cDNA-AFLP Combined with Functional Analysis Reveals Novel Genes Involved in the Hypersensitive Response

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Submitted 19 September 2005. Accepted 27 January 2006.

To identify genes required for the hypersensitive response (HR), we performed expression profiling of tomato plants mounting a synchronized HR, followed by functional analysis of differentially expressed genes. By cDNA-AFLP analysis, the expression profile of tomato plants containing both the *Cf-4* resistance gene against *Cladosporium fulvum* and the matching *Avr4* avirulence gene of this fungus was compared with that of control plants. About 1% of the transcript-derived fragments (442 out of 50,000) were derived from a differentially expressed gene. Based on their sequence and expression, 192 fragments, referred to as *Avr4*-responsive tomato (*ART*) fragments, were selected for VIGS (virus-induced gene silencing) in *Cf-4*-transgenic *Nicotiana benthamiana*. Inoculated plants were analyzed for compromised HR by agroinfiltration of either the *C. fulvum Avr4* gene or the *Inf1* gene of *Phytophthora infestans*, which invokes a HR in wild-type *N. benthamiana*. VIGS using 15 of the *ART* fragments resulted in a compromised HR, whereas VIGS with fragments of *ART* genes encoding HSP90, a nuclear GTPase, an L19 ribosomal protein, and most interestingly, a nucleotide binding-leucine rich repeat (NB-LRR)-type protein severely suppressed the HR induced both by *Avr4* and *Inf1*. Requirement of an NB-LRR protein (designated NRC1, for NB-LRR protein required for HR-associated cell death 1) for *Cf* resistance protein function as well as *Inf1*-mediated HR suggests a convergence of signaling pathways and supports the recent observation that NB-LRR proteins play a role in signal transduction cascades downstream of resistance proteins.

Additional keywords: CC-NB-LRR, defense.

The hypersensitive response (HR) is a swift reaction of plants to resist pathogen invasion (Dangl and Jones 2001; de Jong et al. 2004). Although a number of key players involved in the HR have been identified, many steps in the signal transduction cascade that is activated upon recognition of a pathogen by a resistant host remain obscure. To identify novel components of the HR signaling pathway, the interaction between tomato (*Lycopersicon esculentum*) and the biotrophic fungal pathogen *Cladosporium fulvum* is employed (Thomma et al. 2005). Resistance to the fungus depends on the presence of plant resistance (*Cf*) genes matching to pathogen *Avr* genes that encode race-specific elicitors. Five *Cf* genes (Takken et al. 1998; Thomas et al. 1998) and four of the matching *Avr* genes (Joosten et al. 1994; Luderer et al. 2002; Van den Ackerveken et al. 1992; Westerink et al. 2004) have been cloned. In this typical gene-for-gene interaction, a strict correlation exists between the triggering of a HR and resistance, as the various *Avrs* induce a specific HR in tomato genotypes carrying the matching *Cf* resistance gene. In a susceptible tomato that lacks the matching *Cf* gene, such a HR does not occur. Furthermore, loss of an *Avr* by the fungus results in evasion of *Cf*-specific resistance (Joosten and De Wit 1999; Joosten et al. 1994; Van den Ackerveken et al. 1992).

To study HR signaling at the whole-plant level and to identify genes involved in this defense response, we have crossed near-isogenic tomato lines carrying a *Cf* gene with transgenic tomato plants that lack functional *Cf* genes and express the matching *Avr* gene. The resulting *Cf/Avr* seeds germinate similar to seeds of the parental lines. However, within two to four days after emergence of the hypocotyl, the seedlings mount a systemic HR and express typical defense-related genes (Cai et al. 2001). Interestingly, these defense responses are suppressed by incubating the seedlings at an elevated temperature (33°C) immediately after germination. A subsequent shift to a permissive temperature (20 to 25°C) results in a synchronized HR that becomes visible within 36 h (de Jong et al. 2002). However, already within 1 to 4 h after the temperature shift, expression of the HR marker gene *Lehsr203* (Pontier et al. 1998) and various other defense-related genes, such as pathogenesis-related (*PR*) genes and *NPR1*, are induced (de Jong et al. 2002). Therefore, this synchronized activation of *Cf*-mediated signaling allows us to study early transcriptional changes in plants that mount a HR. In the absence of the pathogen itself, all differentially expressed genes, except for the genes present

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on the T-DNA carrying the *Avr* and selection markers, originate from the plant. Furthermore, the constitutive production of the *Avr* protein continuously triggers HR signaling throughout the plant, facilitating the detection of the genes involved.

To identify *Avr4*-responsive tomato (*ART*) genes, *Cf-4/Avr4* tomato plants undergoing synchronized HR were used for RNA fingerprinting using cDNA-AFLP (Vos et al. 1995). By comparing the cDNA-AFLP patterns of *Cf-4/Avr4* and *Cf-4* control plants, 442 differential *ART* transcript-derived fragments (TDF) were identified. A subset of these fragments was used for virus-induced gene silencing (VIGS) (Ratcliff et al. 2001) in *Cf-4*-transgenic *Nicotiana benthamiana*, and the plants were challenged either with *Avr4* of *C. fulvum* or the *Inf1* elicitor of *Phytophthora infestans* (Kamoun et al. 1997, 1998). This resulted in the identification of 20 *ART* TDF for which VIGS compromises the HR. Inoculation with *Tobacco rattle virus* (TRV) recombinants carrying fragments of the *ART* genes encoding an HSP90, a nuclear GTPase, an L19 ribosomal protein, and most interestingly, a nucleotide binding-leucine rich repeat (NB-LRR)-type protein had the most pronounced effect on the HR.

