***Nicotiana plumbaginifolia* plants silenced for the ATP-binding cassette transporter gene *NpPDR1* show increased susceptibility to a group of fungal and oomycete pathogens**

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SUMMARY

The behaviour of *Nicotiana plumbaginifolia* plants silenced for the ATP-binding cassette transporter gene *NpPDR1* was investigated in response to fungal and oomycete infections. The importance of *NpPDR1* in plant defence was demonstrated for two organs in which *NpPDR1* is constitutively expressed: the roots and the petal epidermis. The roots of the plantlets of two lines silenced for *NpPDR1* expression were clearly more sensitive than those of controls to the fungal pathogens *Botrytis cinerea*, *Fusarium oxysporum* sp., *F. oxysporum* f. sp. *nicotianae*, *F. oxysporum* f. sp. *melonis* and *Rhizoctonia solani*, as well as to the oomycete pathogen *Phytophthora nicotianae* race 0. The *Ph* gene-linked resistance of *N. plumbaginifolia* to *P. nicotianae* race 0 was totally ineffective in *NpPDR1*-silenced lines. In addition, the petals of the *NpPDR1*-silenced lines were spotted 15%–20% more rapidly by *B. cinerea* than were the controls. The rapid induction (after 2–4 days) of *NpPDR1* expression in *N. plumbaginifolia* and *N. tabacum* mature leaves in response to pathogen presence was demonstrated for the first time with fungi and one oomycete: *R. solani*, *F. oxysporum* and *P. nicotianae*. With *B. cinerea*, such rapid expression was not observed in healthy mature leaves. *NpPDR1* expression was not observed during latent infections of *B. cinerea* in *N. plumbaginifolia* and *N. tabacum*, but was induced when conditions facilitated *B. cinerea* development in leaves, such as leaf ageing or an initial root infection. This work demonstrates the increased sensitivity of *NpPDR1*-silenced *N. plumbaginifolia* plants to all of the fungal and oomycete pathogens investigated.

INTRODUCTION

The ATP-binding cassette (ABC) transporters are proteins found widely in the biological membranes of all living organisms. They

couple ATP hydrolysis with transport across the membrane of various structurally unrelated substrates. The ‘full size’ ABC transporters comprise two copies each of two basic elements: a highly hydrophobic transmembrane domain (TMD), believed to play a key role in substrate translocation across the membrane, and a hydrophilic nucleotide-binding domain (NBD) that hydrolyses ATP (Holland and Blight, 1999; Locher, 2004; Rea, 2007; Verrier *et al.*, 2008). Among the ABC superfamily, the pleiotropic drug resistance (PDR) family occurs exclusively in fungi and plants. In the *Arabidopsis*, rice and *Lotus japonica* genomes, 15, 23 and 12 PDR genes, respectively, have been identified and tentatively organized into five clusters according to their phylogenetic relationship (Crouzet *et al.*, 2006; Jasinski *et al.*, 2003; Rea, 2007; Sugiyama *et al.*, 2006; van den Brûle and Smart, 2002).

Some PDR genes have been shown to be involved in the response to biotic stress. The expression of *AtPDR12* is detected mainly in leaves and flowers and is strongly up-regulated by different pathogens (*Alternaria brassicicola*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum* and *Pseudomonas syringae* pv. *tomato* DC3000), as well as methyl jasmonate, ethylene and salicylic acid, signalling molecules implicated in plant defence (Campbell *et al.*, 2003; Lee *et al.*, 2005). *AtPDR8* (also known as *PEN3*) is expressed in plant roots, stems and leaves, and is up-regulated by *P. syringae* infection. *AtPDR8* knock-out mutants show increased susceptibility to different lifestyle pathogens, such as *Blumeria graminis* f. sp. *hordei*, *Erysiphe pisi* and *Phytophthora infestans*, suggesting that the substrate(s) of this transporter is secreted to protect the plant from these microorganisms (Glombitza *et al.*, 2004; Kobae *et al.*, 2006; Stein *et al.*, 2006). *NtPDR1* from *Nicotiana tabacum* and *GmPDR12* from soybean are up-regulated by microbial elicitors, methyl jasmonate and salicylic acid (Eichhorn *et al.*, 2006; Sasabe *et al.*, 2002).

NpPDR1 (formerly known as *NpABC1*) has been shown to be expressed in the plasma membrane of *Nicotiana plumbaginifolia* suspension cells treated with the diterpenes sclareol, abietic acid and larixol (Grec *et al.*, 2003; Jasinski *et al.*, 2001). Under normal

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growth conditions, *NpPDR1* is expressed in *N. plumbaginifolia* in the leaf short glandular trichomes, root and upper part of the corolla. *NpPDR1* expression is strongly induced in whole healthy leaves of *N. plumbaginifolia* by several *Pseudomonas* strains and by signalling molecules involved in plant defence, such as methyl jasmonate, ethylene and, to a lesser extent, salicylic acid. There is evidence that the diterpene sclareol, which has antimicrobial properties, is a substrate of *NpPDR1* (Jasinski *et al.*, 2001; Stukkens *et al.*, 2005), as well as of *AtPDR12* (Campbell *et al.*, 2003) and *SpTUR2* (van den Brle *et al.*, 2002). Silencing *NpPDR1* expression by RNA interference causes increased susceptibility of *N. plumbaginifolia* plantlets to infections by *Botrytis cinerea* at the root level, resulting in the premature mortality of some plants (Stukkens *et al.*, 2005). However, until now, the induction of *NpPDR1* expression in response to a fungal infection had been demonstrated only after 10 days in cut pieces of leaf material in the presence of *B. cinerea* (Stukkens *et al.*, 2005).

Among the *N. tabacum* fungal diseases, grey mould is caused by *B. cinerea*, a typical necrotrophic pathogenic fungus (Rowe and Kliebenstein, 2008). *Botrytis cinerea* is generally considered to be a tobacco disease of low importance that becomes more severe only in favourable humid conditions. It is mainly a disease of seedlings in seed beds. In the USA, it can result from infection on the lower leaves, which are more susceptible because they are past maturity and etiolated (Wolf, 1931). It has been reported (Wolf, 1931) from early observations made in Java (Peters, 1912) and Germany (Pape, 1921) that the stems of seedlings near the surface of the soil may be involved in decay. Seedling roots can become decayed and large necrotic spots can occur on the leaves. Fading flowers can be infected and the diseased corollas falling and remaining on the leaves can induce leaf infections.

