Rapid and Transient Activation of Transcription of the ERF3 Gene by Wounding in Tobacco Leaves

POSSIBLE INVOLVEMENT OF NtWRKYs AND AUTOREPRESSION*

This study investigated the regulatory mechanism of rapid and transient induction of a transcriptional repressor ERF3 gene by wounding in tobacco (Nicotiana tabacum) leaves. Deletion and mutation analysis of the promoter region have suggested that the proximal W boxes (TGAC(T)/G) and a GCC box, respectively, may be involved in the positive and negative regulation of wound-induced expression of the ERF3 gene. Electrophoretic mobility shift assays indicated that wounding enhanced the specific binding activity of nuclear factors to the W boxes. NtWRKY1, -2, and -4, which are tobacco group I WRKYs, interacted specifically with the W boxes and activated transcription via the W boxes. On the other hand, deletion of the GCC box from \( \text{NsERF3} \) promoter-GUS reporter gene caused a delay in down-regulation of transcription after wounding induction. In addition, ERF3 repressed transcription via the \( \text{NsERF3} \) promoter activated by NtWRKYs. These results suggest the possible involvement of NtWRKYs and autorepression in the rapid and transient expression of the ERF3 gene by wounding.

It is well known that wounding activates the expression of a diverse array of genes that contribute to healing of injured tissues and protection from subsequent invasion by pathogens. Several recent reports have shown that the expression of several wound-responsive genes is activated rapidly after wounding. These genes are called immediate early-responsive genes (2, 3) and often encode transcription factors (3–6). These transcription factors are involved in the immediate early genes by wounding remain unclear. In addition, the regulatory mechanism for transient expression of immediate early genes has not been addressed in plants.

Four ERFs (ERF1, ERF2, ERF3, and ERF4) were originally identified as proteins that bind to a GCC box, an ethylene-responsive element, in tobacco (8). Ethylene is a plant hormone produced in tissues that are responsive to environmental stimuli, such as wounding and pathogen infection, and which regulates the induced expression of various defense-related genes. It has been shown that ERFs are involved in transcription via the GCC box as promoters of defense genes encoding antifungal proteins, including chitinases and glucanases, in response to ethylene and fungal elicitors (8, 9). Elicitors are signaling molecules that are generated on pathogen infection and recognized by plant cells, which then activate defense responses. ERF proteins share a well conserved and plant-specific DNA-binding domain, the ERF domain, and form a large gene family in plant species, as shown in Arabidopsis (10). ERF proteins play important roles in plant responses to various hormones or environmental cues, including biotic and abiotic stress (11).

Transient expression assays with tobacco protoplasts have demonstrated that ERF1, -2, and -4 function as transcriptional activators (12). In contrast, ERF3 has been shown to function in tobacco protoplasts as a transcriptional repressor (12). However, there is no evidence to date that ERF3 functions inherently as a transcriptional repressor in intact cells. Although the repressor ERF3 gene and the other activator ERF genes may have multiple functions, these genes appear to be expressed in common responses, but with different kinetics, to wounding (2).

In addition, the promoter of the \( \text{NsERF3} \) gene was also shown to contain the GCC boxes (7). It is likely that there is a very intricate mechanism for transcription via GCC boxes that is antagonistically regulated by the wound-induced transcriptional repressor and the other activators. Therefore, it is important to unravel and elucidate which cis-acting elements and transcription factors are involved in the immediate early expression of the ERF3 gene induced by wounding.

The present study addresses cis-acting elements and transcription factors involved in rapid and transient activation of transcription of the ERF3 gene in response to wounding. This study revealed the involvement of tobacco WRKY proteins and W boxes in wound-activated transcription of the ERF3 gene. Our results also suggest that transcriptional autorepression of the ERF3 gene by its own product is involved, at least in part, in the transient expression pattern of the ERF3 gene upon wound induction.

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1 The abbreviations used are: ERF, ethylene-responsive transcription factor; GUS, \( \beta \)-glucuronidase; LUC, luciferase; EMSA, electrophoretic mobility shift assay.