RESULTS

Analysis of the transcriptome of *Cf-4/Avr4* seedlings mounting a HR.

To identify the optimal parameters for the cDNA-AFLP analysis, we first compared the transcriptome of *Cf-4/Avr4* and control *Cf-4* plants at different timepoints after the shift to a permissive temperature. RNA was isolated from the above-ground parts and was used to generate *TaqI/MseI* templates, which were amplified using a *TaqI* forward primer and a small set of *MseI* reverse primers with an extension of either two or three selective nucleotides (4 and 16 primer combinations, respectively). This resulted in about 50 TDF per lane for the more-selective primer combinations and about 100 bands per lane for the less-selective combinations. For this limited set of AFLP primers, no changes in gene expression were observed over the first three timepoints (0, 10, and 20 min). However, after 30 min, TDF corresponding to both up- and down-regulated genes were detected. For most of these genes, changes in expression were observed in both the *Cf-4/Avr4* and the control plants, indicating that these differentials are the result of the temperature shift itself, rather than the onset of the HR. *ART* genes can easily be discriminated from temperature-responsive tomato genes, as *ART* TDF differ in the

Cf-4/Avr4 plants when compared with the controls that were similarly subjected to the temperature shift (Fig. 1).

The small-scale cDNA-AFLP screen at the various timepoints eventually resulted in the identification of a total of seven *ART* TDF using the 16 primer combinations, whereas only one of these TDF was found using the four primer combinations. Therefore, to identify a representative set of *ART* TDF, it was decided to carry out the cDNA-AFLP analysis using all 1,024 AFLP primer combinations with an extension of two and three selective nucleotides (discussed below). Furthermore, as we were interested in early transcriptional reprogramming and no substantial additional differences became apparent when timepoints later than 90 min were compared (results not shown), we focused on the analysis of changes in the transcriptome at 0, 30, 60, and 90 min after the temperature shift.

Identification of *ART* fragments, sequence analysis, and selection of *ART* TDF for functional studies.

To identify *ART* genes, for the four timepoints and 1,024 AFLP primer combinations, the presence and intensity of approximately 50,000 bands was compared between the *Cf-4/Avr4* and *Cf-4* samples. TDF that by eye appeared to be differentially expressed (approximately 5,000) were selected, and the band intensities were quantified. These data were used to calculate the difference in expression levels between the *Cf-4/Avr4* and the control plants. Based on these data, 442 *ART* TDF were selected that showed at least a threefold difference in expression level at two timepoints or at least a twofold difference at three timepoints. Remarkably, about 70% of the *ART* genes were already differentially expressed at the timepoint of the temperature shift. In total, 328 *ART* TDF (74%) corresponded to up-regulated genes, whereas 114 (26%) corresponded to down-regulated genes.

Of the 442 *ART* TDF, 405 fragments were successfully cloned and sequenced and were analyzed using the Protein extraction description and analysis tool (PEDANT), to perform BLAST (basic local alignment search tool) searches in public databases and to determine sequence homology at the protein and DNA level (Frishman et al. 2003). Furthermore, the program determines homology based on the predicted three-dimensional protein structure and on domains present in the encoded proteins. Of all *ART* TDF, we found 85% to be represented in expressed sequence tag libraries (E-value < 0.001). To select *ART* TDF for functional analysis and to exclude TDF that are derived from the same gene, BLAST searches on The Institute for Genomic Research tomato database were performed. From the 405 *ART* TDF, 314 fragments appeared to be unique, whereas the remainder corresponded to 34 different tentative contig (TC) sequences, reducing the maximum number of different *ART* genes to 348 (Fig. 2). In addition, five TDF were identified that originated from the T-DNA present in the transgenic *Cf-4/Avr4* plants and from homologs of *Cladosporium* resistance gene *Cf-9* (*Hcr9s*) present in the *Cf0 Milky Way* and *Northern Lights* clusters (Parniske et al. 1999), leaving 343 *ART* TDF for further analysis (Fig. 2). Based on either their putative identity, expression pattern, or both, 192 TDF were selected for functional analysis. This resulted in a selection of 147 TDF corresponding to up-regulated genes (of which 65 showed homology to known genes [E-value < 0.001]) and 45 TDF corresponding to down-regulated genes (of which 21 were homologous to known genes [E-value < 0.001]).

Functional analysis of selected *ART* genes by VIGS.

For functional analysis of the selected *ART* genes, VIGS was performed using the binary TRV vectors described by Ratcliff and associates (2001). To identify genes involved in the HR, TRV:*ART*-inoculated *Cf-4*-transgenic *N. benthamiana* plants (*N.*

Fig. 1. Typical examples of cDNA-AFLP profiles of *Cf-4* and *Cf-4/Avr4* seedlings. The expression profiles at four timepoints (0, 30, 60, and 90 min) after the temperature shift were analyzed. Two types of transcript-derived fragments (TDF) were found, those corresponding to temperature-responsive tomato (*TRT*) genes and those corresponding to *Avr4*-responsive tomato (*ART*) genes. An example of a *TRT*-down TDF (down-regulated in *Cf-4* as well as in *Cf-4/Avr4* plants) and an *ART*-up TDF (specifically up-regulated in the *Cf-4/Avr4* plants) is shown in the left panel. An example of an *ART*-down TDF (in this case constitutively down-regulated in the *Cf-4/Avr4* plants) is shown in the right panel.