Another fungal disease of *N. tabacum* is *Fusarium* wilt caused by several formae speciales of *F. oxysporum*. The *Fusarium* strains that induce wilt in tobacco have still not been defined completely. A general view is that at least several formae speciales would be able to infect tobacco: *F. oxysporum* f. sp. *nicotianae*, *F. oxysporum* f. sp. *batatas*, also pathogenic on sweet potato, and *F. oxysporum* f. sp. *vasinfectum*, also pathogenic on cotton (Armstrong and Armstrong, 1958; Clark *et al.*, 1998; Smith and Shaw, 1943). An isolate from okra has also been noted to be pathogenic to the tobacco cultivar Kentucky (Clark *et al.*, 1998), and an analysis of pathogenic isolates from tobacco fields in Spain revealed two groups of isolates: one genetically homogeneous and the other genetically heterogeneous (Alves-Santos *et al.*, 2007).

A third fungal disease of tobacco is caused by *Rhizoctonia solani* strains from the anastomose groups 1, 2-2 and 4. They are responsible for the sore shin and damping off of *N. tabacum*, one of the most common diseases of tobacco seedlings in glass-houses; they cause damage to the roots via sclerotia and melanized hyphae present in the soil and trays used in seed beds

(Gutierrez *et al.*, 1997). They differ from the *R. solani* strains of anastomose group 3 that are responsible for target spot disease on the leaves of *N. tabacum* (Ceresini *et al.*, 2002).

Phytophthora nicotianae is an oomycete and a soil-borne pathogen. It is responsible for the black shank disease of *N. tabacum* and *N. plumbaginifolia*. It is a serious root, stem and leaf disease of cultivated tobacco worldwide; infection can occur at any stage of development and the symptoms are plant wilting and stunting, chlorosis and black lesions on the stem (Csinos, 1999; Sullivan *et al.*, 2005a, b; Van Jaarsveld *et al.*, 2003). In tobacco, four races have been described depending on the varying sensitivities of reference cultivars (Van Jaarsveld *et al.*, 2002). Race 0 is the most common, very aggressive against *N. tabacum* and fits well in soils, but is non-pathogenic to *N. plumbaginifolia* (Apple, 1962; Carlson *et al.*, 1997; Johnson *et al.*, 2002; Sullivan *et al.*, 2005a). The resistance to race 0 is monogenic and it has been introduced in cultivated cultivars of *N. tabacum* from *N. plumbaginifolia* (*Ph* gene) and *N. longiflora*, resulting in the increased importance of race 1; the other races have been reported in South Africa and Connecticut (Carlson *et al.*, 1997; Csinos, 2005; Johnson *et al.*, 2002; Sullivan *et al.*, 2005b).

In this study, we analysed the behaviour of *NpPDR1*-silenced and wild-type lines of *N. plumbaginifolia* in response to fungal and oomycete infections. A greater susceptibility of *NpPDR1*-silenced lines to the tested pathogens was demonstrated in roots and flowers. We observed, for the first time, the rapid activation (after 2–4 days) of *NpPDR1* expression in *N. plumbaginifolia* and *N. tabacum* plants after infection by fungi and one oomycete: *R. solani*, *F. oxysporum* and *P. nicotianae*. With *B. cinerea*, we observed that *NpPDR1* was not expressed during latent infections, but was expressed when conditions favourable to *B. cinerea* facilitated disease development.

RESULTS

Root infection tests

Root infection tests were conducted on young plantlets of *N. plumbaginifolia* inoculated at transfer time from *in vitro* culture to Jiffy pots. At this time, corresponding to the inoculation time, the development of wild-type and *NpPDR1*-silenced lines was similar in all respects. The root systems were similarly developed and no apparent alteration was observed in the roots of *NpPDR1*-silenced lines.

Botrytis cinerea MUCL 46725, previously isolated from a naturally infected *NpPDR1*-silenced plant (Stukkens *et al.*, 2005), is a poor spore producer on the culture media tested. Therefore, the spores that served as inoculum in root infection tests were produced on infected *Nicotiana* plantlet leaves in Petri dishes. Figs 1A and 2A show the results of *B. cinerea* inoculation of

Fig. 1 Root infection tests showing inoculated (I) and uninoculated (NI) *Nicotiana plumbaginifolia* plantlets of a wild-type (Wt) and two *NpPDR1*-silenced lines (L 6.2 and L 8.2) or wild-type *N. tabacum* plantlets. (A) *Botrytis cinerea* MUCL 46725 test on *N. plumbaginifolia* after 15 days. (B) Part of a *Rhizoctonia solani* B112 test on *N. plumbaginifolia* after 15 days. (C) *Phytophthora nicotianae* 183 test on *N. plumbaginifolia* after 12 days. (D) *Phytophthora nicotianae* 183 test on wild-type *N. tabacum* plantlets after 7 days using an inoculum of 50 sporangia/mL. (E) *Fusarium oxysporum* PB1 test after 8 days, comparative growth of uninoculated and inoculated Wt plantlets, and comparison of pathogen damage: the red arrows show *F. oxysporum* progression in the leaf veins of *NpPDR1*-silenced plantlets.

