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Differential induction of two potato genes, *Stprx2* and *StNAC*, in response to infection by *Phytophthora infestans* and to wounding

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Abstract

To find out more about the interaction between potato and *Phytophthora infestans* at the molecular level, we screened for genes induced early after infection using mRNA differential display. Among the twenty cDNA clones recovered in the screen, two were found to represent plant genes whose transcript levels increased during infection of intact plants. These two genes differed strikingly in their response to wounding. *Stprx2*, a putative peroxidase, responded slowly and transiently to wounding, and its expression pattern was similar to that of *gst1*, a well-described pathogen-induced gene of potato. The second gene, *StNAC*, was induced rapidly and strongly after wounding but not systemically. Transcript levels reached a maximum after around 1 h and returned to basal levels after ca. 24 h. StNAC has strong similarity to the ATAF subfamily of NAC domain proteins, a large family of putative transcriptional activators. *Arabidopsis ATAF1* and *ATAF2* were also shown to be induced by wounding. This implies that the ATAF genes are not merely structurally similar but also share a conserved role in stress responses.

Abbreviations: NAC domain, petunia NAM and *Arabidopsis* ATAF1 ATAF2 and CUC2; a.i., after inoculation

Introduction

Plants exposed to virulent and avirulent pathogens respond with a range of defence- and damage-limiting mechanisms. Many of the responses are also induced by other stress-causing agents, which reflects the common elements in different stress situations. For example, a number of effectors, including pathogens, radiation and heavy metals, all cause oxidative stress, and wounding is associated with the risk of infection by bacteria, viruses and fungi as well as tissue damage. Other responses are more specific; for example, certain genes of *Arabidopsis* are induced by particular strains of pathogenic bacteria (Reuber and Ausubel, 1996), and the PR protein gene *gst1* (previously *prp1-1*) of potato was shown to be induced by infection but not by wounding, darkness, white light or heat shock (Martini *et al*., 1993). Knowledge of the factors controlling expression of the different genes induced by pathogen infection can increase our understanding of the infection process and the ways plants perceive infection.

The oomycete pathogen *Phytophthora infestans* causes late blight in potato, the most important disease of potato world-wide. Although intensively studied (e.g. Cuypers and Hahlbrock, 1988, Gees and Hohl, 1988; Freytag *et al*., 1994; Kamoun *et al*., 1999), we still do not fully understand what controls and constitutes the difference between a resistant and susceptible response to *P. infestans* attack. Nevertheless, it is clear that recognition of *P. infestans* infection occurs during or after penetration of the first epidermal cell, and that the outcome of the interaction, compatibility or

The nucleotide sequence data reported will appear in the EMBL Nucleotide Sequence Database under the accession numbers AJ401150 (*Stprx2*) and AJ401151 (*StNAC*).

incompatibility, is decided soon afterwards (Freytag *et al*., 1994).

A comprehensive picture of potato responses during the early phases of both compatible and incompatible interactions, coupled with knowledge of how the different responses are regulated, would tell us how *P. infestans* attack is perceived and successfully countered. As a first step towards this goal, we screened for genes induced by *P. infestans* in potato during the first few hours after inoculation, using mRNA differential display. One gene, encoding a putative peroxidase, was shown to be induced by infection, but only slowly and to a relatively small degree by wounding. A second gene was also induced on infection but more dramatically by wounding. This gene belongs to a large gene family encoding putative transcriptional regulators.

Material and methods

Plant material and treatment

Axenic potato plants, cv. Bintje, were kindly provided by Dr Patrick Schweizer (then at the University of Fribourg, Switzerland). The plants were grown from internode cuttings in Murashige and Skoog medium containing 2% w/v sucrose, without hormones, for up to 8 weeks. Soil-grown plants were raised from two-week old axenic cuttings in a growth chamber with a 16 h light period and temperatures of 17 °C in the night and 20 °C in the day. Seeds of *Arabidopsis* ecotype Columbia were grown individually in pots in a growth chamber with a 12 h light period at 19 °C in the dark and 23 °C in the day.

P. infestans strain RDA-49 was kindly provided by Dr David Johnston, then at Changins Agricultural Station, Nyon, Switzerland. It was grown on rye agar at 16 °C. Virulence was maintained by infecting leaves and re-isolating every 3–4 months. Zoospores were released by flooding 10–14-day old cultures with 10 ml cold distilled water and incubating at 4 °C for 2–3 h. Potato plants were infected with *P. infestans* by spraying until run-off with freshly isolated spores at 1×10^6/ml and maintained in the conditions described above. To maintain the high humidity required for infection, plants were covered with Plexiglas boxes of which the inner surfaces had been sprayed with water. Control plants were sprayed with water and covered in the same way.

