Systemic Acquired Resistance in *Arabidopsis thaliana* Induced by a Predisposing Infection with a Pathogenic Isolate of *Fusarium oxysporum*

Brigitte Mauch-Mani and Alan J. Slusarenko

Institute for Plant Biology, Zollikerstr. 107, CH-8008 Zürich, Switzerland

Fusarium oxysporum was isolated from lesions on a naturally infected *Arabidopsis* plant, and pathotypic variation in host response was found in different ecotypes. In some ecotypes limited necrotic lesions were produced on inoculated leaves, whereas in susceptible ecotypes a systemic infection leading to plant death was observed. Infection of lower rosette leaves in an ecotype showing a limited lesion response induced resistance in the other rosette leaves to a subsequent challenge with a virulent race of *Peronospora parasitica*. Induction of systemic acquired resistance was associated with the accumulation of transcripts for pathogenesis-related proteins. Histologically, a range of phenotypes was observed, from a phenocopy hypersensitive response to a trailing necrosis which ultimately contains any *Peronospora* hyphal growth.

Additional keywords: dichloroisonicotinic acid, INA.

The small cruciferous weed *Arabidopsis thaliana* has recently become an interesting model host for the investigation of outstanding problems in plant pathology (Mauch-Mani and Slusarenko 1993). Several pathosystems, involving bacterial (Davis *et al.* 1991; Debener *et al.* 1991; Whalen *et al.* 1991), fungal (Koch and Slusarenko 1990; Crute *et al.* 1993), and viral (Li and Simon 1990) plant pathogens have been developed for study. Here we describe a novel pathosystem for *Arabidopsis* with the fungus *Fusarium oxysporum* and the ability of this pathogen to induce systemic acquired resistance to subsequent infection with *Peronospora parasitica*.

F. oxysporum isolates cause vascular wilts and damping-off of various annual vegetables, perennials, and weeds. Different isolates are often specialized for a particular host, e.g., *F. o.* f. sp. *lycopersicae* on tomatoes and *F. o.* f. sp. *conglutinans* on cabbage. *Fusarium* is difficult to control and is responsible for heavy crop losses. It is very persistent as a saprophyte in soil and not easy to eradicate. Some control can be achieved by planting resistant varieties. *F. oxysporum* has been transformed (Kistler and Benny 1988), which makes it a good subject for molecular genetic analysis. An isolate of *F. oxysporum* from diseased *Arabidopsis* varies in virulence on different ecotypes, and its ability to immunize *Arabidopsis* against subsequent infection by *P. parasitica* was investigated.

Plants which are normally susceptible to a particular pathogen can often be made resistant by a predisposing infection with a pathogen which causes necrosis. This phenomenon has been termed systemic acquired resistance (SAR) and has been known for some years (Chester 1933; Kuc 1982). Resistance so induced is typically effective against a wide range of pathogens, including fungi, bacteria, and viruses. SAR has been very well characterized in tobacco; as early as 1961, Ross showed that tobacco reacting hypersensitively to tobacco mosaic virus becomes resistant to various other viruses in untreated leaves (Ross 1961, 1966).

Certain chemicals are also believed to exert apparent broad-spectrum antibiotic effects by inducing SAR in plants rather than having a direct inhibitory effect on the pathogen. Dichloroisonicotinic acid (INA) (Métraux *et al.* 1991) and the commercial preparation Aliette (fosetyl-Al) are thought to act in this manner. INA has been shown not to have any detectable antifungal activity *in vitro*, but although it induces resistance to *Peronospora* in *Arabidopsis* (Uknes *et al.* 1992), direct effects on the pathogen cannot be tested, because the pathogen is an obligate biotroph. In the case of Aliette evidence suggests that *in vitro* antifungal effects are dependent on the composition of the test media (Guest 1984) and are especially pronounced at low phosphate levels (Fenn and Coffey 1984), like those encountered *in planta*. Additionally, mutants resistant to the effects of Aliette *in vitro* at low phosphate concentrations are able to cause disease in Aliette-treated plants (Dolan and Coffey 1988). These results cast doubt on the original hypothesis that Aliette works by inducing SAR. Such complications do not arise when the inducing treatment is biological, i.e., preinfection with a necrosis-inducing pathogen rather than chemical treatment. Thus, it is important to establish such biologically induced SAR model systems in *Arabidopsis* in order to dissect the process by characterizing mutants altered in the SAR response. One such system using turnip crinkle virus (TCV) to induce resistance against further infection by TCV or *Pseudomonas syringae* pv. *tomato* was recently reported (Uknes *et al.* 1993).

