Enhancer Trapping Identifies *TRI*, an *Arabidopsis* Gene Up-Regulated by Pathogen Infection

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Enhancer trap *Arabidopsis thaliana* plants were screened for genes up-regulated by virus infection. The plants carried T-DNA insertions comprising a minimal –60-bp *Cauliflower mosaic virus* 35S promoter fused to the β -glucuronidase (GUS) reporter gene. Approximately 12,000 plants were assayed for GUS activity before and after rub-inoculation with *Tobacco rattle virus* (TRV) tagged with the green fluorescent protein (GFP). One plant and its progeny consistently showed upregulation of GUS activity in response to TRV-GFP infection, indicating that a virus-responsive enhancer element was “tagged” by the T-DNA in this line. Other viruses, bacteria, and oomycetes, but not wounding, up-regulated GUS activity in the enhancer trap line, indicating that the response was not specific to TRV-GFP infection. A pathogen-inducible, alternatively spliced gene was identified, which we have termed *TRI* for TRV-induced gene. A pathogen-responsive element was localized to a 1.1-kb region upstream of the T-DNA insertion, and two different *cis*-acting elements, both implicated in defense responses, were found in the sequence upstream of *TRI*. Sequence analyses revealed that *TRI* is similar to *ACRE169*, a gene that is up-regulated in *Cf-9*-expressing tobacco when treated with Avr-9, the *Cladosporium fulvum* elicitor of the *Cf-9* resistance response.

Additional keywords: gene regulation, WRKY transcription factor, W-box, *as-1/ocs* element.

Virus infection affects the expression of many plant genes both temporally and spatially (Aranda et al. 1996; Cooper 2001; Escaler et al. 2000; Geri et al. 1999; Golem and Culver 2003; Havelda and Maule 2000; Wang and Maule 1995; Whitham et al. 2003; Yoda et al. 2002). Some genes may show altered patterns of gene expression in response to virus infection due to the activation of defense mechanisms against the invading pathogen. In addition, some genes will be induced or up-regulated because they encode host factors allowing the virus to replicate or move through the plant. Likewise, changes in gene expression will occur when cellular functions are redi-

rected to the synthesis of viral nucleic acids and proteins, resulting in changes in plant metabolism and, often, the development of symptoms (Hull 2002). A better understanding of the transcriptional changes that occur during virus infection will provide valuable insight into how plants recognize and respond to viruses and how these pathogens cause disease.

To search for host genes that are induced or up-regulated in response to virus infection, we have exploited gene trapping. There are several types of gene traps (Springer 2000), and we used enhancer trap vectors because they are powerful tools for studying expression patterns and for identifying new genes, especially those that are difficult to identify in loss-of-function screens. Enhancer trap vectors often comprise T-DNA or a transposable element carrying a reporter gene under the control of a minimal promoter (Baxter-Burrell et al. 2003; Campisi et al. 1999; Geisler et al. 2002; He et al. 2001; Klimyuk et al. 1995; Meissner et al. 2000; Topping et al. 1991; Tsugeki and Fedoroff 1999). Expression of the reporter gene is only detected when integration occurs near to an active enhancer element but can be monitored both temporally and spatially. In addition, the regulatory enhancer element is “tagged” with the enhancer trap construct that should facilitate its identification and promote the characterization of the gene that the enhancer regulates.

We used *Arabidopsis thaliana* lines transformed with a T-DNA-based enhancer trap vector (Campisi et al. 1999). The lines were screened for upregulation of a reporter gene in response to the bipartite RNA *Tobacco rattle virus* (TRV), since this pathogen produces a compatible infection on *A. thaliana*. The TRV RNA-2 genome was tagged with the green fluorescent protein (GFP) reporter gene to allow the monitoring of the infection process (Ratcliff et al. 1999).

We demonstrate that enhancer traps can be used to detect changes in gene expression in response to virus infection. Using the trap, we have identified a virus-responsive element and a novel gene that it regulates. We also show that the gene is up-regulated during infection by other pathogens but not in response to wounding. The functional identity of the gene and its putative role in the plant defense response to pathogen attack are discussed.

RESULTS

Screening for virus-induced genes.

In the enhancer trap plants (discussed below) the T-DNA carries the β -glucuronidase (GUS) reporter gene (*uidA*) fused to a minimal –60-bp *Cauliflower mosaic virus* (CaMV) promoter (Campisi et al. 1999). The Nottingham Arabidopsis Stock Centre (NASC) supplied 50 pools of seed, with each

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pool of seed having been generated from 100 plants. A total of 240 seeds from each pool (about 12,000 plants in total) were grown in a controlled environment chamber, which was used to minimize changes in plant gene expression in response to environmental conditions.

At 28 days postgermination, one leaf from each plant was excised and assayed for GUS activity by histochemical staining. In addition, one leaf per plant was rub inoculated with a TRV-GFP infected sap extract. At 4 days postinoculation (dpi), virus infection was confirmed by detection of GFP under UV light on the inoculated leaf. This leaf was subsequently assayed for GUS activity by histochemical staining.