Crushing wounds were inflicted on fully expanded leaves with a haemostat, one wound per *Arabidopsis* leaf and two or three per potato leaf. When wounding was directly compared with infection, plants were also sprayed with water and covered as above.

Leaves were removed and immediately frozen in liquid nitrogen, then stored at −80 °C prior to RNA extraction (Chomczynski and Sacchi, 1987).

Differential display

Leaves from ca. 6-week old axenically grown plants were excised and placed on moist filter paper in petri dishes. Four to five 50 μl drops of zoospore suspension, 1×10^6/ml, or water (as control) were placed on the adaxial surface of the leaves. The petri dishes were sealed and placed in a 16 °C incubator. Infected and control leaves were collected immediately after inoculation and after 1, 2, 4 and 24 h for RNA extraction. In each experiment, a sample of leaves was maintained for four days to ensure that the infection was successful.

RNA was extracted from leaves by the method of Chomzcynsky and Sacchi (1987) and differential display (Liang *et al*., 1994) was performed on RNA from each time point with an RNAimage kit from GenHunter (Brookline, MA) with thirteen 5p primers. The PCR reactions were run on Sequagel XR (National Diagnostics) denaturing polyacrylamide gels and differential bands were re-amplified and cloned into pGEM-T (Promega). Differential display gel bands can contain more that one cDNA fragment. Clones carrying the differentially amplified fragments were identified as described by Vögeli-Lange *et al*. (1996).

RNase protection assays

Antisense RNA probes were prepared with a MAXIscript kit (Ambion). The appropriate strand was chosen by assuming that the anchored oligo-dT primers had annealed to the poly(A) tail in the mRNA differential display experiments. High-specific-activity probes for the differential display clones were made with 6 μM ^{32}P-UTP at ca. 15 TBq/mmol in the reaction. Control 18S rRNA probes were made with 500 μM ^{32}P-UTP at ca. 375 MBq/mmol in the reaction. RNase protection assays were performed using reagents from Roche on 10 μg total RNA hybridized with both antisense probes in the same tube essentially as described by Goodall *et al*. (1990). The denaturing polyacrylamide gels were analysed with a Storm II phosphorimager (Molecular Dynamics). Transcript levels of the clone of interest were normalized to the 18S rRNA level, and fold induction was calculated

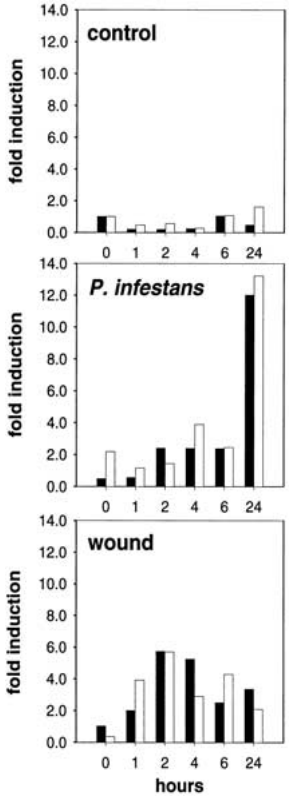

Figure 1. Clone *Stprx2* and *gst1* are induced by *P. infestans* infection and minimally by wounding. RNase protection assays were performed on 10 μg total RNA with either *Stprx2* (original 350 bp clone) or *gst1* probe together with 18S rRNA probe. The transcript levels (*Stprx2*, black; *gst1*, white) have been normalized to the 18S rRNA level, and fold induction relative to the time zero level in the control (water-treated) plants is shown.

relative to the time zero level in the plants treated with water as control.

RNA gel blots

Total RNA samples (10 μg) were separated on formaldehyde-agarose gels and transferred onto positively charged nylon membrane (Zeta-probe, BioRad) (Sambrook *et al*., 1989). DNA probes were labelled with the Prime It II random primed labelling kit (Stratagene), and hybridization and washing were carried out at 65 °C as described by Church and Gilbert (1984).