Fig. 2 Means and standard deviations of three repetitions of *Nicotiana plumbaginifolia* root infection tests for different pathogens. Each test included a wild-type (Wt) and two *NpPDR1*-silenced lines (L 6.2 and L 8.2). (A) *Botrytis cinerea* MUCL 46725 test after 8 days. (B) *Rhizoctonia solani* B112 test after 23 days. (C) *Phytophthora nicotianae* race 0 183 test after 11 days. (D–F) *Fusarium oxysporum* test after 11 days: *F. oxysporum* PB1 (D), *F. oxysporum* f. sp. *nicotianae* Ft-Rob (E) and *F. oxysporum* f. sp. *melonis* MUCL 31367 (F).

plantlet roots of the wild-type line Wt and the *NpPDR1*-silenced lines L 6.2 and L 8.2. Characteristic grey sporulation was observed on diseased plants (Fig. 1A), which confirmed the involvement of *B. cinerea* in the observed symptoms. Clear differences in mortality were observed among inoculated plantlets: the silenced lines showed increased susceptibility to the pathogen, although some mortality in the wild-type line was also observed. Death occurred relatively soon after inoculation (less than 10 days). The uninoculated control plantlets did not show any mortality during the test periods. They grew similarly in all lines, without any apparent difference in vigour between wild-type and *NpPDR1*-silenced plants. This was consistently observed

in all infection tests performed (Fig. 1A,C,E). Reduction of the *B. cinerea* inoculum concentration from 3×10^4 to 3×10^3 spores/mL resulted in fewer dead plants in all lines, but the differences between wild-type and *NpPDR1*-silenced lines remained clear (data not shown).

Figures 1B and 2B show the results obtained with *R. solani* B112. Typical damping off symptoms were observed (Fig. 1B), which confirmed the involvement of *R. solani* in the disease. Although the pathogen had a clear effect on the wild-type line, greater susceptibility was observed in *NpPDR1*-silenced lines. Plantlet death occurred more slowly with *R. solani* (only after 23 days for some plants) than with *B. cinerea*.

The *N. plumbaginifolia* wild-type line was not strongly affected 12 days after inoculation of *P. nicotianae* race 0 183 (Figs 1C and 2C). This was expected because *N. plumbaginifolia* is naturally resistant to race 0 of *P. nicotianae*. Indeed, it possesses the *Ph* gene conferring resistance to race 0 of *P. nicotianae*. However, the increased sensitivity of *NpPDR1*-silenced lines of *N. plumbaginifolia* was very clear after 12 days (Fig. 1C). On the other hand, the susceptibility of wild-type *N. tabacum* plantlets lacking the *Ph* gene was very clear after 6 days (all eight inoculated plantlets were killed), despite the use of a twice reduced inoculum (50 sporangia/mL; Fig. 1D). Moreover, a single wild-type plant was still surviving after 14 days when a 10-fold reduced inoculum (10 sporangia/mL) was used (data not shown). These different behaviours of wild-type *N. plumbaginifolia* and *N. tabacum* plantlets in relation to the presence or absence, respectively, of the *Ph* gene, as well as the shank darkening and similar aspect of the diseased plants of *NpPDR1*-silenced *N. plumbaginifolia* and wild-type *N. tabacum* (Fig. 1C,D), confirmed the involvement of *P. nicotianae* in the disease in both cases.

When *NpPDR1*-silenced *N. plumbaginifolia* plants were grown in the glasshouse, *Fusarium* sp. and *B. cinerea* naturally present in the soil caused significant mortality. *Fusarium oxysporum* PB1 was isolated from a dead *NpPDR1*-silenced *N. plumbaginifolia* plant and used in our tests. The inoculated wild-type plantlets showed reduced growth compared with the uninoculated control plantlets, but were still alive (Figs 1E and 2D). However, the *NpPDR1*-silenced plantlets showed white mycelium growth on the leaves and apparent progression of the pathogen via leaf veins. These typical symptoms of *Fusarium* wilt confirmed the involvement of inoculated *F. oxysporum* in the disease. All the *NpPDR1*-silenced plantlets were dead after 8 days. Similar results were obtained with the pathogens *F. oxysporum* f. sp. *nicotianae* Ft-Rob (Fig. 2E) and *F. oxysporum* f. sp. *melonis* race 0 MUCL 31367 (Fig. 2F) after 11 days. Interestingly, the reduced growth of wild-type plantlets observed in the presence of *F. oxysporum* PB1 (Fig. 1E) was also observed in the presence of the two other *Fusarium* strains, indicating a general susceptibility of wild-type *N. plumbaginifolia* plantlets to the tested *F. oxysporum* strains under our test conditions.

Leaf infection tests

The infiltration of *B. cinerea* MUCL 46725, *F. oxysporum* PB1, *R. solani* B112 and *P. nicotianae* 183 inoculum through leaf stomata in the intercellular space of mature plants of *N. plumbaginifolia* did not result in any significant difference in behaviour between *N. plumbaginifolia* wild-type and *NpPDR1*-silenced lines. The symptoms observed over time were absent, involved fading in the infiltrated zones or involved both fading and limited necrosis in the infiltrated zones, but without a clear increased sensitivity of *NpPDR1*-silenced lines (data not shown).

Induction of *NpPDR1* expression by pathogens in *N. plumbaginifolia* and *N. tabacum*

Leaves were used to demonstrate the induction of *NpPDR1* expression by pathogens. Indeed, *NpPDR1* is constitutively expressed in the roots of *N. plumbaginifolia*, and the damage caused to the roots by the pathogens prevented the use of Western blotting analyses (data not shown). However, in leaf tissues, the constitutive expression of *NpPDR1* is restricted to the short trichomes. The infiltration of pathogen suspensions through the leaf stomata of mature wild-type plants of *N. plumbaginifolia* induced *NpPDR1* expression (monitored by Western blotting) for *F. oxysporum* PB1 and *R. solani* B112 (after 4 days) and *P. nicotianae* 183 (after 2 days) (Fig. 3A). We also observed that expression of the *NpPDR1* orthologue in *N. tabacum* leaves of mature wild-type plants was activated by the same pathogens (Fig. 3B). At the time of analysis (2 or 4 days), no symptoms were observed in the infiltrated zones for *R. solani* B112 and *P. nicotianae* 183 in *N. plumbaginifolia*. Fading (leaf tissues becoming light green or yellow) to various extents was observed in the other cases. The results were different for *B. cinerea* MUCL 46725. Indeed, no *NpPDR1* expression could be detected after 4 days in either *N. plumbaginifolia* (Fig. 4A) or *N. tabacum*

Fig. 3 *NpPDR1* expression in leaves of wild-type mature plants of *Nicotiana plumbaginifolia* (A) and *Nicotiana tabacum* (B) infiltrated for 48 or 96 h with *Phytophthora nicotianae* 183, *Fusarium oxysporum* PB1 and *Rhizoctonia solani* B112 or with water. In (B), *NpPDR1* represents the *N. tabacum* orthologue of *NpPDR1*. Western blotting analysis was performed using antibodies raised against *NpPDR1* and the plasma membrane H⁺-ATPase.