Approximately 11,300 plants showed no detectable GUS activity before or after virus infection, indicating that either the T-DNA had not integrated adjacent to a regulatory element or that the enhancer was inactive. None of these plants were analyzed further.

Approximately 100 plants showed uniform staining for GUS activity both before and after inoculation, indicating that the T-DNA had integrated adjacent to enhancer elements that were capable of directing expression of the reporter gene during normal development of the plant. A 10 more plants showed tissue-specific expression of GUS in the midribs or veins. However, none of the plants displaying uniform or tissue-specific GUS activity showed any change after virus infection and were not analyzed further.

A total of 523 plants showed upregulation of GUS activity in response to rub-inoculation with TRV-GFP (Fig. 1A), indicating that the T-DNA had “trapped” an enhancer element in each plant. However, these preliminary screens (performed on pools of seed) were not sufficient to determine whether the enhancer was responsive to rub-inoculation of TRV-GFP, to exposure to the UV light used to monitor infection, to wounding as a result of the inoculation technique, or to a developmental switch. Therefore, self-pollinated seed was collected from 466 of the 523 plants to permit further analyses.

GUS activity in all but one line was induced by wounding.

Ten progeny plants from each of the 466 parental plants were assayed for GUS activity before treatment. Consistent with the observations made in the initial screen, all of these plants were GUS negative before treatment. The plants were then either rub inoculated with TRV-GFP, rub inoculated with water as a control for wounding responses, or left untouched to assess whether upregulation of GUS was in response to a developmental switch. Of the 466 lines tested, all but one showed upregulation of GUS activity in response to rub-inoculation with water (Fig. 1B), indicating that the trapped enhancer sequences were responsive to wounding rather than to virus infection. Polymerase chain reaction (PCR) analysis of plants that showed no GUS activity before or after rub-inoculation revealed that these represented segregants that had lost the enhancer trap transgene (data not shown).

One line (GS393) showed upregulation of GUS activity in response to TRV-GFP infection at 4 dpi but not in response to rub-inoculation with water (Fig. 1C). We never detected GUS activity at 1 or 2 dpi in the inoculated leaves. The levels of GUS activity at 4 dpi, as determined by 4-methyl umbelliferyl glucuronide (MUG) assays, ranged from 0.3 to 1.6 nmol of 4-methylumbelliferone (4-MU) produced per min per mg of total leaf protein in TRV-infected leaves (in untouched or water-inoculated leaves, all GUS activities were below 0.1 nmol of 4-MU per min per mg). Control treatments (i.e., exposure to UV light and inoculation with noninfected sap) confirmed that the upregulation of GUS activity in GS393 was specifically due to TRV-GFP infection (data not shown).

To determine whether the upregulation of GUS was restricted to the inoculated leaves or followed the course of infection throughout the plant, GS393 plants were inoculated with TRV-GFP or water (as a control). Systemically infected leaves were

Fig. 1. β -glucuronidase (GUS) activity in enhancer trap lines. All leaves were assayed for GUS activity by histochemical staining and were cleared of chlorophyll in 70% (vol/vol) ethanol. **A**, A Total of 523 plants were negative for GUS activity before inoculation (Before) and positive for GUS activity after virus inoculation (After) (the inoculated leaf is shown). One representative enhancer trap line is shown (GS167). **B**, In 465 enhancer trap lines, GUS activity was up-regulated in response to the rub-inoculation procedure, i.e., rub-inoculation with water or virus-induced GUS (the inoculated leaves are shown). UT indicates untouched. A representative line is shown (GS82). **C**, In one line (GS393), upregulation of GUS activity was in response to *Tobacco rattle virus*-green fluorescent protein (TRV-GFP) infection and not in response to the inoculation procedure. GUS activity in the inoculated leaves of several GS393 plants in response to inoculation with water (Water) or TRV-GFP (TRV) or left untouched (UT). The three treatments were each repeated on several occasions on more than 300 GS393 plants. Little or no GUS activity was observed in the untouched or water-inoculated plants but 95 to 100% of plants in each experiment showed induction of GUS activity in response to TRV-GFP infection. In A, B, and C, a hole in the leaf indicates an inoculated leaf. **D**, Systemically infected leaf of GS393, imaged under UV light at 5 days postinoculation (dpi). Green fluorescence indicates TRV replication, whereas red fluorescence is due to chlorophyll. **E**, Same leaf as shown in D, stained for GUS activity and cleared of chlorophyll. **F**, Systemically infected leaf of GS393, imaged under UV light at 7 dpi. **G**, Same leaf as shown in F, stained for GUS activity. **H**, Induction of GUS activity in response to TRV RNA-1 infection. The plant on the left is healthy; the plant on the right is infected with TRV RNA-1, at 7 dpi. **I**, Induction of GUS activity in response to *Turnip mosaic virus* (TuMV) infection. The plant on the left is healthy; the plant on the right is infected with TuMV, at 6 dpi. **J**, Induction of GUS activity in response to *Peronospora parasitica* infection using isolate Noco2 (virulent on Col-*gl*) or isolate Cala (avirulent on Col-*gl*). Plants were spray-misted with 10^4 spores per ml. Control plants were spray-misted with water (Healthy). Plants were stained at 7dpi. A representative set of samples is shown.