5′ RACE and sequence analysis

5′ RACE of the original *Stprx2* clone was carried out with two forward primers: RACE I, 5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTT-TTTT-3′ and RACE II, 5′-GACCACGCGTATCGAT-GTCGAC-3′, and three clone-specific reverse primers: *Stprx2* I, 5′-CAACAACATCAATGGGT-3′; *Stprx2* II, 5′-CAACGTTACCTATACCCT-3′ and *Stprx2* III, 5′-TCACCCACCTAATGAAATGA-3′. Reverse transcription was carried out on 5 μg of total RNA extracted from potato leaves 24 h after infection with *P. infestans*, with primer *Stprx2* I and SuperscriptII RNase H-Reverse Transcriptase (Life Technologies) under the recommended conditions. The first-strand cDNA was then purified by QIAquick PCR purification (Qiagen) and ethanol precipitation. Poly(A) tailing of the first-strand cDNA was performed with terminal transferase from Boehringer Mannheim (Roche Molecular Biochemicals). PCR was then carried out with the RACE I and *Stprx2* II primers and a 250-fold final dilution of the tailed cDNA. Secondary PCR was performed on a 100-fold final dilution of the primary PCR product using primers RACE II and *Stprx2* III. PCR was performed with Clontech's Advantage PCR enzyme in a 25 μl reaction volume under the following conditions: 94 °C for 1 min and 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 3 min. 5′ RACE of the original *StNAC* clone was carried out in the same way with the following three clone-specific reverse primers: *StNAC* I, 5′-TGAAGAATGATAACTTGATA-3′; *StNAC* II, 5′-AGGCTCATTTGAGCTGT-3′; and *StNAC* III, 5′-CCACCAAATTTGACTTGCAT-3′. The single PCR products were isolated from an agarose gel with the QIAquick Gel Extraction Kit (Qiagen) and cloned into the pGEM-T vector (Promega).

Sequencing was performed with an ABI PRISM 377 DNA Sequencer, and sequence data were analysed by DNASIS (Hitachi Software) and the GCG package (Wisconsin Package Version 10, Genetics Computer Group, Madison, WI). Similarity searches were made with BLAST programs at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Amino acid motif and cellular localization signal searches were made with MOTIF (http://motif.genome.ad.jp) and PSORT (http://psort.ims.u-tokyo.ac.jp). The protein sequence alignment shown in Figure 3 was generated with the ClustalW program (Thompson *et al*., 1994; at http://circinus.ebi.ac.uk:6543/cgi-bin/clustalw.cgi) and displayed with BOXSHADE 3.21 (Kay Hofmann and Michael D. Baron, ISREC Bioinformatics group, Epalinges s/Lausanne, Switzerland; at http://www.ch.embnet.org/software/BOX_form.html).

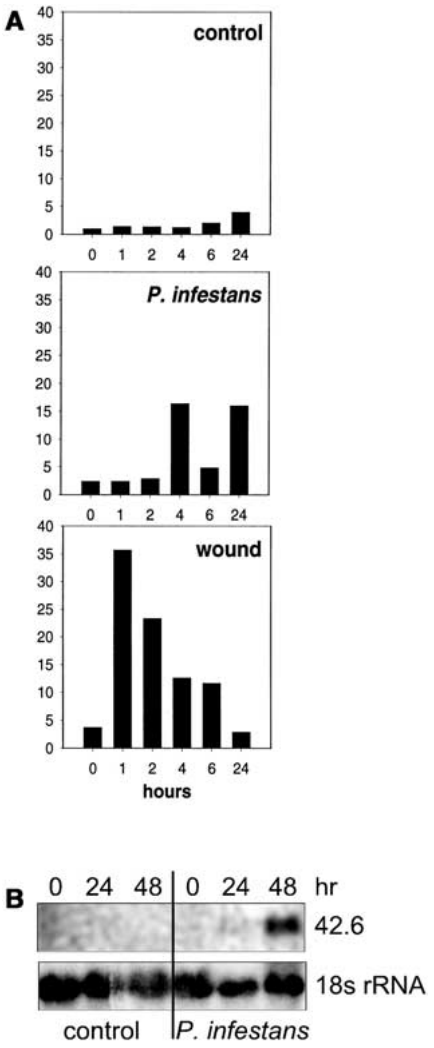

Figure 2. Clone *StNAC* is induced by *P. infestans* infection and by wounding. A. RNase protection assays were performed on 10 μg total RNA with both *StNAC* (original 198 bp clone) and 18S rRNA probes. The *StNAC* transcript levels have been normalized to the 18S rRNA level, and fold induction relative to the time zero level in the control (water-treated) plants is shown. B. RNA gel blot analysis of *StNAC* expression during 48 h a.i. with *P. infestans*.