benthamiana::Cf-4) were analyzed for compromised HR. This HR was induced by agroinfiltration of *C. fulvum* Avr4. Furthermore, we included the *Inf1* gene of the potato late-blight pathogen *Phytophthora infestans*, as *Inf1* is an elicitor of the HR in wild-type *N. benthamiana* (Kamoun et al. 1997, 1998). Agroinfiltration of Avr4 or *Inf1* in noninoculated or TRV:00-inoculated *N. benthamiana::Cf-4* plants resulted in a HR within 3 to 5 days (Figs. 3 and 4, panels 1 and 2), indicating that both elicitors induce a clear HR in spite of the TRV infection. To test whether this response is compromised by silencing of genes known to be involved in either the Avr4 or the *Inf1* response, or both, TRV:Cf-4, containing a fragment of *Cf-4*, and TRV:SGT1 (Peart et al. 2002) were used for VIGS. TRV:Cf-4-inoculated *N. benthamiana::Cf-4* plants showed a decreased HR upon agroinfiltration of Avr4, whereas the HR induced upon agroinfiltration of *Inf1* was not compromised (Figs 3 and 4, panel 3). The TRV:SGT1-inoculated plants displayed stunted growth (Fig. 5, panel 4) and did not mount a HR upon agroinfiltration of either Avr4 or *Inf1* (Figs. 3 and 4; panel 4), confirming that the downstream component SGT1 is required for both Avr4- and *Inf1*-induced HR.

To test the involvement of the selected *ART* genes in the HR, the 192 fragments, varying in length between 50 and 500 bp, were cloned into the TRV vector and were inoculated onto *N. benthamiana::Cf-4* plants. In general, TRV-inoculated plants were smaller than noninoculated plants (Fig. 5, compare panel 1 with panels 2, 3, 12, and 13). In addition, inoculation with a number of TRV:ART constructs resulted in chlorosis, leaf deformations, or stunted growth (Fig. 5, panels 5 through 11 and 14). The plants were subsequently challenged with Avr4 and *Inf1* at three weeks after TRV:ART inoculation. Initially, VIGS using 78 TDF appeared to compromise the induction of a HR. Retesting eventually resulted in 20 TDF for which VIGS reproducibly compromised either the Avr4-induced, *Inf1*-induced HR or both. VIGS using five of these fragments—470, 424a, 614, 653, and 222—resulted in a severely compromised HR (Figs. 3 and 4, panels 5 through 9), indicating that the orthologous genes in *N. benthamiana* are essential for the HR induced by both elicitors. Fragments 614 and 653 are nearly identical in sequence, indicating that they correspond to the same gene (discussed below). For these five fragments, we have not been able to confirm ‘knock-down’ of the corresponding *ART* orthologs in *N. benthamiana* when using primers designed on the original tomato cDNA sequences for the reverse transcription-polymerase chain reaction (RT-PCR) assays. However, after isolation of a full-length cDNA for *ART424a* (discussed below), we could identify a sequence that is highly conserved in tomato, potato, and tobacco. Primers designed in the most conserved region allowed us to show by RT-PCR that *ART424a* transcript levels are significantly lower in the TRV:424a-inoculated plants.

Characterization of the four *ART* genes required for Avr4- and *Inf1*-induced HR.

VIGS using the five tomato *ART* TDF 470, 424a, 614, 653, and 222 resulted in a pronounced suppression of both the Avr4- and *Inf1*-induced HR. To allow a more reliable BLAST search, the full-length tomato cDNAs corresponding to the various TDF were isolated and analyzed (Table 1). Furthermore, the cDNA-AFLP analysis for these particular TDF was repeated once more, now including Cf0 plants and the transgenic Cf0 parental line expressing Avr4 (Cf0/Avr4) as controls.

According to the cDNA-AFLP analysis, the gene corresponding to *ART* TDF 470 is up-regulated in the *Cf-4/Avr4* tomato plants as compared with the various control plants (result not shown). *ART* TDF 470 matches the 3' end of the open reading frame (ORF) of the tomato gene encoding heat shock protein (LeHSP) 90-1 (Liu et al. 2004) (Table 1). This gene

was recently shown to be required for plant development, *R* gene-mediated resistance, and nonhost resistance to *Pseudomonas cichorii* (Kanzaki et al. 2003; Lu et al. 2003; Van Bentem et al. 2005).

Our cDNA-AFLP analysis revealed constitutive expression of *ART* gene 424a in *Cf-4/Avr4* plants at all timepoints, including before the temperature shift, whereas the gene was not expressed in the control plants (result not shown). TDF 424a has a size of 373 bp, and the full-length cDNA was isolated from a tomato cDNA library using primers specific for the 424a sequence (Table 1). BLAST Analysis of the complete ORF revealed that the *ART* gene matching TDF 424a encodes a nuclear GTPase with an N-terminal nuclear localization signal. In *N. benthamiana*, inoculation with TRV:424a results in stunted plants that also have curled leaves (Fig 5, panels 9 through 11).