monitored for GFP expression and then were stained for GUS activity at 5 and 7 dpi. Little or no GUS activity was detected on the systemic leaves from plants inoculated with water (data not shown). In contrast, at 5 dpi, GFP was visible as intermittent strips of fluorescence along the petiole and midrib of systemically infected leaves from plants inoculated with TRV-GFP, indicating that the virus was emerging from the vascular tissue (Fig. 1D). In these leaves, the pattern of GUS expression was similar to that observed for GFP in that sporadic spots of blue coloration were visible along the midrib and petiole (Fig. 1E). At 7 dpi, systemically infected leaves showed extensive fluorescence spreading out from the midrib and veins, characteristic of TRV infection (Fig. 1F). The pattern of GUS activity was similar to that observed for GFP but was less extensive (Fig. 1G), confirming that the enhancer trap was also responsive to a systemic TRV-GFP infection.

GUS activity is up-regulated by other viruses.

When transiently expressed, the GFP reporter gene can alter plant gene expression (Page and Angell 2002). To investigate whether the upregulation of GUS activity was in response to the GFP expressed from TRV RNA-2, GS393 was infected with wild-type TRV. All plants infected with wild-type TRV showed GUS activity (data not shown), confirming that the upregulation of GUS activity was in response to virus infection and not to GFP.

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) to monitor levels of mRNA in GS393 and nontransformed plants. Tracks 1 and 3 represent GS393 samples. Tracks 2 and 4 represent nontransformed (NT) samples. Plants were either left untouched (UT) or inoculated with *Tobacco rattle virus*-green fluorescent protein (TRV). The results for water-inoculated samples were similar to the untouched samples (data not shown). RNA was extracted at 4 days posttreatment, and cDNA prepared from DNase-treated RNA using an oligo d(T) primer. RT-PCR was performed using gene-specific primers, indicated to the right of each panel. Products are shown as ethidium bromide-stained bands on an agarose gel. All RT-PCR were performed on the same batch of cDNAs. The cycle number for all PCR using gene-specific primers was 35. **A**, RT-PCR using β -glucuronidase (GUS)-specific primers, **B**, At1g78420-specific primers, **C**, At1g78400-specific primers, and **D**, At1g78410-specific primers. The two PCR bands represent two *TRI* (TRV-induced) transcripts. **E**, RT-PCR using AC007651-specific primers and **F**, ubiquitin5-specific primers.

To identify whether TRV RNA-2 was necessary for the upregulation of GUS activity, GS393 was inoculated with TRV RNA-1 alone, which is capable of independent replication and systemic spread in plants. GUS activity was observed in systemically infected leaves at 7 dpi (Fig. 1H), showing that neither TRV RNA-2 nor any proteins encoded by it were required to mediate the upregulation.

GS393 was inoculated with three other unrelated viruses. During the course of infection, GUS activity was observed in plants infected with *Turnip crinkle virus* (TCV), a species in the *Carmovirus* genus (data not shown), *Turnip mosaic virus* (TuMV), a species in the *Potyvirus* genus (Fig. 1I), and CaMV, a DNA virus in the *Caulimovirus* genus (data not shown), indicating that the upregulation of the reporter gene in GS393 was a general response to virus infection.

GUS activity is up-regulated by other pathogens.

GS393 was inoculated with virulent or avirulent *Pseudomonas syringae* pv. *maculicola* or with *Peronospora parasitica* isolate Noco-2 or Cala. All *Pseudomonas syringae*-infected plants showed GUS activity at 1 dpi, whereas all water-inoculated controls were GUS negative (data not shown). All *Peronospora parasitica*-infected plants showed GUS activity at 7 dpi (Fig. 1J). Little or no GUS activity was observed on the water-inoculated control plants (Fig. 1J). Taken together, these data indicated that the upregulation of the reporter gene in GS393 was a general response to pathogen infection.

Transcription of *uidA* is up-regulated in GS393.

To confirm the GUS activity assays, reverse transcription (RT)-PCR was performed using primers specific for *uidA* on cDNA prepared from GS393 and nontransformed plants, which were each inoculated with either TRV-GFP or left untouched (as controls). A GUS RT-PCR band was observed in virus-infected GS393 but not in healthy GS393 (Fig. 2A, tracks 1 and 3), indicating that expression of GUS activity was regulated at the level of transcription. As expected, there was no RT-PCR product detected in either the healthy or the infected nontransformed plants (Fig. 2A, tracks 2 and 4).

Our data indicated that a virus-responsive enhancer sequence was tagged by the T-DNA in line GS393 and that this regulatory element directed transcription of a pathogen-inducible gene. This regulatory element was likely to be in close proximity to the gene that it regulated, and so we used the enhancer trap T-DNA sequence to locate the open reading frame (ORF) that was up-regulated during virus infection.

Identification of the T-DNA-tagged gene in GS393.