In this work we describe biological induction of SAR in *Arabidopsis* by a predisposing fungal infection and provide details of an important new pathosystem for *Arabidopsis* with a fungus that is amenable to molecular analysis.

RESULTS

Infection of *Arabidopsis* by *F. oxysporum*.

F. oxysporum isolated from naturally infected *A. thaliana* was used to induce resistance against a virulent race of *P.*

parasitica in a normally susceptible ecotype of *A. thaliana*. *Arabidopsis* plants resistant to *F. oxysporum* were found by testing a range of ecotypes for their reactions to leaf infection by *Fusarium* (Table 1). Plants were classified as resistant if no macroscopically visible symptoms could be seen within 2 wk of infection. Susceptible plants started to yellow a few days after infection, and then the tissue became water-soaked, and profuse aerial mycelium could be observed with the naked eye. To confirm the macroscopic disease rating, plants were stained with trypan blue (Keogh *et al.* 1980), in a staining procedure which allows the visualization of fungal structures inside plant tissue and at the same time stains plant cells which are dead or have damaged membranes. In an incompatible interaction, the plants reacted to attempted infection by *Fusarium* with a hypersensitive necrosis affecting one or a few epidermal cells (Fig. 1A). The *Fusarium* spores began germinating after a few hours but did not germinate synchronously. In order to allow time for germination, penetration, and host response to be observed in a reasonable number of cells, microscopy was routinely carried out starting 3 days after inoculation. In a compatible interaction, the fungus penetrated the epidermis directly or via stomata (illustrated in Mauch-Mani and Slusarenko 1993), grew intracellularly through the plant tissue, and formed numerous chlamydospores in the macerated tissue. About 2 wk after infection, *Fusarium* sporulated on the remaining plant debris.

SAR induction by *F. oxysporum*.

To test the ability of *F. oxysporum* to induce resistance in *A. thaliana*, the three lower leaves of resistant plants (Wei-0) were inoculated with a conidial suspension of this fungus 7, 4, and 1 day prior to a challenge infection of the whole rosette with *P. parasitica*. This schedule of 7, 4, and 1 day prior to the test inoculation with *P. parasitica* is the same as that used by Uknes *et al.* (1992) for treatment of *Arabidopsis* with INA and is described here in this way to facilitate direct comparison.

Table 2 shows that inoculation of *A. thaliana* Wei-0 with *F. oxysporum* 7 days before challenge with *P. parasitica* totally inhibited asexual sporulation of the latter fungus. Exposure to *Fusarium* 4 days before inoculation with *P. parasitica* markedly reduced asexual sporulation: 19 of the 25 inoculated

Table 1. Reactions of different *Arabidopsis thaliana* ecotypes to leaf inoculation with *Fusarium oxysporum*

Ecotype	Reaction[a]
WS	S
La-er	S
Kelsterbach 4	S
Col-0	S
RLD	R
Tsu-0	R
Fel-0	R
Oy-0	R
Kil-0	R
C-24	R
Pla-0	R
Pi-0	R
Te-0	R
Mt-0	R
Hi-0	R
Wei-0	R

[a] R = resistant; S = susceptible.

plants showed no sporulation at all, and six plants had only up to three conidiophores per infected leaf. Exposure to *Fusarium* 1 day before the challenge infection led to only a minimal reduction of asexual sporulation: 16 of the 25 plants were fully susceptible, and nine plants showed a slight reduction in susceptibility, scored as the density of mycelial colonization and sporulation. Water-treated control plants showed heavy asexual sporulation after inoculation with *P. parasitica*. The fungus colonized the leaves intercellularly, forming haustoria in the cells, and developed numerous oospores as described by Koch and Slusarenko (1990). These symptoms were uniform throughout the infected plants.

At the microscopic level a whole array of reactions to the challenge inoculation could be observed in plants showing SAR. Colonization and degree of sporulation by *P. parasitica* on plants with SAR were inversely proportional to the time from the inducing infection with *F. oxysporum*. In plants induced 7 days before the challenge inoculation, in most cases, resistance was expressed as a phenocopy hypersensitive response (HR) of the penetrated plant cell (Fig. 1B). That is, the HR normally occurs as a result of the interaction between incompatible genotypes of host and pathogen, but in plants with SAR, although the genotypes are compatible, the HR phenotype is apparently expressed. Thus, to distinguish this "acquired HR" response we use the term *phenocopy HR*. Another, less frequent, type of reaction was the deposition of material, presumably of host origin, around the infection hypha (data not shown), which then failed to build the first haustorium. In these cases, no host cell death was observed.