Results

To identify transcripts accumulating rapidly after infection, we performed differential display on RNA from axenically grown potato leaves harvested during the first few hours after inoculation with zoospores of *P. infestans*. These time points were chosen because *P. infestans* penetrates the first cell 1–2 h after inoculation and the first reactions in the invaded plant cell and surrounding tissues can be detected by 3 h after infection (a.i.) (Freytag *et al.*, 1994). Differential

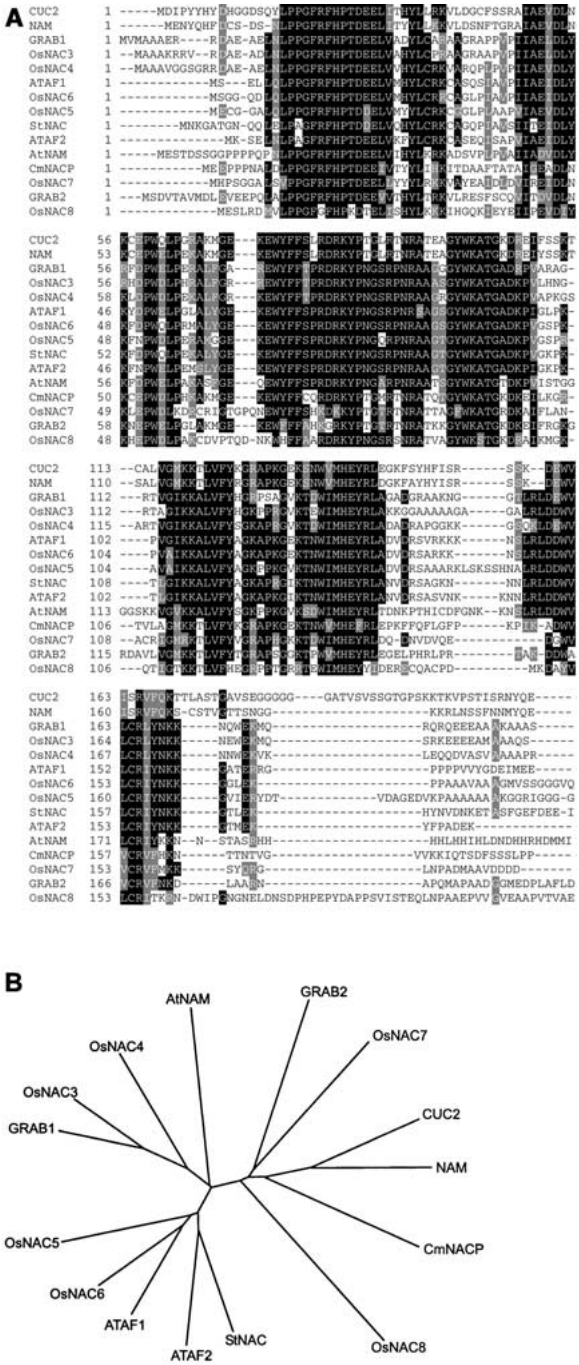

Figure 3. A. Alignment of StNAC with with related NAC domain proteins: ATAF1 (translated from mRNA, X74755), ATAF2 (translated from mRNA, X74756), AtNAM (AAD17313) and CUC2 (Aida *et al.*, 1997) from *Arabidopsis*, petunia NAM (Souer *et al.*, 1996), GRAB1 and GRAB2 from wheat (Xie *et al.*, 1999), CmNACP from pumpkin (Ruiz-Medrano *et al.*, 1999) and OsNAC3 to OsNAC8 from rice (Kikuchi *et al.*, 2000). Only the N-terminal part of the proteins including the highly conserved NAC domain is shown. Shading shows amino acids that are identical (black) or similar (grey) in at least 50% of the sequences. Dashes indicate gaps introduced to create the alignment. B. Unrooted tree derived from the same ClustalW alignment, and drawn using Phylodendron (D.G. Gilbert, Version 0.8 d, beta) at http://iubio.bio.indiana.edu/treeapp/treeprint-sample2.html.

display gel bands were identified that increased in intensity over the time course in at least two completely independent experiments. The cDNA fragments isolated from differential display gels were first analysed by Southern blotting to identify those of potato origin (not shown). The cDNA clones from potato were then analysed by RNase protection assays to determine which were really induced by infection (not shown). Of the twenty potato clones analysed, most were not differentially expressed, not detectable or could not be analysed by RNase protection because the probes were self-protecting. Three clones appeared to be induced and were further analysed for induction using infected and wounded soil-grown plants.