To elucidate the genome location of the enhancer trap T-DNA in GS393, thermal asymmetric interlaced (TAIL)-PCR was performed using a T-DNA right-border-specific primer in combination with a primer that would bind randomly to genomic DNA. One right-border-specific PCR product was obtained and sequenced. BLASTN analysis (Altschul et al. 1997) indicated that the T-DNA had integrated between two divergently transcribed genes (gene identifiers At1g78400 and At1g78410) on chromosome I (BAC clone F3F9; accession number AC013430) (Fig. 3). These genes are predicted to encode a putative polygalacturonase (At1g78400) and a hypothetical protein (At1g78410). TAIL-PCR using a left-border-specific primer and random primers produced a single product, and sequence analysis of this confirmed the insertion site. Based on the location and orientation of the T-DNA, it was likely that the enhancer trap had integrated into a regulatory region (Fig. 3).

To identify the virus-induced gene, RT-PCR was performed using primers specific for each of the two divergently tran-

scribed genes (Atlg78400 and Atlg78410) and an additional gene downstream of Atlg78410 predicted to encode an expressed unknown protein (Atlg78420). Ubiquitin5 primers were also used as an internal control. Although the TAIL-PCR data indicated that the T-DNA had not disrupted any ORF, it was possible that gene expression was affected in GS393, due to insertion into a regulatory region. To monitor expression of the three most likely candidate genes, we analyzed the RNA levels of each in both GS393 (homozygous line) and nontransformed plants.

RT-PCR was performed on cDNA prepared from plants either inoculated with TRV-GFP or left untouched (as controls). RT-PCR using ubiquitin5 primers as a control revealed similar levels of PCR product in each sample, indicating standardization of the RT-PCR and RNA (Fig. 2F). RT-PCR using Atlg78420 gene-specific primers revealed similar amounts of PCR product in all plants (GS393 healthy or infected and nontransformed healthy or infected) (Fig. 2B), indicating that Atlg78420 mRNA was expressed in all samples and was not up-regulated in response to TRV-GFP infection.

RT-PCR using Atlg78400 gene-specific primers revealed PCR products in all samples. However, in GS393, irrespective of the treatment used, the level of PCR product was consistently less than that observed in nontransformed plants (Fig. 2C, tracks 1 and 3 compared with tracks 2 and 4). This observation indicated that TRV-GFP infection did not change expression of Atlg78400 but that the T-DNA insertion in GS393 (Fig. 3) reduced accumulation of Atlg78400 mRNA.

In contrast, when Atlg78410 primers were used, we observed and were able to reproduce RT-PCR products in both GS393 and nontransformed plants when inoculated with TRV-GFP (Fig. 2D, tracks 3 and 4) but little or no product in the control samples (Fig. 2D, tracks 1 and 2). These data showed that this gene was up-regulated by TRV-GFP infection. We have termed the gene *TRI*, for *Tobacco rattle virus* induced. Two RT-PCR products were consistently observed in samples from TRV-GFP-infected plants (Fig. 2D), indicating that *TRI* encodes two transcripts.

RT-PCR using Atlg78410 primers was repeated on cDNA prepared from plants inoculated with either TRV-GFP or water or on plants left untouched. Consistent with our previous data (Fig. 2D), we observed RT-PCR products in plants inoculated with TRV-GFP and little or no product in the untouched control samples (data not shown). Similarly, we observed little or no product in the water-inoculated plants, although we occasionally observed faint bands, but this was inconsistent between experiments (data not shown).

Taken together, our data show that the expression of the GUS reporter gene and *TRI* correlate; both are up-regulated by TRV-GFP infection. Furthermore, the expression pattern of *TRI* in GS393 accurately reflects that observed in nontransformed plants, indicating that the T-DNA insertion does not disrupt expression of *TRI*.

Localization of the virus-enhancer regulatory element.

To identify the region containing the virus-responsive enhancer element, approximately 1.1 kb of genomic DNA immediately upstream of the T-DNA integration site (Fig. 3) was cloned upstream of a minimal 35S promoter fused to *uidA* on the T-DNA of a binary vector, to generate plasmid pA351. The ability of this DNA to activate transcription of *uidA* in the presence of TRV-GFP was determined, using a transient expression assay based on *Agrobacterium* inoculation ("agro-infiltration") of *Nicotiana benthamiana* leaves (adapted from the promoter assay described by Yang and associates 2000). Leaves were agro-infiltrated with pA351 or a control construct (pA353) that carried just the minimal promoter fused to *uidA*.

The infiltrated leaves were inoculated with TRV-GFP or water (as a control). The leaves were assayed for virus infection by monitoring GFP and then were stained for GUS activity. All leaves inoculated with water showed little or no GUS activity, irrespective of the agro-infiltration construct used (Fig. 4A and C). As expected, leaves expressing the control construct pA353 showed little or no GUS activity after inoculation with TRV-GFP (Fig. 4B). In contrast, leaves expressing pA351 showed patches of GUS activity, indicating an upregulation of *uidA* expression (Fig. 4D). These areas of intense blue coloration coincided with regions expressing GFP (Fig. 4E and F), indicating that the upregulation of *uidA* was dependent on the presence of replicating virus. In the absence of virus, we occasionally observed very faint blue coloration across the infiltrated patch (Fig. 4C). This low expression of GUS activity was never observed in leaves agro-infiltrated with pA353, suggesting that the 1.1-kb region of genomic DNA in pA351 was capable of activating the minimal promoter in *N. benthamiana* even in the absence of the virus, but upon virus infection, promoter activity was significantly increased.