Fig. 4 NpPDR1 expression in leaves, wounded or not, of wild-type mature *Nicotiana* plants infiltrated with *Botrytis cinerea*. (A) *Nicotiana plumbaginifolia* 4 days after infiltration. (B) *Nicotiana tabacum* 4 days after infiltration. (C) *Nicotiana tabacum* 33 days after infiltration. In (B) and (C), NpPDR1 represents the *N. tabacum* orthologue of NpPDR1. Western blotting analysis was performed using antibodies raised against NpPDR1 and the plasma membrane H⁺-ATPase.

(Fig. 4B) leaves, additionally wounded or not in the infiltrated zones. Symptoms such as small localized fading or necrosis were restricted to the wounded leaves. These symptoms remained constant for days. However, as fading was eventually observed in a wounded *B. cinerea* infiltrated zone in an *N. tabacum* plant

after 33 days, additional Western blotting analysis was performed. A distant zone in the same leaf was used as a control. Higher NpPDR1 expression in the *B. cinerea* infiltrated zone was clear in this case (Fig. 4C). Basically, the same general observations on NpPDR1 expression were made by histochemical analysis of a mature NpPDR1-*gusA* *N. tabacum* transgenic plant expressing the β -glucuronidase (*gusA*) gene under the control of the NpPDR1 transcription promoter (Fig. 5). An analysis was performed 2 (*P. nicotianae* 183), 4 and 6 days (*F. oxysporum* PB1, *R. solani* B112 and *B. cinerea* MUCL 46725) after infiltration. GUS expression was almost absent with *B. cinerea*, but clearly present with *P. nicotianae*, *F. oxysporum* and *R. solani* (Fig. 5).

Western blotting analysis was then performed in order to determine whether NpPDR1 was expressed in the leaves of wild-type *N. plumbaginifolia* plantlets grown in Jiffy pots infected by *B. cinerea* following an initial *B. cinerea* infection at the root level (Fig. 6). NpPDR1 expression was clear in leaves that were still dark green with a firm texture, as well as in collapsing light green to yellow leaves.

Flower infection tests

NpPDR1-silenced plants of *N. plumbaginifolia* grown in non-sterile soil very often did not attain maturity because of infections caused by *Fusarium* and *Botrytis* spp. naturally present in the soil. However, wild-type and NpPDR1-silenced *N. plumbaginifolia* plants protected from infections by the initial use of sterilized Jiffy pots, pots and soil were easily grown until flower induction in a non-sterile growth chamber. The plants behaved similarly in all lines, without any apparent phenotypic difference in the silenced lines compared with the control. Flowers of *N. plumbaginifolia* are alternatively closed during the day and open during the night. However, detached closed flowers of *N. plumbaginifolia* (wild-type and NpPDR1-silenced plants) cut near their final expansion stage were open and remained open after overnight incubation at 25 °C in the dark. After inoculation with 150 μ L of sterile water, the petals of open flowers maintained upright in sterile water in a closed Falcon tube were devoid of brown spots after 1 day and generally for more than 2 days. Almost all such flowers inoculated with a suspension (100 spores/mL) of *B. cinerea* MUCL 46725 presented typical brown spots caused by *B. cinerea* after 2 days and *B. cinerea* sporulation in the following days. To verify that such detached open flowers were still expressing NpPDR1, the upper (pigmented on one side) and lower (white on both sides) parts of the corolla from detached flowers of wild-type *N. plumbaginifolia* were analysed by Western blotting after overnight incubation at 25 °C designed to open flowers (the period corresponding to *B. cinerea* inoculation) and 24 h later (the period corresponding to pathogenicity test reading). NpPDR1 was strongly expressed only in

Fig. 5 β -Glucuronidase (GUS) expression in leaves of a mature plant of transgenic *NpPDR1-gusA* *Nicotiana tabacum* after infiltration with suspensions of pathogens. For each pathogen, two leaf fragments are presented. (A) Leaf infiltrated with *Botrytis cinerea*, punctured with a needle and incubated for 4 days. (B) Leaf infiltrated with *B. cinerea* incubated for 4 days. (C) Leaf infiltrated with *Fusarium oxysporum* incubated for 4 days. (D) Leaf infiltrated with *Phytophthora nicotianae* incubated for 2 days. (E) Leaf infiltrated with *Rhizoctonia solani* incubated for 4 days. (F) Leaf infiltrated with water incubated for 4 days. Bars correspond to 1 mm.

Fig. 6 Plantlets of *Nicotiana plumbaginifolia* infected by *Botrytis cinerea* at the root level with resulting infections of leaves (A) and activation of *NpPDR1* expression in the two types of leaf observed in these conditions, but not in control healthy leaves of non-inoculated plantlets (B). Western blotting was performed using antibodies raised against *NpPDR1* and the plasma membrane H^+ -ATPase.

the upper part of the corolla (Fig. 7A), in total agreement with the observations made on corollas on the plant (Stukkens *et al.*, 2005). Test reading 1 day after inoculation consisted of the detection of the brown spots caused by *B. cinerea* on the petals, as shown in Fig. 7B for a flower from an *NpPDR1*-silenced plant. Similar brown spots appeared on inoculated wild-type and *NpPDR1*-silenced flowers without preliminary cell death (Fig. 7B). The uninoculated flowers, *NpPDR1*-silenced or wild-type, remained intact for the duration of the test. The results of

the three tests, presented as the percentages of spotted flowers, are shown in Fig. 7C. An initial observation was that the general level of spotted petals after 1 day varied from one test to another. This was because spot appearance was a continuous process and its timing was slightly variable depending on the test. Another observation was that the percentage of spotted flowers was always inferior for the wild-type line compared with the *NpPDR1*-silenced lines. This was more apparent when the data were expressed as a percentage compared with the wild-