TDF 614 is 197 bp in length, and the 3' 179 bp are identical to TDF 653, except for four single nucleotide polymorphisms (SNP), one of which is present in the *MseI* restriction site used for cDNA-AFLP analysis. TDF 614 is only present in the Cf0 background, whereas TDF 653 is specific for *Cf-4* (result not shown). There are no differences in the expression of the corresponding genes at the various timepoints, indicating that TDF 614 and 653 do not originate from an *ART* gene, but rather, that the SNP at the *MseI* restriction site has resulted in the generation of a differential TDF. We were not able to clone a cDNA exactly matching either of the two *ART* TDF. However, one full-length cDNA clone was identified that differed in two SNP from TDF 614 and also in two SNP from TDF 653 (Table 1). The encoded protein is an L19 ribosomal protein, which is a subunit of ribosomes. In *Arabidopsis*, for example, five isoforms of L19 are present that show distinct targeting signals.

According to the cDNA-AFLP profile, the *ART* gene corresponding to TDF 222 is slightly up-regulated in *Cf-4/Avr4* when compared with the control plants (result not shown). Interestingly, the full-length cDNA matching *ART* TDF 222 encodes a protein of the coiled coil (CC)-NB-LRR class of resistance proteins (Table 1). As VIGS using TDF 222 results in suppression of both the Avr4- and *Inf1*-induced HR, we designated the corresponding protein NRC1 (for NB-LRR protein required for HR-associated cell death 1).

Fig. 2. Flow chart showing the selection of Avr4-responsive tomato (*ART*) fragments for functional analysis. Eventually, based on either their putative identity, expression pattern, or both, 192 transcript-derived fragments (TDF) were selected for functional analysis by virus-induced gene silencing in *Nicotiana benthamiana*. TC = tentative contig sequences of The Institute for Genomic Research tomato database.

DISCUSSION

Expression profiling of *Cf-4/Avr4* plants by cDNA-AFLP.

Analysis by cDNA-AFLP resulted in the generation of 50,000 fragments, of which about 1% corresponded to *ART* TDF. This proportion is similar to the percentage of differential TDF (260 fragments) identified amongst the 30,000 fragments that were

analyzed by Durrant and associates (2000), who used a transgenic *Cf-9* tobacco cell suspension treated with *Avr9*. Surprisingly, 70% of the *ART* genes are already differentially expressed before the shift to a lower temperature, when no HR-related gene expression is apparent on RNA gel blots (de Jong et al. 2002) (data not shown). However, when the *Cf-4/Avr4* plants are kept at 33°C more than four weeks, yellowing of the oldest

Fig. 3. Inoculation of *Nicotiana benthamiana* plants expressing *Cf-4* with various *Tobacco rattle virus*–*Avr4*-responsive tomato (TRV:ART) recombinants affects the *Avr4*-induced hypersensitive response (HR). *Avr4* was agroinfiltrated three weeks after virus inoculation on the leaf half marked by a white dot, and pictures were taken four days after agroinfiltration. The HR was not compromised in noninoculated and TRV:00-inoculated plants (panels 1 and 2), whereas virus-induced gene silencing using TRV:Cf-4, TRV:SGT1, TRV:470, TRV:424a, TRV:614, TRV:653, and TRV:222 severely suppressed the *Avr4*-induced HR (panels 3 through 9).

leaves and stunted growth can be observed (data not shown), suggesting that HR signaling is not completely eliminated at this temperature. This observation is in agreement with data of de Jong and associates (2002), who used a *Cf-9*-transgenic tobacco cell suspension that had lost Avr9 responsiveness at 33°C but did not show a complete loss of the Avr9-binding capacity at this temperature, suggesting that residual elicitor perception occurs

at 33°C. Thus, suppression of HR signaling at an elevated temperature is not complete and some *Cf* signaling still occurs. However, this seems not to be sufficient to trigger actual execution of the HR, thereby allowing rescue of the plants. Recently it was shown that an elevated temperature in combination with high humidity (95%) results in complete blocking of the HR (Wang et al. 2005). Shifting the *Cf-4*/Avr4 and control *Cf-4*

Fig. 4. Inoculation of *Nicotiana benthamiana* plants expressing *Cf-4* with various *Tobacco rattle virus*-Avr4-responsive tomato (TRV:ART) recombinants affects the *Inf1*-induced hypersensitive response (HR). *Inf1* was agroinfiltrated three weeks after virus inoculation on the leaf half marked by a white dot, and pictures were taken four days after agroinfiltration. The HR was not compromised in noninoculated, TRV:00-, and TRV:Cf-4-inoculated plants (panels 1 through 3), whereas inoculation with the other TRV recombinants severely suppressed the *Inf1*-induced HR (panels 4 through 9).

Fig. 5. Symptoms of *Nicotiana benthamiana* plants expressing *Cf-4*, resulting from inoculation with various *Tobacco rattle virus*–*Avr4*–responsive tomato (TRV:ART) recombinants. Phenotype of noninoculated plants (panel 1) and plants inoculated with TRV:00 (empty vector; panel 2), TRV:Cf-4 (panel 3), TRV:SGT1 (panel 4), and various TRV:ART recombinants (panels 5 through 14), at 24 days postinoculation. Note that most of the TRV:ART-inoculated plants shown appear chlorotic or stunted, whereas the TRV:424a-inoculated plants also have curled leaves (panels 9 through 11). Inoculation with TRV:614 and TRV:653 did not result in changes in morphology (panels 12 and 13).

seedlings from 33°C and 95% humidity to a permissive temperature and humidity might enable us to identify additional *ART* genes.

In addition to *ART* TDF corresponding to genes differentially expressed due to the specific *Cf-4/Avr4*-mediated response, various TDF were identified that originate from known differences between the *Cf-4* control plants and *Cf-4/Avr4* cross. Retrieval of a TDF corresponding to *Avr4* and *Hcr9s* derived from the *Cf0* background (Parniske et al. 1999), together with the observed redundancy of various identified TDF, indicates that differential expression profiling by cDNA-AFLP is very sensitive and close to saturation.