2 NsERF genes, which are ERF orthologs in Nicotiana sylvestris, an ancestor of Nicotiana tabacum (7), in transgenic tobacco plants. At present, however, cis-acting elements and transcription factors involved in the rapid induction of these genes by wounding remain unclear. In addition, the regulatory mechanism for transient expression of immediate early genes has not been addressed in plants.
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EXPERIMENTAL PROCEDURES

Construction of ERF3 Promoter-GUS Fusions—In all ERF3 promoter-E27 constructs, the NeERF3 promoter is transcriptionally fused to the gene for β-glucuronidase in the pH101. A common reverse primer (−30 to −5 from 5′ ATG) was prepared with the addition of a BamHI site. Each forward primer was synthesized with the addition of a SalI site at the 5′ termini. The gels were then dried and autoradiographed. Electro-Fluorometric Assays of GUS Activity—In vitro protein synthesis of NtWRKY proteins—In vitro protein synthesis was performed using TNT quick master mix (Promega), and then a total of 50 μl of reaction mixture was incubated at 30 °C for 60 min. Each protein for EMSAs used 1 μl of the resultant 50-μl reaction mixture(s). We used the TransTrend™-non-radioactive translation colorimetric detection system (Promega) to confirm synthesized proteins. Biotinylated UssRNA was incorporated into nascent protein during translation. We used SDS-PAGE for 1 μl of the reaction and then transferred it to a polyvinylidene difluoride membrane. The membrane was probed with streptavidin-AP and then incubated with Western Blue substrate to develop colored bands.

Transient Assay—Transient assays were performed using a particle gun bombardment method. We coated 510 μg of 1-μm gold biotinylated particles with reporter plasmid and effector plasmid or blank plasmid (total 2 μg of plasmid). The ratio of effector-reporter is noted in the figure legends. As a reference, the Renilla LUC gene under the control of the CaMV35S promoter was used as a reference/reporter plasmid ratio of 1:10. These particles were introduced into tobacco leaves using a PDS-1000/He machine (Bio-Rad). After bombardment, the samples were incubated on filter paper moistened with 50 mM phosphate buffer (pH 7.0) for 18 h and then frozen in liquid nitrogen for luciferase assays. LUC assays were performed with the dual-luciferase reporter assay system (Promega) using a luminescence reader (TD-20/20; Promega). The Renilla LUC gene (Promega) under the control of the CaMV35S promoter was used as an internal control. To normalize values after each assay, the ratio of LUC activity (firefly LUC/Renilla LUC) was calculated.

RESULTS

Involvement of W Boxes of the NeERF3 Promoter in Its Wound-responsive Expression—Our previous study showed that a 1.4-kbp 5′-flanking region of the NeERF3 gene was sufficient to mediate wound-inducible transcription of the GUS reporter gene in transgenic tobacco plants (6). To identify the cis-acting element for wound-responsive expression of the ERF3 gene, we first analyzed the wound-induced expression of the GUS reporter gene, which was transcriptionally fused to a deletion series of the 5′-flanking region of the NeERF3 promoter (6) (Fig. 1). Fig. 1 shows that wounding induced GUS activity in tobacco leaves of three independent lines of E3P1.4G plants. The removal of −1458 to −782 caused a decrease in activity of GUS in the transgenic plants, whereas the ratio of wound-induced activity to basal activity in unwounded tissue did not change. This region may contain cis-elements(s) that are required for transcriptional enhancement. Although further deletion to −163 bp engendered a partial decrease in wound-inducible GUS activity, wound responsiveness remained evident in E3P0.163G plants. By contrast, wound responsiveness was no longer observed after deletion to −123 bp in two independent lines (Fig. 1). An inverted repeat of TGAC/CT) sequences (W boxes) was found between the −163 and −124 region of this promoter. The W box was previously shown to function as the elicitor-responsive element in parsley by specific interaction with transcription factors, namely WRKYs (17). We prepared an E3P0.163WG construct to examine loss of function of these W boxes in wound-inducible transcription of the ERF3 gene. In the construct, core sequences of two W boxes were mutated by nucleotide substitution, as shown in Fig. 3A (mut A). In transgenic tobacco transformed with this construct, both the basal activity and wound-inducible activity of GUS were significantly decreased (Fig. 1). This result indicates that the W boxes of the NeERF3 promoter are necessary for basal and wound-responsive transcription.