Intact 4–6-week old soil-grown plants were wounded or sprayed with *P. infestans* zoospores and covered to maintain high humidity (control and wounded plants were sprayed with water). Under these conditions, the first symptoms of late blight were visible after two days. After four days all the fully expanded leaves were chlorotic with necrotic brown patches or had fallen off. RNase protection assays with RNA extracted from these plants showed that two clones were indeed from mRNAs induced by infection *in planta*.

Clone Stprx2

One clone, *Stprx2*, derives from an mRNA that accumulated early after infection and only minimally after wounding, as demonstrated by RNase protection assays (Figure 1). The expression pattern was compared to that of *gst1*, a well-described pathogen-induced gene (e.g. Martini *et al*., 1993; Hahn and Strittmatter, 1994; Strittmatter *et al*., 1996). The *gst1* cDNA clone was provided by Dr Michel Schneider, University of Fribourg, Switzerland, and the potato 18S rRNA clone used to normalize transcript levels was kindly provided by Dr Ferenc Nagy, then at the Friedrich Miescher Institute, Basel, Switzerland.. The changes in *gst1* transcript levels after infection and wounding were found to be similar, although the transcript levels of *gst1* were substantially higher.

The 350 bp *Stprx2* clone obtained by differential display includes the last 16 codons of the ORF. This short stretch of peptide sequence is up to 80% identical to a handful of peroxidases from *A. thaliana* (Justesen *et al*., 1998). A cDNA clone with a complete ORF of 1337 bp was isolated by 5′ RACE. The ORF is predicted to encode a secreted peroxidase of 331 amino acids with a pI of 8.33. Similarity is highest (70% identical and 83% similar) to a recently described peroxidase from white clover, Trprx2, that is constitutively expressed in roots but down-regulated on infection by virulent *Pseudomonas syringae* (Crockard *et al*., 1999), and to Msprx2 of alfalfa (69% identical, 81% similar), which was isolated in a screen for peroxidases in a cDNA library derived from leaves infected with avirulent *P. syringae* (El-Turk *et al*., 1996). We therefore named this putative potato peroxidase Stprx2. We also observed expression of Stprx2 in roots (not shown).

Clone StNAC

The second differential display clone, *StNAC*, was observed to be induced by infection, but also rapidly after wounding (Figure 2A). Transcript levels increased 24 h a.i. in three independent experiments. The high transcript level observed in the experiment shown in Figure 2A at 4 h a.i. was not seen in other experiments and is probably due to inadvertent wounding when plants were uncovered and re-covered to take leaf samples. In a separate infection experiment analysing the response to infection up to 48 h a.i. by RNA gel blot, the *StNAC* transcript was barely detectable at 24 h but continued to accumulate and reached much higher levels after 48 h (Figure 2B). The weak signal in the 24 h time point in Figure 2B is due to the lower sensitivity of RNA gel blot analysis cf. RNase protection assay.

StNAC *derives from an NAC gene*

The original *StNAC* clone was a 198 bp fragment of the 3′ end of the cDNA, and it had no significant homologies with any entries in the databases. A cDNA clone comprising a complete ORF was obtained by 5′ RACE. The 1295 bp cDNA clone contains a single ORF that is predicted to encode a protein of 301 amino acids and a pI of 7.80. The protein has very strong similarity to a group of plant-specific proteins that share a conserved NAC domain at their N-termini, and was therefore named StNAC. ‘NAC domain’ was coined by Aida *et al*. (1997) and stands for petunia NAM and *Arabidopsis* ATAF1 ATAF2, and CUC2. Figure 3A shows an alignment of the amino-terminal half of the predicted StNAC protein with other putative NAC proteins. The highly conserved boxes of the NAC domain are clearly seen. The C-termini of the proteins are very different. The unrooted tree in Figure 3B shows that the NAC domain proteins form three main subfamilies, as reported by Kikuchi *et al*. (2000). StNAC falls into the ATAF subfamily. At the DNA level, *StNAC*