Functional analysis of *ART* genes by VIGS.

To test the involvement of *ART* genes in the HR, 192 TDF that were selected based on their putative identity and expression profile were subjected to VIGS in *N. benthamiana::Cf-4*. For 10% of the fragments (20 out of 192), VIGS resulted in a decreased HR upon agroinfiltration of *Avr4*, *Inf1*, or both. Lu and colleagues (2003) identified 79 cDNAs (1.6%) in a VIGS screen for genes involved in *Pto/AvrPto*-induced signaling, using a normalized library of *N. benthamiana* consisting of 4,992 clones. The higher percentage found here suggests that the cDNA-AFLP screen enriches for genes involved in the HR. Still, we identified only 20 possible candidates, indicating that many additional genes that are expected to be involved in the HR apparently are not identified. This could be explained by the fact that expression of these genes does not change during the induction or execution of the HR or that a lower number of genes is involved in *Avr4*- or *Inf1*-induced HR as compared with *AvrPto*-induced HR. A third possibility is that the homology of the heterologous tomato TDF used for VIGS is not sufficient to trigger silencing of the endogenous genes in *N. benthamiana* (Thomas et al. 2001). The latter problem may be overcome by performing the VIGS screen in tomato.

For five *ART* genes, severe HR suppression upon agroinfiltration of *Avr4* and *Inf1* in TRV:*ART*-inoculated plants was obtained, indicating that these genes or their close homologs are essential for the HR. These five *ART* genes encode a heat-shock protein 90 (HSP90) (TDF 470), a nuclear GTPase (TDF 424a), an L19 ribosomal protein (TDF 614 and 653), and a CC-NB-LRR protein (TDF 222) (Table 1).

ART fragment 470 is derived from the gene encoding HSP90-1, a member of the HSP90 family (Miloni and Hatzopoulos 1997). HSP90 is a unique molecular chaperone, as most of its known substrates are signaling proteins (Young et al. 2001). Support for the biological relevance of HSP90 in disease resistance comes from the analysis of *N. benthamiana* plants in which the corresponding gene has been silenced. HSP90 proved to be involved in plant development, *N*-, *Rx*-, *Pto*-, and *I-2*-mediated resistance pathways, *Inf1*-induced HR, and nonhost resistance to *Pseudomonas cichorii* (Kanzaki et al. 2003; Lu et al. 2003; Van Bentem et al. 2005). HSP90 associates with the resistance proteins N and I-2 (Liu et al. 2004;

Van Bentem et al. 2005), and in *Arabidopsis*, its binding to RAR1 and SGT1 (Shirasu and Schulze-Lefert 2003; Takahashi et al. 2003) provides further evidence for the involvement of HSP90 in disease resistance signaling. Our results confirm that the protein is required for plant development and *Inf1*-induced HR (Fig. 5, panel 8), and we also show that HSP90 plays a role in *Cf-4/Avr4*-induced HR (Figs. 3 and 4, panel 5). We observed a milder growth phenotype than that described by others (Kanzaki et al. 2003; Lu et al. 2003), as in our experiments, the plants are stunted but still develop up to four normal leaves (Fig. 5, panel 8). Probably silencing using a heterologous fragment is less efficient and only results in (partial) silencing of the closely related HSP90-1 and HSP90-2-encoding genes and not of the other HSP90 isoforms (Liu et al. 2004).

The gene corresponding to TDF 424a is specifically and constitutively expressed in the *Cf-4/Avr4* plants and has high homology to genes encoding nuclear GTPases. In animals, members of the nuclear GTPase family are named nucleostemins and prevent stem cells from differentiation. Interestingly, overexpression of a human nucleostemin induces p53-mediated apoptosis, a form of cell death comparable to HR-like cell death (Tsai and McKay 2002). In yeast, a homologous gene (*NUG1*) is essential for viability, and the GTPase it encodes is physically associated with preribosomal particles (Bassler et al. 2001). VIGS using TRV:424a not only affects the HR but also influences leaf shape and plant size (Fig. 5, panels 9 through 11), indicating that the gene or a close homolog is also involved in developmental processes in addition to the HR.

The sequences of *ART* TDF 614 originating from *Cf0* and 653 from *Cf-4* are identical except for four SNP and a 5' extension of 18 bp in *ART* fragment 614. One SNP is located in the *MseI* site used for cDNA-AFLP analysis, resulting in two TDF of different sizes that discriminate between a *Cf0* and *Cf-4* background. Both TDF were independently identified in the VIGS screen for decreased HR. Isolation of the full-length cDNA from tomato cultivar RZ52201 resulted in a clone that has two SNPs comparable to TDF 614 and two SNP comparable to TDF 653. The gene corresponding to this cDNA codes for an L19 ribosomal protein. In a VIGS screen of random cDNAs of a normalized library of *N. benthamiana*, Lu and associates (2003) found that 22 of the 79 cDNAs suppressing *Pto/AvrPto*-induced HR have homology to various ribosomal proteins. Although we identified the gene only due to differences in the genomic background, ribosomal proteins are differentially expressed in *Pti4*-expressing plants in which defense responses are activated (Chakravarthy et al. 2003). Probably the massive transcriptional upregulation of various defense-related genes in the tomato seedlings mounting the HR (Cai et al. 2001; de Jong et al. 2002) requires a similar increase in translational machinery. Furthermore, the results confirm the importance of de novo protein synthesis for activation of the HR (Greenberg 1997).