Fig. 7 *Nicotiana plumbaginifolia* flower infection tests. (A) NpPDR1 expression in the upper and lower parts of the corolla of six detached flowers of wild-type *N. plumbaginifolia* at the *B. cinerea* inoculation time [day 0 (d 0); flowers 1–3] and 1 day later (d 1; flowers 4–6). Western blotting was performed using antibodies raised against NpPDR1 and the plasma membrane H⁺-ATPase. (B) Infected flower of a *NpPDR1*-silenced plant 1 day after petal inoculation with *B. cinerea*; the arrow indicates spots on petals. (C) Percentage of spotted flowers for wild-type (Wt) and *NpPDR1*-silenced (L 6.2 and L 8.2) lines 1 day after *B. cinerea* inoculation (triplicates). (D) Means and standard deviations of percentage differences between each line (Wt, L 6.2, L 8.2) and the wild-type line (Wt) for the three tests. As a result of the variable quantities of flowers obtained daily in groups of plants of different heights, the quantities of flowers used in each test were as follows: test 1, 24 for line Wt, 25 for line L 6.2 and nine for line L 8.2; test 2, 21 for line Wt, 32 for line L 6.2 and seven for line L 8.2; test 3, 34 for line Wt, 34 for line L 6.2 and 11 for line L 8.2.

type line (Fig. 7D): the petals of the *NpPDR1*-silenced lines were 15%–20% more frequently spotted than those of the wild-type line.

DISCUSSION

NpPDR1 is expressed in the roots of *N. plumbaginifolia* under normal growth conditions (Stukkens *et al.*, 2005). The use in this study of the fungal pathogens *B. cinerea*, *F. oxysporum*, *F. oxysporum* f. sp. *nicotianae*, *F. oxysporum* f. sp. *melonis* race 0, *R. solani* and the oomycete *P. nicotianae* race 0 greatly extends the conclusion reached in a previous study using *B. cinerea* (Stukkens *et al.*, 2005) that *NpPDR1* apparently plays a major role in *N. plumbaginifolia* plant defence against fungi and oomycetes present in the soil. Indeed, clear differences of susceptibility were established between *NpPDR1*-silenced lines and a wild-type line of *N. plumbaginifolia* in soil infected with each of these pathogens (Figs 1 and 2). The reduced growth of wild-type *N. plumbaginifolia* observed in the presence of three *F. oxysporum* strains or formae speciales indicated the broad sensitivity of *N. plumbaginifolia* plantlets to *F. oxysporum* under our test condi-

tions. However, the *NpPDR1*-silenced lines were clearly more sensitive. This broad sensitivity of *N. plumbaginifolia* is not surprising because *N. tabacum* is known to be sensitive to *F. oxysporum* isolates from tobacco, cotton, sweet potato and okra (Alves-Santos *et al.*, 2007; Armstrong and Armstrong, 1958; Clark *et al.*, 1998; Smith and Shaw, 1943).

Phytophthora nicotianae race 0 is non-virulent on *N. plumbaginifolia* (Apple, 1962) and, indeed, the wild-type line of *N. plumbaginifolia* was resistant in our tests to *P. nicotianae* race 0 183. However, the *NpPDR1*-silenced lines were very sensitive to *P. nicotianae* 183. The reason why the *Ph* gene-related resistance of *N. plumbaginifolia* to *P. nicotianae* race 0 was no longer effective in the absence of *NpPDR1* expression remains unclear. The *Ph* gene has not been isolated and its molecular function remains unknown. NpPDR1 is an ABC transporter probably involved in the secretion of antimicrobials out of the cell. The *Ph* gene could be involved in the synthesis of a compound secreted by NpPDR1, or *Ph* gene expression could depend on a product secreted by NpPDR1. In addition, the *Ph* and *NpPDR1* genes could act independently against *P. nicotianae* race 0, and *Ph* gene activity alone may not be efficient enough.

In the leaf tissues, constitutive *NpPDR1* expression was restricted to the leaf glandular trichomes. However, it was induced after 2–4 days in the whole leaf of healthy *Nicotiana* plants by infiltration of bacteria and, only after 10 days, in detached leaf discs treated *in vitro* by *B. cinerea* (Stukkens *et al.*, 2005). However, there was no evidence that fungi and oomycetes activated *NpPDR1* expression rapidly in healthy *Nicotiana* plants. In this study, challenging mature healthy leaves of *N. plumbaginifolia* and *N. tabacum* with *P. nicotianae*, *F. oxysporum* and *R. solani* induced *NpPDR1* expression in the whole leaves after 2–4 days. This was not observed with *B. cinerea*.

Wolf (1931) reported that *B. cinerea* was pathogenic to *N. tabacum* leaves only in the presence of films of moisture on the leaves for extended periods and in leaves weakened by age or shading. This may explain the absence of symptoms observed in this study in mature healthy leaves of wild-type and *NpPDR1*-silenced lines of *N. tabacum* up to 30 days after infiltration of a *B. cinerea* spore suspension, as well as the absence of *NpPDR1* expression after 4 and 6 days. The influence of leaf age could explain why fading in the infiltrated zone and *NpPDR1* expression were observed 33 days after the infiltration of the *B. cinerea* spore suspension in the wild-type line. The leaf possibly became susceptible to *B. cinerea* infection, which induced a plant reaction and *NpPDR1* activation. The other pathogens tested were rapidly virulent in the leaf because they induced fading in the infiltrated zones in a few days, and *NpPDR1* expression was observed after 2 days for *P. nicotianae* and after 4 days for *F. oxysporum* and *R. solani*.

NpPDR1 expression was found in the leaves of *N. plumbaginifolia* plantlets infected by *B. cinerea* through the roots. This expression probably results from stress signalling from damaged roots that might trigger *B. cinerea* infection at the leaf level. Leaves of grapes treated with paraquat were also far more sensitive to *B. cinerea* than were untreated ones (Holz *et al.*, 2003). In addition, *B. cinerea* infection and *NpPDR1* expression, reported by Stukkens *et al.* (2005) in leaf discs of *N. plumbaginifolia* maintained *in vitro* for 10 and 14 days, might be related to stress. In this system, *B. cinerea* spores were deposited on the leaf surface, and the *NpPDR1*-silenced line tested clearly displayed higher susceptibility than the wild-type (Stukkens *et al.*, 2005). Similar results were not observed in this study when leaves were infiltrated with suspensions of *B. cinerea*, *F. oxysporum*, *R. solani* or *P. nicotianae*. The different infection systems (leaf surface vs. intercellular space) could explain these different observations.