We also analyzed the kinetics of wound-responsive transcription via the promoter fragments of the ERF3 gene. Since GUS mRNA, unlike GUS protein, has a relatively short half-life, it is suitable for reporting rapid and transient changes in gene transcription. We examined wound-inducible changes in mRNA levels of GUS in these transgenic plants: E3P1.4G-2, E3P0.78G-17, E3P0.22G-4, E3P0.16G-3, and E3P0.16WG-1 (Fig. 2). A basal activity of the transcription was not detectable based on the mRNA level of the GUS gene (Fig. 2). The relative degrees of wound-inducible mRNA levels at their peaks in the
transgenic plants were consistent with the results shown in Fig. 1. This result confirmed the requirement of the W boxes for the wound-responsive transcription. In addition, deletion from /H11002\textsuperscript{224} to /H11002\textsuperscript{164} caused a delay in the decrease of GUS mRNA level after wound induction (Fig. 2). This result implies that the /H11002\textsuperscript{224}/H11002\textsuperscript{164} region containing a single GCC box is involved in down-regulation of wound-activated transcription of the ERF3 gene.

As described above, W boxes (TGAC(C/T)) and a GCC box, respectively, which are localized at the proximal region of the promoter of the ERF3 gene, are likely to be involved in the positive and negative regulation of wound-induced expression of the ERF3 gene. On the other hand, W boxes and a GCC box are also present in the upstream region between /H11002\textsuperscript{1458}/H11002\textsuperscript{783} (Fig. 1). However, it is unlikely that these elements are required for the wound-responsive transcription of the ERF3 gene.

**Wound-activated Specific Interaction between Nuclear Factors and W Boxes of the NsERF3 Promoter in Tobacco Leaves**—We performed EMSA to investigate tobacco nuclear factors that interact with the /H11002\textsuperscript{163}/H11002\textsuperscript{136} region containing the inverted repeat of W boxes (Fig. 3A). As shown in Fig. 3B, two retarded complexes were formed by incubation of the labeled fragment A with nuclear protein extracts from unwounded leaves. Enhanced formation of these complexes was observed in wounded leaves. We prepared a mut A fragment in which TGAC core elements were replaced by CCTT sequences; we then performed competition analysis to examine the involvement of the two W boxes in the formation of these complexes (Fig. 3A). Formation of complexes was efficiently inhibited in the presence of unlabeled fragment A, whereas it was reduced only slightly in the presence of unlabeled mut A fragment. Moreover, a wound-inducible increase in the complex formation was still observable (Fig. 3B). We therefore inferred that these wound-activated nuclear factors specifically interact with the W boxes.
W boxes of the NsERF3 promoter. Enhanced binding activity of nuclear factors to these W boxes is consistent with the view that W boxes function as wound-responsive elements (Figs. 1 and 2). The quality of these nuclear proteins was verified using a −95/−77 fragment containing a TATA box as a control probe. Two preparations showed a similar ability to form specific retarded complexes using the labeled TATA box-containing probe (Fig. 3C).