The protein encoded by the full-length cDNA corresponding to TDF 222 has high homology to CC-NB-LRR resistance proteins and appears to be involved in the HR induced by at

Table 1. Characteristics of the *Avr4*-responsive tomato (*ART*) transcript-derived fragments (TDF)^a

<i>ART</i> TDF no.	Size (bp)	Size of cDNA ORF (bp)	Location of TDF in ORF ^b	cDNA accession no.	Encoded protein ^c
470	373	2,100	1,637 to 2,009	AY368906	LeHSP90-1
424a	373	1,827	1,379 to 1,751	DQ304482	Nuclear GTPase
614	197	750	583 to 750*	DQ304483	L19 ribosomal protein
653	179	750	601 to 750*	DQ304483	L19 ribosomal protein
222	252	2,664	1,793 to 2,044	DQ304484	CC-NB-LRR protein

^a Those TDF whose inoculation onto transgenic *Nicotiana benthamiana::Cf-4* as a TRV:*ART* recombinant severely compromises both the *Avr4*- and *Inf1*-induced hypersensitive response.

^b Asterisks (*) indicate that the remainder of the TDF corresponds to the 3' untranslated region.

^c CC-NB-LRR = coiled coil-nucleotide binding-leucine rich repeat.

least two nonrelated elicitors (Avr4 and Inf1). The protein, that we have designated NRC1 also appears to be involved in development, as VIGS with a fragment of the encoding gene also causes growth defects (Fig. 5, panel 14). The expression of *NRC1* is slightly up-regulated in *Cf-4/Avr4* plants, and silencing of the gene results in a decreased HR, indicating that the encoded protein is a positive regulator of the HR. Recently, Peart and associates (2005) identified a CC-NB-LRR protein that is specifically required for functionality of the *N* gene that provides resistance to TMV. They suggested that NB-LRR proteins also function in signaling downstream of resistance proteins and that, possibly, many resistance proteins require NB-LRR proteins to be functional. The requirement of a NB-LRR protein downstream of the extracellular receptor-like protein Cf-4 and also downstream of the protein mediating perception of Inf1 in *N. benthamiana* supports this hypothesis. Our observation could also indicate that NRC1 is acting at a point of convergence of various HR signaling pathways. Furthermore, the CC-NB-LRR requirement for Cf protein function provides a possible direct link to SGT1 and HSP90. The latter proteins have both been shown to be associated with CC-NB-LRR resistance proteins (Liu et al. 2004; Lu et al. 2003; Peart et al. 2002; Shirasu and Schulze-Lefert 2003; Takahashi et al. 2003; Van Bentem et al. 2005), whereas silencing of SGT1- and HSP90-encoding genes also compromises the *Cf-4/Avr4* response (Fig. 3, panels 4 and 5). Further studies focused on NRC1 should reveal whether the protein is also required for other defense signaling pathways and should provide information on the stage at which the protein acts in the signal transduction cascade leading to HR.

In conclusion, the combination of transcriptional profiling of plants undergoing a synchronized HR and VIGS using the identified differential TDF is a powerful method for gene discovery. Still, 90% of the selected ART TDF does not appear to affect the HR when used for VIGS in *N. benthamiana*. Either these ART genes are indeed not absolutely required for the HR or the additional genes that play a role in *N. benthamiana* lack sufficient homology to the tomato TDF. To circumvent the latter problem, the TDF will be used for VIGS in tomato, as this will allow us not only to study their role in the HR itself but also in actual resistance against *C. fulvum*.

MATERIALS AND METHODS

Plant material and growth conditions.

The near-isogenic line of tomato cultivar MoneyMaker containing the *Cf-4*-introgressed region providing resistance against *C. fulvum* (referred to as *Cf-4*) was crossed to Cf0 plants (lacking functional *Cf* resistance genes) homozygous for the *Avr4* transgene (de Jong et al. 2002). To synchronize germination, the *Cf-4/Avr4* seeds that were obtained and seeds from *Cf-4* control plants were surface-sterilized with 20% Glorix for 20 min and then thoroughly rinsed with water. After germination under standard greenhouse conditions, the seedlings were transferred to an incubator (Elbanton, Kerkdriel, The Netherlands) at 33°C under a 16-h-light and 8-h-dark regime. After 10 days the plants were transferred to room temperature (20 to 25°C), and at 0, 10, 20, 30, 60, 90 min and 2, 4, 8, and 24 h after transfer, whole plants except for the roots were collected. *Nicotiana benthamiana* plants that were used for VIGS studies were grown under standard greenhouse conditions.

RNA isolation and cDNA-AFLP analysis.

About 100 mg (fresh weight) of plant material obtained at the various timepoints was homogenized for 2 min at 1,800 rpm in a Micronic deep-well tube, using a Retsch MM200 bead mill and 3-mm stainless steel beads. The lysate was

cleared using a Qiashredder homogenizer (Qiagen, Venlo, The Netherlands), and subsequently, total RNA was isolated using RNeasy 96 plates (Qiagen). Poly A⁺ RNA was isolated using magnetic oligo(dT) 25-Dynabeads (Dyna, Oslo, Norway) according to the manufacturer's instructions. First- and second-strand cDNA synthesis was carried out according to standard protocols, using Superscript II (Sambrook and Russell 2001).