Two independent *A. thaliana* lines stably transformed with plasmid pA351 were generated and were inoculated with water or TRV-GFP or were left untouched (as a control). Staining for GUS activity at 4 dpi revealed positive GUS activity in lines SA04023 (Fig. 4G) and SA04068 (data not shown) when inoculated with TRV-GFP but not when inoculated with water or left untouched.

Taken together, these data show that the 1.1-kb region of genomic DNA in pA351 can enhance transcription of *uidA* in a virus-dependent manner and, therefore, must contain a virus-responsive enhancer element. The transient expression data also indicate that the one or more transcription factors that direct transcription of this *A. thaliana* gene are also present and functional in *N. benthamiana*.

Analysis of the *TRI* promoter region.

We scanned the sequence containing the virus-responsive enhancer element for conserved DNA-binding motifs commonly identified in regulatory regions and found the hexamer sequence "TTGACC," which is a W-box *cis*-acting element. This DNA-binding motif is regulated by a superfamily of WRKY transcription factors and has been implicated in pathogen defense, wounding, and senescence (Eulgem et al. 2000). The motif is located approximately 120 nucleotides upstream of the T-DNA insertion site in GS393.

Surprisingly, examination of the 1-kb sequence between the T-DNA insertion site and the *TRI* start codon revealed the se-

Fig. 3. T-DNA integration site in line GS393. A schematic diagram indicating the location of the T-DNA insertion in GS393. The bar indicates approximately 1 kb; the diagonally hatched box indicates the β -glucuronidase (GUS) reporter gene on the T-DNA (T-DNA not drawn to scale). The stippled box indicates Atlg78400 (exopolysaccharase); the shaded box indicates Atlg78410 (hypothetical protein (*TRI*)); and the vertically hatched box indicates Atlg78420 (unknown protein). Arrows indicate the direction of transcription. No other genes are predicted to lie in this part of the genome or in 4.5 kb flanking this region. The dotted line indicates the 1.1-kb region that was cloned upstream of a minimal promoter-GUS construct (pA353) to generate plasmid pA351.

Fig. 4. Reporter gene expression in leaves expressing the *TRI* (*Tobacco rattle virus* [TRV]-induced gene) enhancer sequence fused to β -glucuronidase (GUS). **A** through **F**, GUS expression in *Nicotiana benthamiana* leaves agro-infiltrated with pA351 or pA353 and then rub-inoculated with water or TRV-green fluorescent protein (GFP). **A**, Leaves were agro-infiltrated with pA353 (minimal promoter fused to GUS) and were rub-inoculated with water at 2 days postinoculation (dpi) and **B**, with TRV-GFP at 2 dpi. **C**, Leaves were agro-infiltrated with pA351 (1.1 kb genomic DNA cloned upstream of the minimal promoter in pA353) and were rub-inoculated with water at 2 dpi and **D**, TRV-GFP at 2 dpi. **E**, Leaf transiently expressing pA351 and rub-inoculated with TRV-GFP, imaged under UV light at 6 dpi. Green fluorescence indicates replication of TRV RNA-2. **F**, Leaf shown in panel **E** stained for GUS activity. TRV infection colocalizes with induction of *uidA* from pA351. Leaves were stained for GUS activity at 6 days post-infiltration. **G**, GUS activity in leaves from SA04023 (*A. thaliana* line stably transformed with pA351). Plants were inoculated with water, TRV-GFP (TRV), or left untouched (UT). Leaves were stained for GUS activity at 4 dpi. All leaves were cleared of chlorophyll in 70% ethanol. A representative set of leaves is shown.

quence “TGACGT” approximately 345 nucleotides upstream of the ATG. This sequence is present in the *as-1/ocs* element which, like the W-box, has also been found in the regulatory elements of pathogen-inducible genes but is regulated by basic leucine zipper (bZIP) transcription factors (Rushton and Somssich 1998). Therefore, two regulatory *cis*-acting elements both implicated in defense responses are present in the sequence upstream of *TRI*, located either side of the T-DNA in GS393.

The *TRI* gene transcript is alternatively spliced.

Database predictions for the At1g78410 (*TRI*) ORF suggested that the gene comprised three exons and two introns and that the predicted fully processed mRNA was 579 nucleotides encoding 193 amino acids (Fig. 5A).

Sequence analysis of the two *TRI* RT-PCR products (Fig. 2D) showed that both introns were retained in the larger band (Fig. 5B), indicating that one transcript was unspliced, encoding a predicted peptide of 108 amino acids, due to an in-frame stop codon at nucleotide position 327 (Fig. 5A). This predicted peptide matches the 2001 NCBI submission for the protein, although the full-length cDNA sequences do not match—we detected a 3' untranslated region of more than 500 nucleotides.