The level of aggression of *B. cinerea* in *Nicotiana* leaves influenced *NpPDR1* expression: no expression when *B. cinerea* behaved apparently as a non-pathogen and expression when it became more aggressive. In the former case, *B. cinerea* could either remain undetected or be able to block plant defence

reactions until conditions become more favourable. The initial infection of strawberry and grape flowers, and of strawberry leaves, by *B. cinerea* also stops during a latent period before mature fruits or senescent leaves become more susceptible organs for *B. cinerea* development (Braun and Sutton, 1988; Keller *et al.*, 2003; Powelson, 1960). As *B. cinerea* is a highly variable fungal species, it is possible that different timing of *NpPDR1* expression would have been obtained with another strain. Indeed, in grapes, a low-virulent strain induced the accumulation of many defence products in leaves, whereas a highly virulent strain induced no secondary metabolite synthesis and delayed chitinase and glucanase accumulation (Derckel *et al.*, 1999). Another typically necrotrophic pathogen, *Sclerotinia sclerotiorum*, is able to block the oxidative burst and the defence response of the host plant (Cessna *et al.*, 2000).

NpPDR1 is constitutively expressed in the epidermis of the upper corolla of the flowers of *N. plumbaginifolia* (Stukkens *et al.*, 2005), and this was also observed with detached flowers in this study. The petals of *NpPDR1*-silenced lines were more sensitive to *B. cinerea* than those of the wild-type line. Therefore, the results of this study indicate the importance of *NpPDR1* in plant defence in two organs in which it is constitutively expressed: roots and flowers. The soil is rich in pathogenic organisms and roots also need protection after wounding. Petals are fragile organs close to the reproductive cells and undergo rapid degeneration, which makes them very sensitive to pathogens, such as *B. cinerea*. *NpPDR1* is also constitutively expressed in the leaf glandular trichomes, a location in direct contact with airborne pathogens. Stukkens *et al.* (2005) reported observations on detached leaf discs superficially inoculated with spores of *B. cinerea*, indicating that *NpPDR1* is also involved in plant defence at the leaf surface level.

The transporter nature of *NpPDR1* enables several hypotheses to be formulated about the way it could interact with plant defence. Blocking the expression of *NpPDR1* could lead to accumulation in the plant cell of normally secreted toxic products. Cell death has been observed previously in senescent leaves of *NpPDR1*-silenced plants of *N. plumbaginifolia* (Stukkens *et al.*, 2005). However, it has also been shown previously (Stukkens *et al.*, 2005), and confirmed in this study, that uninoculated wild-type and *NpPDR1*-silenced plants do not display different phenotypes and grow at the same rate (Fig. 1) until flowers develop. Cell death would favour necrotrophs such as *B. cinerea*, but increased susceptibility of *NpPDR1*-silenced plants to the hemibiotroph *P. nicotianae* was also observed in this study. Moreover, cell death was not observed at the time of inoculation in the roots of *NpPDR1*-silenced plants. In addition, cell death was not observed in the petals of *NpPDR1*-silenced plants before infection by the necrotroph *B. cinerea* (Fig. 7B). Taken together, these data do not support the hypothesis that cell death is responsible for the infection process as observed in this study.

Another possibility is that cells are weakened by the accumulation of secondary metabolites. However, the similar growth of uninoculated *NpPDR1*-silenced and wild-type plants suggests that the former apparently does not suffer from physiological disorders. Alternatively, the products secreted by *NpPDR1* could have direct toxic effects on the pathogens. These toxic compounds would then be accumulated in and at the surface of roots and petals, which are sensible organs, but also in contact with the air (secretions by leaf glandular trichomes). In addition, a compound secreted by *NpPDR1* may have some regulatory function in plant defence. Finally, *NpPDR1* may be able to excrete fungal toxins belonging to the sesquiterpene family (structurally similar to plant-produced secondary metabolites), such as botrydial produced by *B. cinerea* and deoxynivalenol produced by *Fusarium* species (Deighton *et al.*, 2001; Masuda *et al.*, 2007). More detailed studies, for example involving metabolite analysis, are required to evaluate these hypotheses.

EXPERIMENTAL PROCEDURES

Pathogens

Botrytis cinerea MUCL 46725 was isolated in Belgium from a naturally infected *N. plumbaginifolia* *NpPDR1*-silenced plant (Stukkens *et al.*, 2005). *Rhizoctonia solani* B112 belonging to anastomose group 4 was received from M. A. Cubeta (Center for Integrated Fungal Research, Raleigh, NC, USA). *Phytophthora nicotianae* 183 belongs to race 0, which is non-pathogenic on *N. plumbaginifolia*, and was received from M.-T. Esquerié-Tugayé (CNRS, University Paul Sabatier, Toulouse, France). *Fusarium oxysporum* PB1 was isolated in Belgium from a naturally infected *N. plumbaginifolia* *NpPDR1*-silenced plant; it was identified at the species level on the basis of morphological characteristics. *Fusarium oxysporum* f. sp. *nicotianae* Ft-Rob was obtained from C. A. Clark (Louisiana State University, Baton Rouge, FL, USA). *Fusarium oxysporum* f. sp. *melonis* race 0 MUCL 31367 was obtained from the MUCL culture collection (Belgian Coordinated Collections of Microorganisms, Louvain-la-Neuve, Belgium).

Plant material

Nicotiana plumbaginifolia wild-type plants, *N. tabacum* cv. Petit Havana SR1 wild-type plants (Maliga *et al.*, 1973), transgenic *NpPDR1*-silenced plants of *N. plumbaginifolia* (Stukkens *et al.*, 2005) of the seventh generation (lines L 6.2 and L 8.2) and *NpPDR1-gusA* *N. tabacum* transgenic plants in which the 1282-bp sequence upstream of the *NpPDR1* transcription initiation site was fused to the *gusA* reporter gene (Stukkens *et al.*, 2005) were used in this study.