The final volume after cDNA synthesis was 50 µl, from which 20 µl (10 to 500 ng of cDNA) was subjected to the standard AFLP template production protocol (Bachem et al. 1996; Vos et al. 1995). Restriction enzymes used for template preparation were *MseI* as frequent cutter and *TaqI* as rare cutter. The AFLP adaptor primers 5'-CTCGTAGACTGCGTAC-3' and 5'-CGGTACGCAGTCT-3' (*TaqI*-adaptor primers, 5' protruding end underlined) and 5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3' (*MseI*-adaptor primers, 5' protruding end underlined) were ligated onto the restriction fragments. A nonselective preamplification was performed using the nonselective adaptor primers without additional nucleotides. The selective PCR amplification steps were performed using ³³P-dATP-labeled AFLP primers with additional nucleotides complementary to the *TaqI* or *MseI* protruding ends of the cDNA fragments (underlined) and with selective nucleotides (indicated by N, representing an A, C, G, or T): 5'-GTAGACTGCGTACCGANN-3' (*TaqI* primer; a +2 primer) and 5'-GATGAGTCCTGAGTAANN-3' (*MseI* primer; a +3 primer). The initial small-scale screen using four and 16 AFLP primer combinations was done using a *TaqI* forward primer with an AA extension in combination with either four +2 *MseI* reverse primers (extension AA, AC, AG, or AT) or 16 +3 *MseI* reverse primers (extension AAN, ACN, AGN, or ATN), respectively. The large-scale cDNA-AFLP analysis was carried out essentially as described by Vos and associates (1995), using all possible (4² × 4³ = 1,024) AFLP primer combinations.

All amplification reactions were performed on a PE-9600 thermocycler, using *Taq* DNA polymerase (PE Biosystems, Foster City, CA, U.S.A.); the oligonucleotides were obtained from MWG Biotech AG (Ebersberg, Germany) and *MseI* and *TaqI* were from NE-Biolabs, Inc. (New Brunswick, NE, U.S.A.). After the selective PCR, the samples were denatured and separated on 4.5% polyacrylamide sequencing-type gels, which were fixed by placing them in 10% acetic acid for 30 min. After washing for 10 min in water, the gel was dried on a glass plate at 80°C, after which a phosphor-imaging screen was exposed to the gel for 20 h. Labeled DNA fragments were visualized by scanning of the screens with a Fuji BAS-2000 phosphor-imager analysis system (Fuji Photo Film Co. Ltd., Tokyo).

Isolation, sequencing and analysis of ART TDF.

Fragments corresponding to genes differentially expressed in *Cf-4/Avr4* seedlings as compared with *Cf-4* were excised from the dried gel and were eluted in 200 µl of Tris-EDTA (pH 8.0), after which 5 µl was reamplified using the same primer set as for the initial PCR amplification. The fragments were cloned in the pCR2.1 vector using the HTP TOPO TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.) and were transformed to *Escherichia coli* DH5α. Four independent colonies were chosen, and the insert sizes were determined on a sequencing gel. One clone containing an insert of the correct size was sequenced using ABI Big-Dye cycle sequencing (Amersham, Roosendaal, The Netherlands). The sequences were analyzed for homology to various databases (Altschul et al. 1997), using the PEDANT program (Frishman et al. 2003).

Plasmid constructs.

From the 426 ART TDF, 192 TDF were reamplified from the pCR2.1 vector, using M13 primers with *HindIII* and *Asp718*

sites. After purification, the PCR fragments were cloned into *HindIII*/*Asp718*-digested pTV:00, the binary TRV RNA2 vector that facilitates VIGS in *N. benthamiana* (Ratcliff et al. 2001), resulting in TRV:ART recombinants. As 15 TDF contained internal *HindIII* or *Asp718* sites, the fragments used for VIGS of the corresponding genes were smaller than the original TDF. For VIGS of *Cf-4*, a *Cf-4* fragment was PCR-amplified from vector pCf4 (pRH48 containing 35S:*Cf-4*) (Van der Hoorn et al. 2000) using Expand-*Taq* polymerase and the primers 5'-GTGACGGATCCCTCCGAATCACTCCTAAAC C-3' (*Bam*HI site underlined) and 5'-CTCTGGGAATCCTGT ACTCTCATC-3'. As the resulting PCR fragment contains an internal *HindIII* site, *Bam*HI/*HindIII* digestion results in a 404-bp fragment (bp 1,163 to 1,566; covering the part of the ORF encoding LRR 15 to 21 [Thomas et al. 1997]), which was cloned into the *Bam*HI and *HindIII* sites of pTV:00.

Transformation of *Agrobacterium tumefaciens*.

The various TRV:ART binary constructs were transformed to *Agrobacterium tumefaciens* GV3101, containing the helper plasmid pSoup (Hellens et al. 2000), by electroporation (Takken et al. 2000).

Transformation of *N. benthamiana* with the *Cf-4* resistance gene.

To obtain *N. benthamiana* plants that respond to the Avr4 elicitor of *C. fulvum*, the plants were transformed with plasmid pFT44. This binary vector is derived from pCGN1548 (Calgene, Davis, CA, U.S.A.) and contains a 5.8-kb *Pst*I fragment encompassing the *Cf-4* gene. The *Pst*I fragment was isolated from a λ clone identified in a genomic λ gem11 library from *Cf-4* tomato plants. In addition to the *Cf-4* coding sequence, it contains 2.9 kb of promoter sequence and 0.8 kb of 3' sequence (Takken 1999). Plant transformation was performed following the protocol of Horsch and associates (1985).