The sequence of the smaller RT-PCR product revealed the presence of exons 1 and 3, with the loss of exon 2 and intron 2 due to the retention of part of intron 1 spliced to exon 3 (Fig. 5C). That is, nucleotides TG at positions 313 and 314 were spliced next to nucleotide A at position 643 of exon 3 to generate an in-frame UGA stop codon (Fig. 5C). This transcript would produce a peptide identical to the one translated from the larger cDNA (Fig. 5B), except that it would be missing the last four amino acids (WSEY).

TRI is similar to *N. tabacum* ACRE169.

BLASTX analysis (Altschul et al. 1997) of the *TRI* cDNA sequences revealed significant homology to a hypothetical protein in *A. thaliana* (AC007651) (BLASTX score 1e-19) and to ACRE169, which encodes *N. tabacum* Avr9/Cf-9 rapidly elicited protein 169 (AF211534) (BLASTX score 1e-10). ACRE169 was identified in a cDNA-amplification fragment length polymorphism (AFLP) analysis performed on transgenic *N. tabacum* cell cultures expressing the tomato Cf-9 resistance gene as a mRNA that was rapidly induced following treatment with Avr9, the *Cladosporium fulfum* elicitor of the Cf-9 resistance response (Durrant et al. 2000).

RT-PCR using primers specific to the *A. thaliana* hypothetical protein (accession numbers AC007651 and AAD50018) revealed that the other ACRE169 orthologue was expressed in all healthy or TRV-infected plants (GS393 or nontransformed) (Fig. 2E), indicating that this gene, unlike *TRI*, is not up-regulated by TRV-GFP infection.

DISCUSSION

Enhancer traps have previously been used to identify developmentally important genes (Klimyuk et al. 1995), tissue-specific regulatory elements (Tsugeki and Fedoroff 1999), senescence-associated genes (He et al. 2001), and oxygen deprivation-regulated genes (Baxter-Burrell et al. 2003). Here, we have demonstrated their use to identify a gene, *TRI*, that is up-regulated during virus infection. Our screen was not exhaustive and a more efficient screen might have revealed other virus-responsive genes. The use of pools of seeds slowed the identification and further development of useful lines. Spray inoculation would require less labor than the manual rub method used here. A further improvement would be the use of a noninvasive reporter, such as GFP or luciferase, that would

allow the expression assay to be automated with real-time imaging. To our knowledge, this is the first report that documents the identification of a virus-responsive enhancer and the gene that it regulates; *TRI* was not identified in a screen for genes up-regulated by virus infection using *A. thaliana* microarrays (Golem and Culver 2003; Whitham et al. 2003). Cooper (2001) reported an increase in gene expression for a *TRI* homologue in *Chenopodium amaranticolor* in response to *Tobacco mosaic virus* (TMV) infection in a cDNA-AFLP analysis. However, the observed upregulation was not reproducible when the experiment was repeated (Cooper 2001).

We have shown that *TRI* is regulated by a virus-responsive enhancer that we localized to a 1.1-kb region immediately upstream of the T-DNA, using stably transformed *A. thaliana* plants and a transient expression assay in *N. benthamiana*. The finding that the enhancer element was also responsive to TRV-GFP when transferred to *N. benthamiana* suggests that the one or more transcription factors that direct transcription of *TRI* in *A. thaliana* were also functional in *N. benthamiana*. We found a *cis*-acting W-box element in the region containing the virus-responsive enhancer. This motif is a DNA-binding site for WRKY proteins and is found in the regulatory regions of genes associated with defense and other stress responses (Eulgem et al. 2000). Although many WRKY proteins have been found (Chen et al. 2002, Eulgem et al. 2000), their target genes remain largely unknown. The defense-related genes characterized to date that have a W-box in their promoter regions include tobacco *CHN50* (Fukuda and Shinshi 1994), parsley *PR-1* (Rushton et al. 1996), and *A. thaliana* *NPR1* (Yu et al. 2001). We propose that *TRI* is likely to be regulated by a member of the family of WRKY transcription factors and that the *N. benthamiana* transient expression assay should provide a useful tool for further dissection of the *TRI* enhancer region to determine whether the W-box element alone is sufficient to mediate responses to viruses.

The expression pattern for *TRI* in GS393 was identical to *uidA*, indicating that the “trapped” virus-responsive enhancer coordinately regulated the minimal promoter driving expression of *uidA* and the *TRI* promoter. This demonstrated that the enhancer could act not only on sequences immediately adjacent to it but also on those located on the other side of the T-DNA insertion, a distance of more than 5 kb. The ability to activate promoters at a distance is a common characteristic of enhancer elements (Latchman 1998). Neither of the genes flanking *TRI* was regulated in a virus-responsive manner, but if the *TRI* enhancer can act over large distances, it is possible that it could also regulate other genes. Identification of such genes would reveal a group of coordinately regulated proteins involved in the plant response to pathogen infection.