In plants exposed to *Fusarium* 4 days or 1 day prior to challenge with *P. parasitica*, by far the most frequent reaction observed was a trailing necrosis of host cells in the wake of the growing hyphae (Fig. 1C). In this case the hyphae penetrated the plant tissue to varying degrees, sometimes only by a few cell diameters and in other cases quite substantially. The hyphae then became surrounded by necrotic, collapsed host cells, and the fungus formed very few haustoria. The distribution of cytoplasm in the hyphae was irregular, as judged by the trypan blue staining pattern (Fig. 1C). Some hyphae were able to outgrow the trailing necrosis and give rise to a few conidiophores.

In contrast to the trailing necrosis, some plants exposed to *Fusarium* 1 day or 4 days prior to challenge with *Peronospora* also showed an additional type of necrotic reaction. Initially, the *Peronospora* hyphae grew normally through the plant tissue, and then, a few days after inoculation, the haustoria became encased and the cells containing the haustoria became necrotic (Fig. 1D), but no cell collapse like that in the trailing necrosis was observed. There was some variability in host response among the plants exposed to *Fusarium* 1 day or 4 days prior to challenge. Different leaves on the same plant and even different regions of the same leaf showed varying degrees of colonization and necrotic responses (Table 2). The resistance reactions described above were never observed in uninduced Wei-0 control plants inoculated with the WELA isolate of *P. parasitica;* these plants always showed complete susceptibility (Koch and Slusarenko 1990).

PR transcript accumulation in plants with SAR.

The accumulation of specific groups of proteins called pathogenesis-related (PR) proteins in plants in response to

pathogens has long been known (Van Loon 1985; Stintzi *et al.* 1993). Recently, the accumulation of PR transcripts and proteins has been associated with the induction of SAR by predisposing infections or abiotic treatments (Ward *et al.* 1991). Therefore, we decided to see if the biological induction of SAR in *Arabidopsis* caused the accumulation of PR transcripts, as in the immunization of *Arabidopsis* by INA (Uknes *et al.* 1992). Gel blots of RNA samples from the upper, uninoculated leaves of plants induced with *F. oxysporum* for different lengths of time were probed for transcripts of *Arabidopsis* PR-1 and PR-2 proteins (Uknes *et al.* 1992). As shown in Figure 2, the accumulation of both PR-1 and PR-2 transcripts was strongly induced by inoculation with *F. oxy-*

sporum. The level of transcript accumulation correlated with the intensity of resistance in plants with SAR (see Table 2), i.e., 7 days > 4 days > 1 day.