Specific Interaction of NtWRKY Proteins with W Boxes in NsERF3 Promoter—It has been demonstrated that elicitor-inducible transcription is mediated by specific interaction of the W boxes with WRKY transcription factors (17). We recently isolated cDNAs for three WRKY proteins, NtWRKY1, NtWRKY2, and NtWRKY4, from tobacco and demonstrated the possible involvement of transcription factors in the transcriptional activation of genes for class I basic chitinase in response to a fungal elicitor (13). The deduced amino acid sequences of NtWRKY1, -2, and -4 contained two WRKY domains and belonged to group I of the WRKY gene family (18). Three NtWRKY proteins were synthesized in vitro with a rabbit reticulocyte transcription/translation system. Biotinylated lysine residues were incorporated into nascent proteins during translation to confirm synthesis of NtWRKY proteins. Fig. 4A shows that each synthesized NtWRKY protein was detected as a single band with the expected molecular weight. Synthesized WRKY proteins were then assayed for binding activity to the inverted repeat of W boxes of the NsERF3 promoter. Fig. 4B reveals that synthesized NtWRKY1 protein interacted strongly with probe A (Fig. 3A). Similarly, retarded complexes were also formed when probe A was incubated with both NtWRKY2 and NtWRKY4 proteins. Formation of such complexes was not observed with incubation of the probe mut A with these proteins, suggesting that these NtWRKY proteins specifically interact with the inverted repeat of W boxes of the NsERF3 promoter.

NtWRKY Proteins Activate Transcription from W Boxes in the NsERF3 Promoter—To investigate the ability of NtWRKYs to regulate transcription from W boxes of the NsERF3 promoter, we performed co-bombardment experiments using a −163 NsERF3 promoter-firefly LUC construct as reporter, CaMV35S-NtWRKY1, -2, or -4 construct as effector, and a CaMV35S-Renilla LUC construct as reference reporter into tobacco leaves (Fig. 5). Transient expression of NtWRKY1 or NtWRKY2 led to a significant (over 3-fold) increase in relative activity of firefly LUC normalized with Renilla LUC. A slight increase in firefly LUC activity due to expression of NtWRKY4 was also detected. By contrast, mutation of TGAC core motifs of two W boxes in the −163 NsERF3 promoter resulted in the disappearance of increase in firefly LUC activity by these factors (Fig. 5). Therefore, these results indicate that these transcription factors can activate transcription mediated by W boxes in the NsERF3 promoter in tobacco leaf cells.

Expression Patterns of NtWRKY Genes after Wounding—We investigated the effects of wounding on the respective expressions of the three NtWRKY genes in tobacco leaves. Fig. 6 shows that the levels of transcripts of genes for NtWRKY1 and NtWRKY2 began to increase after 10 min of wounding, reached a maximum at 30 min, and then gradually declined. By contrast, the gene for NtWRKY4 was constantly expressed at a very low level (Fig. 6).

Possible Autorepression of Wound-induced Expression of the ERF3 Gene—The experiments presented in Fig. 2 show that the removal of a −224/+164 region containing a GCC box delayed the decrease in GUS mRNA after wound induction, suggesting that the GCC box is required for rapid reduction of the transcription level of the ERF3 gene induced by wounding. These clues prompted us to hypothesize that ERF3 autorepresses its own gene because ERF3 was shown to be a transcriptional repressor. To test this hypothesis, we examined the
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FIG. 4. NtWRKY proteins interacted specifically with W boxes of the NsERF3 promoter. A, in vitro syntheses of NtWRKY proteins were performed using the Tnt quick coupled transcriptional/translation system (Promega) according to the instructions provided by the manufacturer. Biotinylated lysine RNA was incorporated into nascent proteins during translation. We used 1 μl of the 50-μl reaction for SDS-PAGE; synthesis of each protein was confirmed by Transcend™ non-radioactive translation colorimetric systems. The mobility of the molecular mass marker is also shown in the left panel. B, probe A and probe mut A were used for EMSAs as depicted in Fig. 3A. Each synthesized NtWRKY protein used 1 μl of the 50-μl reaction for binding assay of EMSA. The binding reaction was performed in the absence (no protein) and presence of each synthesized NtWRKY protein.

In this study, we showed that an inverted repeat of W boxes in the proximal region of the promoter of the NsERF3 gene is required for the basal activity of transcription and at least in part for the wound-responsive transcription (Figs. 1 and 2). By contrast, the distal W box-like sequences were not required. It was shown that a synthetic promoter containing W boxes conferred elicitor- and wound-responsive expression to the reporter gene in Arabidopsis plants (19). Our results consistently show that the proximal W boxes in the promoter of the ERF3 gene may function as cis-acting elements for wound-inducible immediate early expression, which differs from previously described wound-responsive elements in promoters of plant genes (20, 21).