Seeds of wild-type and transgenic *N. plumbaginifolia* and *N. tabacum* were sterilized for 1 min in 70% (v/v) ethanol and for 5 min in 50% (v/v) commercial bleach, and then washed five

times in sterile water. The seeds were then treated with 500 µg/mL of 0.22-µm filtrated gibberellic acid. The seeds were germinated and the plantlets were grown *in vitro* on solid Murashige and Skoog (MS) medium [4.4 g/L of Murashige and Skoog salts (MP Biomedicals Europe, Illkirch, France), pH 5.6 (KOH), 3% sucrose, 0.9% agar] supplemented with 100 mg/L kanamycin at 25 °C under 16 h light (50 µmol photons/s/m²). After 6 weeks, the *in vitro* *N. plumbaginifolia* plants were transferred to a chamber under 16 h light at a temperature of 23 °C during the day that fell to 20 °C during the night.

NpPDR1-gusA *N. tabacum* plantlets were transferred to soil in individual pots and grown in a growth chamber under 16 h light (200 µmol photons/s/m² at soil level) at 25 °C and 8 h darkness at 19 °C. During pathogen sensitivity tests, plantlets were grown in a growth chamber under 12 h light (200 µmol photons/s/m² at soil level) and 12 h darkness at 25 °C.

Nicotiana plumbaginifolia plantlets were transferred at different ages, depending on the pathogenicity test, to twice-sterilized (1 kg gas water pressure/cm² for 30 min) hydrated Jiffy pots (Jiffy Products Nederland BV, Hoek van Holland, Netherlands). Four Jiffy pots were placed in a plastic box containing sterilized water and six plastic boxes were placed in a small glasshouse to keep the air humidity high. The glasshouses were placed in a growth chamber [12 h light (200 µmol photons/s/m² at soil level) and 12 h darkness at 25 °C]. For the analysis of higher plants, the plants in Jiffy pots were adapted to lower air humidity, transferred to sterilized pots containing sterilized soil and grown in a growth chamber or glasshouse.

Root infection tests

Two *NpPDR1*-silenced and one wild-type line of *N. plumbaginifolia* and one wild-type line of *N. tabacum* were used. Inoculated plantlets consisted of eight plants per line. Non-inoculated plantlets consisted of four plants per line. The inoculation of the pathogens was performed during the transfer of plantlets from *in vitro* culture to Jiffy pots. One millilitre of inoculum was spread in the Jiffy pot hole before transfer of the plantlet; the inoculated plant material consisted of intact roots and of roots broken during plant transfer. The inoculum and plantlet age at the transfer time varied according to the pathogen. Plants were co-cultivated with pathogens in the small glasshouses described above. The number of surviving plants was scored over time. For each pathogen, three repetitions of the test on *N. plumbaginifolia* were performed.

For spore formation, *B. cinerea* MUCL 46725 was cultivated on sterile *N. plumbaginifolia* *NpPDR1*-silenced plants cultured in Petri dishes containing a water–agar gel (15 g/L of Kalys HP 696 agar). The plant was infected with an agar block of YMPGA medium [0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) peptone, 1% (w/v) glucose, 0.8% (w/v) agar] con-

taining *B. cinerea* MUCL 46725 mycelium, incubated at 15 °C without light until spores appeared and then kept at 4 °C. The spores were collected in sterile water, filtered (224 µm), counted using a Thoma-type grid and diluted at 3×10^4 spores/mL. The tests were initiated on plantlets aged 10 weeks.

Fusarium oxysporum PB1, *F. oxysporum* f. sp. *nicotianae* Ft-Rob and *F. oxysporum* f. sp. *melonis* race 0 MUCL 31367 were grown on potato dextrose agar (PDA) medium [20% (w/v) infusion from potato, 2% (w/v) dextrose, 1.5% (w/v) agar] at 28 °C for 4 days. Spores were collected, filtered (224 µm), counted using a Thoma-type grid and diluted at 10^6 spores/mL. The tests were initiated on plantlets aged 8 weeks.

Rhizoctonia solani B112 mycelium was grown in 75 mL of liquid potato dextrose broth (PDB; Sigma-Aldrich, Bornem, Belgium) medium in 125-mL Erlenmeyer flasks at 28 °C for 9 days with constant agitation (220 r.p.m.). Forty millilitres of culture were then transferred to a sterile Falcon tube (50 mL) and allowed to sediment until approximately 10 mL of medium in the upper part of the suspension could be differentiated visually from the more sedimented fraction and collected. The concentration of the small mycelium pieces was evaluated using a Thoma-type grid, and the mycelium was then adjusted to 10^4 mycelium pieces/mL. The tests were initiated on plantlets aged 7 weeks.

Phytophthora nicotianae 183 was grown on V8 juice agar for oospore production (V8-O) containing per litre: 354 mL of V8 juice, 5 g of CaCO_3 , 132.32 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 30 mg of *b*-sitosterol, 20 mg of tryptophan, 1.12 mg of thiamine.HCl and 15 g of Kalys HP 696 agar. V8 juice and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were initially mixed and centrifuged for 20 min at $1753 \times g$. The collected supernatant was adjusted to 1 L before addition of the remaining ingredients and autoclaving. *Phytophthora nicotianae* 183 was grown at 20 °C for 20–30 days until sporangium formation. Sporangia were suspended in sterile osmoted water by removing mycelium slants from the agar surface, followed by vigorous shaking by hand and using a vortex. In the tests on *N. plumbaginifolia*, the suspension was filtered (500 µm), the sporangia were counted using a Thoma-type grid and diluted to 100 sporangia/mL; the tests were initiated on plantlets aged 10 weeks. Tests were also performed with wild-type *N. tabacum* plantlets using reduced inoculum concentrations of 50 and 10 sporangia/mL.

The root material of wild-type plantlets of *N. plumbaginifolia* cultured in Jiffy pots infected by pathogens was frozen in liquid nitrogen, stored at –20 °C and analysed by Western blotting to detect *NpPDR1* expression.