DISCUSSION

The genetic basis of the pathotypic variation in host response to infection with *F. oxysporum* (Table 1) is currently under investigation in selected crosses. If resistance appears to be conditioned at a single locus, this would be very interesting, because a source of resistance to *Fusarium* could be agronomically very important. In addition, this pathogen can easily be cultured axenically and has been genetically trans-
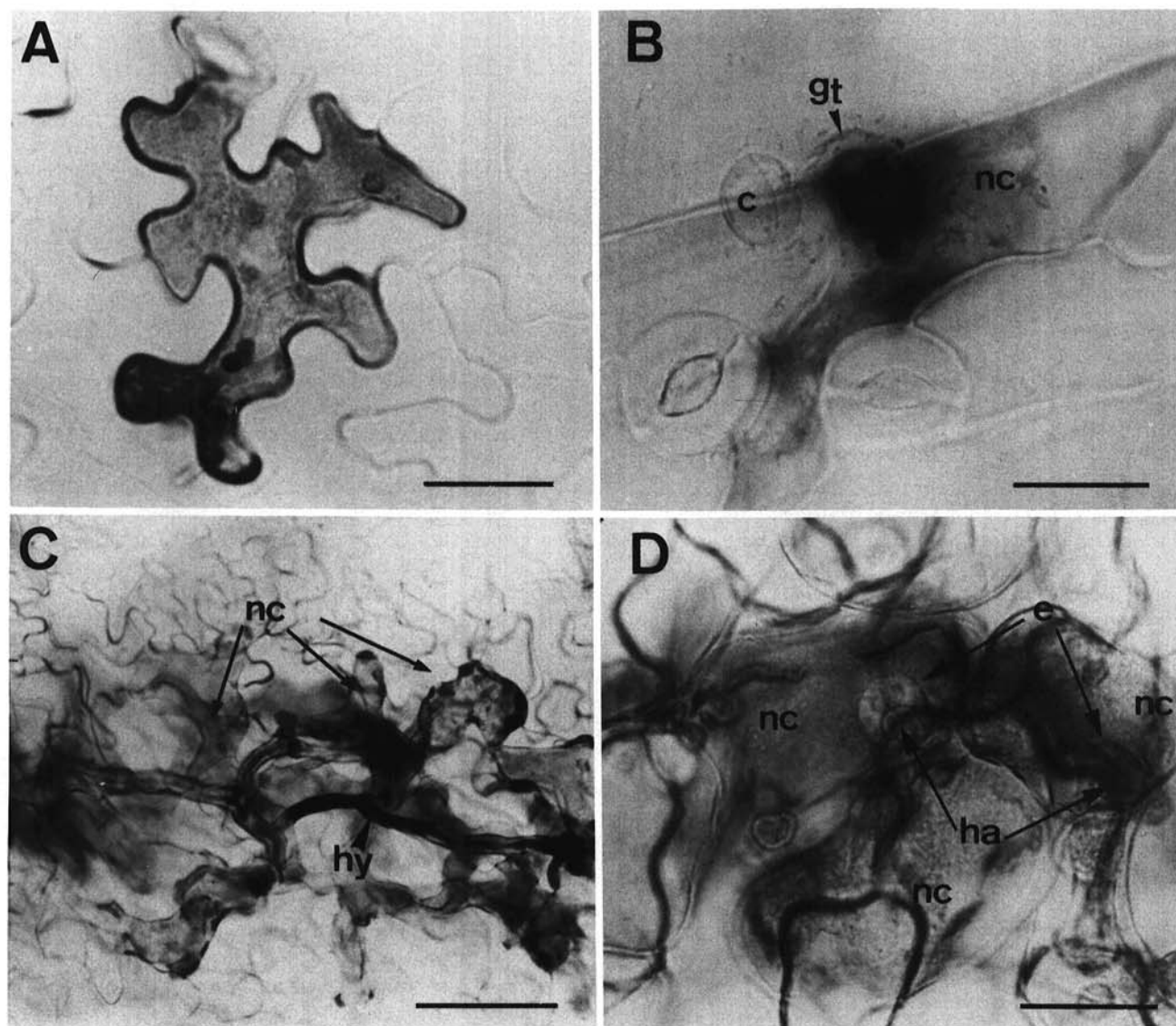

Fig. 1. Bright-field light microscopy of *Arabidopsis thaliana* Wei-0 stained with trypan blue after infection with *Fusarium oxysporum* or *Peronospora parasitica*. **A,** Single-cell necrosis of an epidermal cell induced by *F. oxysporum* on a leaf of *A. thaliana* Wei-0, observed 3 days after inoculation. Bar = 21 μm. **B** and **C** were photographed 6 days after inoculation with *P. parasitica*. **B,** Phenocopy hypersensitive response caused by the attempted penetration of *P. parasitica* into an epidermal cell of *A. thaliana* Wei-0 challenge-inoculated 7 days after exposure to *F. oxysporum*. c = Conidium; gt = germ tube; nc = necrotic cell. Bar = 19 μm. **C,** Trailing necrosis in the mesophyll of a leaf of *A. thaliana* Wei-0 inoculated with *P. parasitica* 4 days after exposure to *F. oxysporum*. The intercellular hypha (hy) of *P. parasitica* is surrounded by necrotic, collapsed cells (nc). The distribution of the cytoplasm within the hypha is irregular. Bar = 75 μm. **D,** Encased haustoria inside necrotic cells of a leaf of *A. thaliana* Wei-0 exposed to *F. oxysporum* 4 days before inoculation with *P. parasitica*. ha = Haustorium; e = encasement; nc = necrotic cells. Bar = 27 μm.

formed (Kistler and Benny 1988). Thus, this pathosystem could be developed as one in which both partners are amenable to genetic and molecular analysis.