Interestingly, we found another *cis*-acting element in the region between the T-DNA and *TRI*. This second motif, an *as-1/ocs* element, is a binding site for bZIP transcription factors, which also regulate gene expression during pathogen infection and other stresses (Rushton and Somssich 1998). The *A. thaliana* bZIP protein TGA1 recognizes TGACG and preferentially binds to this motif, if it is followed by a T residue (Schindler et al. 1992). Thus, based on the hexamer sequence found upstream of *TRI* (TGACGT), it is likely that this gene can be regulated by TGA1. The presence of two pathogen-responsive elements located either side of the T-DNA insertion may provide an alternative explanation for why *uidA* and *TRI* are coordinately regulated in GS393. It is possible that the W-box upstream of the T-DNA insertion regulates *uidA* and the *as-1/ocs* element regulates *TRI*. We

propose that, in nontransformed plants, both elements probably regulate *TRI*.

In TRV-GFP-infected GS393, we observed regions in which the patterns of GUS activity coincided with GFP activity (Figs. 1 and 4). However, it was difficult to determine whether upregulation of *uidA* was occurring behind, at, or in advance of the infection front. It is probably impossible to resolve this issue with the system used here, since the GFP represented a “snapshot” of cells in which the virus was replicating, whereas the expression of *TRI* was monitored by GUS, which had to accumulate to levels detectable by histochemical staining.

BLAST analysis indicated that *TRI* is similar to *ACRE169*, a gene rapidly induced during the *Avr-9/Cf-9*-mediated hypersensitive response (Durrant et al. 2000). However, unlike *ACRE169*, *TRI* is not up-regulated in response to wounding. The BLAST analysis also revealed that *TRI* is similar to an *A. thaliana* gene predicted to encode a hypothetical protein of 89 amino acids. Both *TRI* and the hypothetical protein are very similar at the N- and C-termini but show less identity in the central region. Our data show that this unknown gene is not up-regulated in response to virus infection; the levels of expression were similar in healthy and noninfected plants (Fig. 2E). That is, this gene is unlikely to be regulated by a virus-responsive enhancer similar to the one that regulates *TRI*.

We have shown that the *TRI* transcript is alternatively spliced. The *N* gene from tobacco that confers resistance to TMV also undergoes alternate splicing (Dinesh-Kumar and Baker 2000). However, unlike the *N* transcripts, the ratio of *TRI* transcripts did not change after virus infection—both *TRI* transcripts were in low abundance (i.e., below the levels

Fig. 5. Transcript profiles for *TRI* (*Tobacco rattle virus*-induced gene). *TRI* encodes two alternatively spliced RNAs. **A**, A schematic diagram showing the predicted exons (E1, E2, and E3, white boxes) and introns (shaded boxes) for *TRI*. Exon and intron junctions are numbered 1 to 4 above the diagram. Nucleotides are indicated below the diagram. The asterisk indicates an in-frame stop codon in intron 1 at nucleotide position 327. The predicted protein from a fully spliced mRNA (193 amino acids) is shown below as a white box. **B**, A schematic diagram showing the mRNA detected by reverse transcription-polymerase chain reaction (RT-PCR). Both intron sequences have been retained. The predicted peptide (108 amino acids) is shown below as a white box. **C**, A schematic diagram showing the second transcript detected by RT-PCR. Splicing at junction 1 has not occurred. However, a splice site 11 nucleotides upstream of the stop codon indicated in A has been spliced to junction 4. The resulting sequence has created a new in-frame stop codon. The predicted peptide (104 amino acids) is shown below as a white box.

of detection by RT-PCR) in healthy plants and both were up-regulated by TRV-GFP infection. In *A. thaliana*, most alternatively spliced genes encode products involved in gene regulation and stress responses (Kazan 2003), and the splicing usually leads to the generation of structurally or functionally distinct proteins, or both. However, our sequence data indicated that the *TRI* products were identical, except that one had an extra four amino acids (WSEY) at the C-terminus. At present, we cannot rule out the possibility that the UGA stop codon in the shorter transcript was suppressed; read-through of UGA as tryptophan is common in bacteria and viruses (Beier and Grimm 2001). We were unable to find any other examples in the databases of proteins ending in WSEY. The tryptophan is especially interesting as they are often buried within the protein structure, but in this case, because it is so close to the C-terminus, it is most likely to be on the surface of TRI. Tryptophan residues on the surface of proteins may be involved in ligand binding (M. Durrant, *personal communication*). The biological significance of the two transcripts remains to be determined.

Consistent with our findings that *TRI* has sequence similarity to other genes up-regulated during pathogen infection, we have shown that the *TRI* enhancer is responsive to infection by a diverse group of pathogens. In future, functional studies of the products of *TRI* by gene silencing or overexpression strategies may reveal more information about the roles of these gene products in plant defense. Similarly, characterization of the proteins that bind to the *TRI* *cis*-acting elements and understanding how these pathogen-dependent transcription factors are activated will reveal the regulatory pathways of transcription and provide further insight into the host response to pathogen attack.

MATERIALS AND METHODS

Plant material and growth conditions.

Enhancer trap lines (ecotype Col-*glabrous1*) were obtained from NASC as pools of seeds. *A. thaliana* plants were grown in a controlled environment chamber at 22°C with 8-h-day length. The enhancer trap T-DNA vector was pD991 (Campisi et al. 1999). Lines SA04023 and SA04068 were generated by transformation of Col-0, using the flower dip transformation procedure (Bechtold et al. 1993), and transformants carrying the T-DNA from pA351 were selected by screening for resistance to the herbicide Challenge (Aventis, Cambridge). GUS activity was assessed on T1 herbicide-resistant plants.

