylvaticum* Campbell and Hendrix.

DISCUSSION

We have attempted to gain further insight into the role of ethylene in plant–pathogen interactions. In the tobacco plants transformed with the *Arabidopsis* mutant *etr1–1* gene under its own promoter, this gene was highly expressed. A homolog of *ETR1* with high sequence similarity to the *Arabidopsis* gene has been identified in tobacco (27), supporting the conservation at the level of ethylene perception seen by others (28). Consequently, clear phenotypic effects were expected. Besides confirming that interference with ethylene perception through transformation with the mutant *etr1–1* gene from *Arabidopsis* abolishes the triple response, retards leaf senescence, and increases flower longevity in a heterologous plant, our results demonstrate novel functions for ethylene in the interaction of the plant with its biotic environment.

First, Tetr plants did not slow growth when encountering neighbors, indicating a role for ethylene in sensing the environment and adjusting growth rate in dense vegetation. Whether physical contact between neighboring plants induces

ethylene production with resultant inhibition of growth and accelerated leaf senescence remains to be elucidated.

Second, Tetr plants showed altered responses to microorganisms, in that transformed tobacco plants were impaired in expression of basic PR defense genes and had become susceptible to normally nonpathogenic soil fungi. *Arabidopsis etr1–1* mutant plants have not been reported to show such behavior, indicating that model plants do not necessarily behave in the same way when containing the same gene constructs. However, growth of the virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 in the *Arabidopsis etr1–1* mutant is substantially increased compared with wild-type ecotype Columbia (C. M. J. Pieterse, unpublished observation). Ethylene antagonists increase and ethylene application reduces nodulation by *Rhizobium* in pea (29–31). The *sickle* mutant of the legume *Medicago trunculata* is defective in perception of the ethylene signal and shows an increase in the number of persistent rhizobial infections (32). Moreover, local ethylene production between xylem poles seems to determine preferential nodulation opposite the latter in *R. leguminosarum*-infected vetch plants (33). Thus, modulating ethylene perception seems to affect susceptibility to microbial infection.

Pythium spp. are widely distributed in soils throughout the world (34) and comprise pathogenic as well as nonpathogenic species and strains. Non- or weakly pathogenic strains are unable to cause disease, because they are believed to lack pathogenicity factors overcoming nonhost resistance. Clearly, nonhost resistance was broken down in the Tetr tobacco plants. Because basic PR-gene expression was shown to be dependent on ethylene perception, it is possible that Tetr plants are susceptible to *Pythium* because of the inability to express these PRs. Notably, the basic PR-1g and -5c possess substantial antifungal activity against oomycete fungi (35, 36). In contrast, the hypersensitive response to the viral pathogen TMV was maintained. Whether ethylene is a determinant of plant resistance against microorganisms apparently depends on the pathogen involved. As control of ethylene responses is being exploited to improve longevity of agricultural products, the possibility of concomitant increased susceptibility to disease should be taken into account.

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