Previous work has shown that chemical treatment of *Arabidopsis* with INA (Uknes *et al.* 1992) or salicylic acid (Uknes *et al.* 1993) immunizes the plants against infection by *P. parasitica*. This phenomenon was associated with a reduction of asexual sporulation and a variety of histological effects not normally seen in a compatible interaction. Thus, after INA treatment and subsequent infection with a virulent isolate of *P. parasitica*, host cell necrosis at the site of fungal penetration in a phenocopy of the HR, trailing necrosis in the wake of hyphal growth, and encasement of haustoria near the fungal growing tip were all observed. Similar histological effects were also seen after the biological induction of SAR by preinfection with *F. oxysporum* (Fig. 1B–D), and sporulation was similarly reduced (Table 2). This suggests that INA does indeed act by mimicking the biological induction of the SAR phenomenon. In addition, these histological effects and the appearance of induced resistance were accompanied by the accumulation of transcripts for PR proteins, both in the INA treatment (Uknes *et al.* 1992) and in predisposing infection with *F. oxysporum* (Fig. 2). This is a further instance correlating the appearance of PR transcripts with resistance and, in light of the recently published observation that overexpression of PR-1a in tobacco makes the plants more resistant to *Peronospora* and *Phytophthora* (Alexander *et al.* 1993), supports a direct role for PR molecules in resistance. Using a necrotizing infection with TCV, Uknes *et al.* (1993) induced resistance in *Arabidopsis* to *Pseudomonas syringae* pv. *tomato* and further TCV infection. In this case SAR was also correlated with an accumulation of transcripts for PR proteins. In addition, these authors showed that levels of endogenous salicylic acid increased in *Arabidopsis* plants showing SAR, as had previously been reported for other species, e.g., tobacco and cucumber (Malamy *et al.* 1990; Métraux *et al.* 1990), and salicylic acid has been shown to be an essential component for the induction of SAR in tobacco (Gaffney *et al.* 1993). Thus, *Arabidopsis* shows all the usual attributes of SAR and is confirmed as an attractive subject for mutational studies which may then have relevance for more important crop species.

In an incompatible combination there is a switch-over of host cell metabolism from normal housekeeping functions to a characteristic defense metabolism (Slusarenko and Longland 1986; Collinge and Slusarenko 1987). After biological induction of SAR by a predisposing infection with *F. oxy-*sporum, the host cells in systemic (i.e., untreated) leaves appear to have been primed to go over to a defense metabolism in response to the normally virulent pathogen isolate. The systemic signal which conditions SAR in tobacco and cucumber appears to be salicylic acid (Malamy *et al.* 1990; Métraux *et al.* 1990). However, very little is known of the way in which this systemic signal is "decoded" in the untreated systemic leaves, enabling them to show effective resistance responses to otherwise virulent pathogens (Malamy and Klessig 1992; Enyedi *et al.* 1992). Recently a salicylic acid binding protein from tobacco has been identified as a catalase, and this may be the putative receptor involved in SAR induction (Chen *et al.* 1993). A subsequent signal transduction pathway presumably then leads to PR gene activation and conditioning of the cells to resistance (immunization) against the virulent pathogen. This resistance is associated with the histological reactions reported here and the direct antimicrobial role of some of the PR proteins themselves (Alexander *et al.* 1993).

Mutants altered in the biological induction of SAR by *F. oxysporum* will be sought, and their analysis should yield new understanding of the molecular basis of plant immunization by defining genetic lesions in the various steps involved.

MATERIALS AND METHODS

Plant material.

A. thaliana seeds were surface-sterilized with bleach (2.5%) for 15 min and washed several times with sterile tap water before sowing. The seeds were sown either singly (for induction of resistance) or densely (for determination of resistance phenotype to *F. oxysporum*) in 30-ml pots containing autoclaved potting compost and were vernalized for 5 days at 4° C in the dark before being transferred to a growth chamber with a photoperiod of 14 hr of light (75 $\mu E\ m^{-2}\ s^{-1}$ at plant level) and 10 hr of darkness and a temperature of 18–20° C.

Fungal cultures.