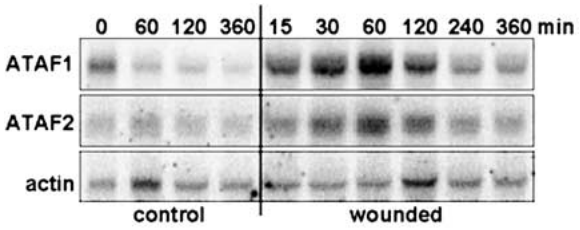

Figure 4. RNA gel blot analysis of *ATAF1* and *ATAF2* mRNA levels after wounding. 10 μg of total RNA extracted from wounded leaves and leaves of control plants were separated and transferred to a membrane. The membrane was successively hybridized with the cDNA probes indicated on the left. The times indicate minutes after infliction of wounds.

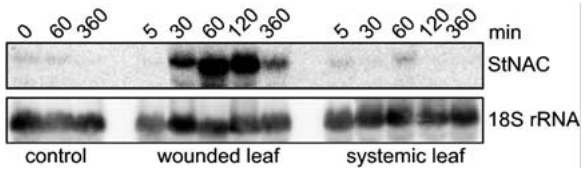

Figure 5. RNA gel blot analysis of *StNAC* mRNA levels in wounded and systemic leaves. 10 μg of total RNA extracted from wounded and systemic leaves and from leaves of control plants were separated and transferred to a membrane. The membrane was successively hybridized with radiolabelled probes from the full-length *StNAC* cDNA and from 18S rRNA cDNA. The times indicate minutes after infliction of wounds.

has significant BLAST scores only with *ATAF2* (highest) and *ATAF1*, which again indicates that this group of NAC domain proteins are orthologues.

Genomic DNA blot analysis (not shown) indicated that there may be at least one other *StNAC*-similar gene in Bintje. No homologous sequences were detected in the *P. infestans* genome.

Wound induction of ATAF1 *and* ATAF2

We analysed whether the closest *Arabidopsis* homologues of *StNAC*, *ATAF1* and *ATAF2*, are also induced by wounding. Three-month old Columbia plants were wounded, and the extracted RNA was analysed by RNA gel blot hybridization. Clones of *ATAF1* (accession number T22869) and of *ATAF2* (T04277), generated by Newman *et al*. (1994), were obtained from the *Arabidopsis* Biological Resource Center. The two clones hybridize to RNA bands of slightly, but distinctly, different sizes. The *Arabidopsis* Actin-1 (Nairn *et al*., 1988) clone was PCR-amplified (Penninckx *et al*., 1996) and provided by Dr Lourdes Gómez Gómez of the Friedrich Miescher Institute. Figure 4 shows that both *ATAF1* and *ATAF2* transcript levels began to increase within 15 min of wounding, reaching a maximum after 60 min. These results indicate that the function of these proteins as well as their sequence may be similar to StNAC.

StNAC *was not induced systemically*

Most wound-responsive genes are induced in other parts of the plant by wounding, not only locally in the wounded leaf. We therefore analysed *StNAC* expression in wounded leaves of potato and in the next, distal leaf on the stem (Figure 5). In the control and in the wounded leaf after 5 min, *StNAC* transcript was barely detectable. Its level increased up to 60 min then began to decline. In the systemic leaf a small amount of transcript was detectable 60 min after wounding, but this was similar to the trace amount seen at 5 min. StNAC is therefore not a classic, systemically induced wound-responsive gene such as proteinase inhibitor I and II (Graham *et al*., 1986; Keil *et al*., 1989) or Twi1 of tomato (O'Donnell *et al*., 1989).

Discussion

Stprx2 *encodes a putative peroxidase with an expression pattern similar to* gst1

The predicted peroxidase gene, *Stprx2*, responded to wounding with a slow and transient increase in transcript level. The transcript level of *gst1* followed a similar pattern. This result was unexpected because *gst1* did not appear to be induced by wounding in previous studies (Martini *et al*., 1993). The discrepancy may arise from variation in response between potato varieties or to differences in experimental procedure: we inflicted crushing wounds to leaves on intact plants, while Martini *et al*. (1993) made cuts in detached leaves. The first increase in transcript level in response to infection could be seen two to four hours a.i. The role of *Stprx2* may be similar to its homologue *Msprx2* since both are expressed in infected leaves but constitutively expressed in roots (Esnault *et al*., 1993; Savouré *et al*., 1997). The induction of specific peroxidases early in infection has been frequently reported (e.g. Harrison *et al*., 1995; Ray and Hammerschmidt, 1998; Kristensen *et al*., 1999), and roles in several plant responses discussed, including lignification, cross-linking of cell wall proteins, and the production of antimicrobial radicals.