Leaf infection tests

Leaves of mature wild-type plants of *N. tabacum* and *N. plumbaginifolia*, transgenic *NpPDR1*-silenced plants of *N. plumbaginifolia* and transgenic *NpPDR1-gusA* plants of *N. tabacum* were infiltrated with individual pathogens through the stomata using

a syringe. The infiltrated zones were marked with a marker. The pathogen concentrations were as described above, except for *R. solani* B112 (3×10^4 mycelium pieces/mL) and *P. nicotianae* 183 (10^3 sporangia/mL); for *B. cinerea*, the spore source was a sporulating sclerotium in a PDA culture. For *B. cinerea*, the wild-type plants were also wounded in an infiltration zone with a needle. Observations of symptoms or GUS and protein analyses were performed over time.

Leaf material of wild-type plantlets of *N. plumbaginifolia* cultured in Jiffy pots, which were infected following an initial infection of *B. cinerea* through the roots (see Root infection tests), was frozen in liquid nitrogen, stored at –20 °C and, finally, analysed by Western blotting to detect *NpPDR1* expression.

Flower infection tests

Nicotiana plumbaginifolia wild-type and transgenic *NpPDR1*-silenced plants were grown in a growth chamber until flower induction. To obtain flower homogeneity, all the mature or nearly mature flowers were cut in the morning of one day and the new mature flowers used in the tests were cut the day after in the afternoon. To obtain permanent flower opening (*N. plumbaginifolia* flowers are closed during the day and open during the night), cut flowers were kept overnight in the dark at 25 °C in Falcon tubes containing 100 µL of sterile osmoted water. The open flowers were then inoculated by placing on the petals 150 µL of a 100 spores/mL *B. cinerea* suspension (see Root infection tests) and incubated in the dark at 25 °C. The flowers showing small brown *B. cinerea* infection spots were counted after 1 day. Three independent repetitions of the test were performed.

At inoculation time, after the initial overnight incubation at 25 °C and at test reading time 24 h later, the corollas from three uninoculated detached flowers were separated into upper (yellow–green on one side) and lower (white) parts with a scalpel, frozen in liquid nitrogen, stored at –20 °C and eventually analysed by Western blotting to detect *NpPDR1* expression.

Plant microsomal fraction preparation and Western blotting

Plant material was ground in 2 vol of cold homogenization buffer [250 mM sorbitol, 50 mM Tris-HCl, pH 8.0, 2 mM ethylenediamine-tetraacetic acid (EDTA), 6 g/L polyvinylpyrrolidone, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonylfluoride (PMSF) and 2 µg/mL each of leupeptin, pepstatin, aprotinin, antipain and chymostatin] using a 1-mL glass 'Dull' grinder (VWR).

Cell debris was removed by centrifugation for 5 min at 3500 *g* and the supernatant was centrifuged for 5 min at 10 000 *g*. The supernatant was then centrifuged for 15 min at 100 000 *g*. Finally, the pellet corresponding to the microsomal fraction was suspended in 5 mM KH_2PO_4 , 330 mM sucrose, 3 mM KCl, pH 7.8 (KOH). Proteins were then quantified (Bradford, 1976).

Proteins (20 µg) were solubilized for 15 min at 37 °C in Laemmli buffer [80 mM Tris-HCl, pH 6.8, 2% (w/v) sodium dodecylsulphate (SDS), 10% (w/v) glycerol, 1% (w/v) DTT and 0.005% bromophenol blue; Laemmli, 1970] containing 1 mM PMSF and protease inhibitors (2 µg/mL each of leupeptin, pepstatin, aprotinin, antipain and chymostatin), and separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [7% (w/v) polyacrylamide] using Mini-protean 3 Cell electrophoresis (Bio-Rad, Hercules, United States). After electrophoresis, proteins were electrotransferred (22 V, 1 h) onto a poly(vinylidene difluoride) (PVDF) membrane (Millipore, Billerica, United States) using the semi-dry system (Bio-Rad) and a transfer solution containing 50 mM Tris, 40 mM glycine, 0.0375% (w/v) SDS and 10% (v/v) methanol. Membranes were saturated for 30 min at room temperature in Tris-buffered saline [50 mM Tris, 150 mM NaCl, pH 7.6 (HCl)] containing 3% (w/v) low-fat dried milk and 0.5% (w/v) Tween 20, washed 3 × 5 min with washing solution [Tris-buffered saline with 0.1% (w/v) low-fat dried milk and 0.5% (w/v) Tween 20], and then incubated for 1 h at room temperature in washing solution containing rabbit primary polyclonal antibodies raised against a peptide corresponding to Ala-212–Arg-335 of NpPDR1 (Stukkens *et al.*, 2005), diluted 1 : 500, or a 1 : 100 000 dilution of a rabbit primary polyclonal anti-plasma membrane H⁺-ATPase antibody (Morsomme *et al.*, 1998). After two 10-min washes, the membranes were incubated for 1 h with sheep secondary anti-rabbit IgG antibodies conjugated to horseradish peroxidase (POD) (Millipore), diluted 1 : 10 000. After three 5-min washes, the antigen–antibody complexes were detected by the enhanced chemiluminescence method (Roche Diagnostics Belgium, Vilvoorde, Belgium).

Histochemical analysis

Hand-cut sections of plant leaves were fixed in 50 mM phosphate buffer, pH 7.2 (NaOH) [34.2 mM Na₂HPO₄, 15.8 mM NaH₂PO₄ and 0.05% (v/v) Triton X-100] containing 4% (v/v) formaldehyde (37%) for 30 min under vacuum, washed three times with 50 mM phosphate buffer pH 7.2, and transferred into a reaction buffer containing 50 mM phosphate buffer pH 7.2 (NaOH), 0.05% (v/v) Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) and 0.02% NaN₃ (w/v). The sections were vacuum infiltrated for 30 min and incubated for 2–16 h at 37 °C in the dark. The reaction buffer was then discarded and chlorophyll was solubilized in 70% (v/v) ethanol twice for 2 h, or overnight if necessary. The stained tissues were preserved in 50% (w/v) glycerol and 0.02% (w/v) NaN₃.

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