Pathogen inocula.

All virus inocula were prepared as infectious sap extracts from *N. benthamiana*. For TRV infection, GFP was imaged using a Leica MZFLIII microscope and digital camera.

Pseudomonas syringae cultures at 10^7 CFU per ml were syringed into GS393 plants and were stained for GUS activity 1 dpi. The control treatment was leaf infiltration with 10 mM $MgCl_2$.

Three-week-old GS393 seedlings were sprayed with water or *Peronospora parasitica* Noco2 or Cala at 10^4 spores per ml, using a spray gun to gently mist leaves with the inoculum. Plants were covered and were incubated at 18°C under short-day conditions for 7 days.

Analysis of GUS activity.

GUS activity was assayed by histochemical staining as described in Angell and Baulcombe (1995). Leaves were cleared of chlorophyll by incubation in 70% (vol/vol) ethanol and were imaged on a Hewlett Packard scanner. MUG assays were

performed as described by Chapman and associates (1992) on 10 leaves per treatment. Total protein was calculated using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hertfordshire, U.K.).

RT-PCR.

Total RNA was extracted from *A. thaliana* leaves at 4 dpi (leaves were inoculated with TRV-GFP or water or were left untouched). RNA was purified by lithium chloride precipitation with an additional DNase treatment. Lack of genomic DNA contamination was confirmed in all samples by checking for the loss of the band that represented the amplification of the genomic fragment and by RT-PCR in the absence of reverse transcriptase. In all experiments, 1 µg of RNA was used to prepare cDNA using an oligo d(T)₁₈ primer and Expand reverse transcriptase (Roche Diagnostics, East Sussex, U.K.). The same cDNAs were used as templates for all of the PCRs shown in Figure 2. RT-PCR was performed on the cDNA, using primers designed to regions close to the predicted start and stop codons for At1g78400, At1g78420, and AC007651. For GUS, primers were designed to a region close to the start codon and to approximately 930 bp downstream of this. For At1g78410, primers were designed to the start codon and to a region close to the predicted stop codon indicated in the original database annotations, i.e., in exon 3 (Fig. 5A). The entire experiment was repeated three times, and all PCR products were sequenced to confirm their identities. The PCR program for all RT-PCRs was 35 cycles with an annealing temperature of 65°C and an extension time of 1 min. Primer sequences were as follows (all 5' to 3').

```
GUS F cgtcctgtagaaaccccaaccctgaaatcaaaaaactc
GUS R gtcgtgcaccatcagcagttatcgaatccttggcc
At1g78420 F ggaagaggcaagtgtgtg
At1g78420 R gtcgtcgtggcatgaacc
At1g78400 F tagtagcatttcagccgcc
At1g78400 R cagaagaagttagagtggtg
At1g78410 F ggagccgatgaaggtgtgttc
At1g78410 R acttggttagcaagacgcc
AC007651 F aaggtgtcttcattaacacac
AC007651 R gaccaagattatagaattgccgacgcg
ubiquitin5 F gaatcatccgacaccatcgac
ubiquitin5 R caactccttcttctgtaaac
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TAIL-PCR.

Approximately 80 ng of genomic DNA from GS393 was used as a template. The TAIL-PCR program was as detailed in Liu and associates (1995), using the random primers TAIL1 (NTCGASTWTSGWGTT) and TAIL3 (WGTGNAG WANCANAGA) in combination with pD991 right border oligo number 156 (CCTATAAATACGACGGATCG) and right border oligo number 123 (GCATGCAAGCTTGGC ACTGG).

Construction of agro-infiltration plasmids for transient expression assays.

The -60-bp minimal promoter sequence fused to GUS was cloned out of the binary vector that had been used to generate the enhancer trap lines pD991 (provided by T. Jack, Dartmouth University, Hanover, NH, U.S.A.) as a PCR fragment incorporating *EcoRI* and *StuI* restriction enzyme sites at the 5' end of the minimal promoter. This cassette was transferred upstream of a transcriptional terminator in pUC19 to generate pA626. The 1.1-kb region immediately upstream of the T-DNA insertion site in GS393 was cloned by PCR into *EcoRI*- and *StuI*-digested pA626 to generate pA319. The expression cassettes were transferred from pA319 and pA626

into pGreen0229 (Hellens et al. 2000) as *EcoRI-HindIII* fragments to generate pA351 and pA353, respectively. Both constructs were confirmed by sequencing and were transferred to *Agrobacterium tumefaciens* GV3101 by electroporation.

Agroinfiltration of *N. benthamiana* leaves.

Nontransformed *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* carrying pA351 or pA353. At 2 days postinfiltration, leaves were rub-inoculated with TRV-GFP sap or water. Four days later the leaves were stained for GUS activity. The experiment was repeated twice on a total number of 16 leaves per treatment.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Dartmouth College Department of Biological Sciences Jack Lab T-DNA generated enhancer traps in *Arabidopsis* website:
www.dartmouth.edu/~tjack/