F. oxysporum Schlechtend.:Fr. was initially isolated from roots of *A. thaliana* accession Landsberg erecta showing

Table 2. Macroscopic rating of asexual sporulation of *Peronospora parasitica* on leaves of *Arabidopsis* after exposure to *Fusarium oxysporum*[a]

	Time from treatment to inoculation		
Treatment	1 day	4 days	7 days
H₂O control	+++++	+++++	+++++
F. oxysporum	+++ to +++++	– to ++	–

[a] Degree of sporulation: – = no conidiophores; ++ = leaves with up to three conidiophores; +++ = blotches of sparse sporulation on leaves, never covering the whole leaf area; ++++ = sporulation on the whole leaf, but not as dense as on leaves with the next rating; +++++ = leaf covered evenly with a dense lawn of conidiophores.

F.oxysporum   **H₂O**

–1 –4 –7   –7

PR-1

PR-2

Fig. 2. Accumulation of transcripts for pathogenesis-related (PR) proteins in *Arabidopsis* in response to inoculation with *Fusarium oxysporum*. Upper, untreated leaves were harvested 1, 4, and 7 days after inoculation of the three lowest rosette leaves with *F. oxysporum*, and RNA gel blots were probed with *Arabidopsis* PR-1 and PR-2 cDNAs. Water-treated plants were used as controls, and the 7-day sample from these plants is shown for comparison.

wilting and yellowing symptoms of the aboveground parts. The identity of the fungus was confirmed by the International Mycological Institute (Egham, Surrey, U.K.). *Fusarium* was grown on potato-dextrose agar (Difco) at 18–20° C. Stock cultures were kept at 4° C. To induce asexual sporulation, the cultures were grown under continuous black light (Leach 1962).

The WELA isolate of *P. parasitica* (Pers.:Fr.) Fr. was maintained by subculturing weekly on *A. thaliana* accession Weiningen (Wei-0) (Koch and Slusarenko 1990).

Induction of resistance by *Fusarium oxysporum.*

A 50-μl droplet of a conidial suspension (2×10^6 conidia per milliliter) was applied to each of the three lowest rosette leaves of Wei-0 plants 7, 4, or 1 day before challenge inoculation with *P. parasitica*. The plants were challenge-inoculated with *P. parasitica* 6 wk after sowing. Control plants were mock-induced with water. At each time point, a set of 25 single plants was used for each treatment.

Plants quick-frozen in liquid nitrogen at the time of the challenge infection with *P. parasitica* were used for RNA extraction.

Inoculation with *P. parasitica.*

Plants (6 weeks old) were inoculated by spraying with a conidial suspension of *P. parasitica* (10^5 conidia per milliliter). The plants were incubated overnight in a humid chamber at 16° C and then returned to the growth chamber. Six days after inoculation the plants were again incubated in a humid chamber overnight to induce sporulation. They were scored for conidiophores under a stereomicroscope (magnification 31×), and then the whole plants were stained with trypan blue to assess fungal colonization of the tissues.

Staining for microscopy.

Whole plants with their roots removed were stained with alcoholic trypan blue solution (Keogh *et al.* 1980) and cleared in chloral hydrate (2.5 g ml^{-1} of H_2O) for approximately 24 hr. Single leaves were mounted in chloral hydrate and viewed under a microscope with bright-field optics.

Determination of resistance or susceptibility of *A. thaliana* to *F. oxysporum.*

Two-week-old *A. thaliana* plants of 15 different accessions were sprayed with a spore suspension of *F. oxysporum* (2×10^6 conidia per milliliter) and incubated for 2 wk under high humidity in a growth chamber. The plants were then scored macroscopically for disease symptoms and microscopically for fungal growth or resistance reactions of the plant tissue.

RNA extraction and hybridization.

Total RNA was isolated from frozen tissue samples as described in Meier *et al.* (1993). Samples of total RNA (10 μg) were separated by electrophoresis through formaldehyde-agarose gels as described in the protocol of the lambda ZAP cDNA synthesis kit (Stratagene, La Jolla, CA). Ethidium bromide was included in the loading buffer at a concentration of 67 μg ml^{-1}, which allowed photography under UV light after electrophoresis to confirm equal sample loading. The gels were blotted onto nylon membranes (Hybond-N, Amersham, Little Chalfont, U.K.) and hybridized to random-prime

^{32}P-labeled *A. thaliana* PR-1 and PR-2 cDNA probes (Uknes *et al.* 1992) by the use of a kit from Boehringer Mannheim. The most stringent wash was with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate for 30 min at 65° C. Autoradiography was performed with Fuji medical X-ray film and intensifier screens at −70° C.

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