VIGS using TRV:ART recombinants and analysis for a decreased HR.

Four-week-old *Cf-4*-transgenic *N. benthamiana* plants were inoculated by coinfiltration of *Agrobacterium tumefaciens* carrying pBintra6 (the binary TRV1 vector [Ratcliff et al. 2001]) and *Agrobacterium tumefaciens* carrying the various TRV:ART recombinants, in a 1:1 ratio. For each of the TRV:ART clones, three or four plants were used. Three weeks after virus inoculation, the third, fourth, and fifth leaves above the primary inoculated leaves were challenged with Avr4 and Inf1 by agroinfiltration, and after another two weeks, the sixth and seventh leaves also were challenged with these elicitors. Agroinfiltrations were performed as described by Van der Hoorn and associates (2000), using either *Agrobacterium* sp. strain GV3101 containing the binary vector pRH87 (35S:Avr4 construct) (Van der Hoorn et al. 2000), at an optical density (OD) of 0.03 or strain MOG101 carrying the binary vector pInf1 (35S:Inf1 construct) (Kamoun et al. 2003) at an OD of 1. The leaves were examined 3 to 5 days postinfiltration for the development of a HR.

RT-PCR.

Three weeks after inoculation, four leaf disks (approximately 100 mg [fresh weight] of tissue) were collected from the third, fourth, and fifth leaves above the primary inoculated leaves of TRV-infected *N. benthamiana* plants. Total RNA was extracted using the QIA-Gen RNAeasy extraction method (Qiagen) and was treated with RNase-Free DNase (Bio-Rad, Veenendaal, The Netherlands). First-strand cDNA was synthesized from 1 μ g of total RNA, using the Bio-Rad cDNA synthesis kit (Bio-Rad) according to the manufacturers' instructions. PCR primers for actin were based on the sequence of TC8012 (ActinF: 5'-TATGGA

AACATTGTGCTCAGTGG-3'; ActinR: 5'-CCAGATTCGTCA TACTCTGCC-3'), generating a fragment of 217 bp. For *PDS* (TC9930), primers PDSF (5'-CCTGAGAGACTTTGCATGCC-3') and PDSR (5'-CTTCAGTTTTCTGTCAAACCATATATG-3') were used, generating a fragment of 294 bp. Primers to amplify the *N. benthamiana* ART424a ortholog were designed on a highly conserved sequence present in tomato ART424a and the ART424a ortholog of potato (TC121093) and tobacco (BP134842) (ART424aF: 5'-AACCAGGAATGAGGGGGAGC ATT-3' and ART424aR: 5'-GTTTAGTGTTCAACATGCCTTC CTC-3'), generating a fragment of 360 bp. PCR assays were performed for each cycle, denaturing at 95°C for 15 s, annealing at 60°C for 45 s, and elongation at 72°C for 60 s.

Isolation of full-length ART cDNAs.

For full-length cDNA isolation, 5' and 3' flanks of the ART TDF were isolated by PCR using ART TDF-specific primers from a cDNA library derived from *L. esculentum* cultivar RZ52201 infected with potato aphids (*Macrosiphum euphorbiae*). In this library, the cDNA was directionally cloned into the *Eco*RI and *Xho*I sites of Lambda ACT, which can be converted into a yeast two-hybrid shuttle plasmid, using Cre-lox excision. Primers were designed matching the ends of the TDF (222F: 5'-AACTTTGGAACGTACAAACCCTCATTGT-3'; 222R: 5'-AGAGAACATACTCAGTGCAGCTTTCTGGT-3'; 614/653F: 5'-AGAGAACATACTCAGTGCAGCTTTCTGGT-3'; 614/653R: 5'-GAGCTAGTTTGTAGTGTTTTTTCAGG GCA-3'; 424aF: 5'-TAAAGTCATCACCGTCCATATCAATG GCA-3'; 424aR: 5'-CGATGAAGTTTATGGAAGTGAATCCT CCA-3'). Because the orientation of the TDF in the cDNA was unknown, each primer was used in combination with both a vector-specific forward primer (5'-GGGATGTTTAATACCAC TACAATGGATGA-3') and reverse primer (5'-AAGTGAAT TGCGGGGTTTTTCAGTATCT-3'). Amplified fragments were inserted into pGEM-T Easy and were subjected to sequence analysis. The 5' and 3' flanks were assigned to one cDNA when the overlapping parts were identical.

ACKNOWLEDGMENTS

We thank L. Perebolte for cloning all TDF in pTV:00 and R. Hooiveld for assistance with the VIGS experiments. R. Jansen and B. Essenstam are acknowledged for excellent plant care, B. Thomma and R. van der Hoorn for valuable suggestions and critical reading of the manuscript. We thank S. Kamoun for providing the *plnfl* construct and D. Baulcombe and J. Peart for providing the TRV vectors and TRV:SGT1. The work described here is the result of a collaborative effort and has been made possible by the generous cooperation of Keygene NV, De Ruiter Zaden CV, ENZA de Enkhuizer Zaadhandel BV, and Vilmorin Clause et Compagnie. F. Takken was supported by a grant from Keygene, S. Gabriëls by the Graduate School Experimental Plant Sciences, and J. Vossen is employed on a VIDI fellowship that was awarded to M. Joosten by The Netherlands Organization for Scientific Research (NWO).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Institute for Genomic Research (TIGR) database: tigrblast.tigr.org/tgi