StNAC *transcript levels increase in response to infection and wounding*

The response of *StNAC* to infection was slower than that of *Stprx2*. An increased message level was detectable at around 24 h a.i. with *P. infestans*, at which point only one or two cells at each infection site have generally visibly reacted to infection. As revealed by the RNA gel blots, *StNAC* mRNA accumulated to much higher levels after 48 h, when larger number of dead cells are seen (Beyer and Collinge, unpublished results; Cuypers and Hahlbrock, 1987). It is not absolutely clear whether the cell death arises from the change from biotrophic to necrotrophic nutrition of *P. infestans* (perhaps once propagation is ensured) and the plant cells are killed or whether this is a late HR-type response. Nevertheless, the levels of *StNAC* transcript appeared to correlate with the numbers of dead cells in infected tissues. The response of *StNAC* to wounding was rapid and apparently restricted to the wounded leaf. Thus the gene could be generally induced by cell or tissue damage.

A subfamily of NAC domain proteins involved in stress responses

NAC proteins have so far been found only in plants. They constitute a large family and Kikuchi *et al*. (2000) recently identified three subfamilies, NAM, OsNAC3 and ATAF, and speculated that the subfamilies may have evolved different roles. *StNAC* and its two *Arabidopsis* homologues, *ATAF1* and *ATAF2*, were rapidly and transiently induced by wounding. This indicates that the members of the ATAF subfamily of NAC domain proteins may share a conserved role in the response to stress, in contrast to other NAC genes, *NAM*, *CUC2* and *NAP*, which are known to be involved in development (Souer *et al*., 1996; Aida *et al*., 1997, 1999; Sablowski and Meyerowitz, 1998). The other two members of the ATAF gene subfamily shown in Figure 3B, *OsNAC5* and *OsNAC6*, are from rice. *OsNAC5* is expressed in roots and embryos and *OsNAC6* is expressed in all tissues (Kikuchi *et al*., 2000), but determination of transcript levels in response to stress has not yet been reported. Entries in the database describe two more NAC domain proteins induced by elicitor or pathogen treatment. One cDNA clone from *Brassica napus* is an expressed sequence tag from the response to *Leptosphaeria maculans* (AI352853) and also has similarity to ATAF2. In contrast, a tobacco cDNA clone induced by elicitor treatment does not fall into the ATAF subfamily, and a number of invariant residues in the NAC domain are not conserved.

NAC domain proteins may be transcriptional activators

The most likely role for NAC domain proteins is as transcriptional activators. *ATAF1* and *2* were isolated in a one-hybrid screen for *Arabidopsis* genes that allowed transcription from the CaMV 35S promoter in yeast (H. Hirt, personal communication). Since they do not specifically bind the promoter, transcriptional activation in concert with yeast transcription factors is the most likely mode of action. The database entry for AtNAM (AAD17314) also assigns a transcriptional activation function. The role would be consistent with the requirement of *CUC1* and *CUC2* for expression of *SHOOT MERISTEMLESS* (Aida *et al*., 1999). Although no nuclear localization signals can be recognized in NAC domain proteins, CmNACP was detected in nuclei (Ruiz-Medrano *et al*., 1999). If NAC proteins do activate transcription, specificity could be conferred by their expression pattern or by the variable C-termini.

The demonstration by Ruiz-Medrano *et al*. (1999) that pumpkin *CmNACP* mRNA is transported in the phloem suggests an additional dimension to the potential control of plant processes by NAC genes. *cmNACP* has a potential role in development as its mRNA was transported to and accumulated in meristems. However, if phloem transport were a general property of NAC gene mRNAs, then the ATAF genes could mediate stress responses at the systemic level as well as in the directly affected cells.

In summary, we have identified two genes that were induced during infection by *P. infestans*, but which had different responses to wounding. Our results show that the early plant responses are regulated differently, but also, as others have observed (e.g. Baron and Zambryski, 1995), that different stress treatments can induce similar responses. This may be because plants respond to a plethora of different effectors when attacked by a pathogen; some of these may be pathogen-derived molecules, others may be derived from the action of the pathogen on the structural components or the metabolism of the host. A better understanding of these complex, interacting processes may lead to the development of improved ways to combat diseases caused by oomycetes and other pathogens.

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