In this report, we showed that NtWRKYs are possible candidates for transcription factors involved in the rapid activation of wound-responsive transcription of the ERF3 gene in tobacco. NtWRKY1 and NtWRKY2 significantly activate proximal W box-mediated transcription (Fig. 5). In addition, the increase in transcripts of genes for NtWRKY1 and NtWRKY2 due to wounding occurred prior to the activation of NsERF3 promoter-mediated transcription (Figs. 2 and 6). The genes encoding WRKY transcription factors form a multigene family in plants. For example, over 70 WRKY genes have been identified and classified into three groups in Arabidopsis (18). WRKYs are involved in the regulation of various biological functions, including sugar response (22), senescence (23), and development of seed coats and trichomes (24), as well as defense responses against pathogen infection (25). Many WRKY genes have also been identified in tobacco (4, 26–28). In most cases, tobacco WRKY proteins have been implicated in the transcriptional regulation of defense genes in response to pathogen infection, elicitors, and salicylic acid. By contrast, WIZZ has been identified as a wound-induced WRKY protein, whereas the WIZZ protein failed to activate the W box-mediated transcription (4). Thus, NtWRKY1 and NtWRKY2 are candidates for transcription factors involved in wound-inducible rapid transcription of the ERF3 gene via W boxes in its promoter, although we cannot exclude the possibility that other tobacco WRKY proteins might be also involved in the wound-inducible transcription of the ERF3 genes.

The proximal GCC box in the promoter region was required for transcriptional down-regulation after wound-induced activation of the ERF3 gene. Co-expression of ERF3 and NtWRKYs in tobacco cells resulted in a decrease in transcription of the ERF3 gene that had been activated by NtWRKYs. These results suggest that transcriptional autorepression of the ERF3 gene by its own gene product might be involved, at least in part, in its transient expression pattern after wound induction. However, no enhancement of the basal or induced activity of transcription of the ERF3 gene was observed by deletion of the proximal GCC box from the −224 promoter region. One possible explanation for this result is as follows. In intact leaves, levels of expression and/or transactivation activity of NtWRKYs may be insufficient to activate transcription via the promoter, irrespective of the influence of transcriptional repression by ERF3. Transcription of the ERF3 gene might be triggered when wound-induced expression of NtWRKYs exceeds a threshold level, after which a certain level of ERF3 protein might repress its own transcription.

Fujimoto et al. (29) showed that AtERF3, which is a repressor-type ERF in Arabidopsis, competitively suppressed the GCC box-mediated transcription activated by AtERF5, which is an activator-type ERF, in Arabidopsis leaves. In addition, they demonstrated, using a synthetic promoter containing the yeast GAL4-binding sites adjacentally fused to the GCC boxes, that AtERF3 repressed the GAL4-binding site-mediated transcription activated by a chimeric protein of the GAL4-binding domain fused with the herpes simplex virus VP16 activation domain, which has constitutive transactivation activity in
The present study showed that ERF3 suppressed NtWRKY-activated transcription mediated by the W box in a fragment of the natural promoter of \( \text{NsERF3} \). This suggests that expression of ERF3 beyond a certain threshold level may repress the expression of genes with a GCC box in their promoter, even in the presence of transcriptional activators, which recognize adjacent cis-acting sequences, provided no additional stimuli cause inactivation of ERF3 and/or hyperactivation of the transcriptional activator(s). In mature tobacco leaves, wounding simultaneously induces a rapid increase in the levels of mRNAs for ERF2, ERF3, and ERF4 (2, 6), whereas activation of genes for class I basic glucanase and GCC box-mediated transcription of genes by wounding was not observed (2). In contrast, expression of chitinase genes and the GCC box-mediated transcription increased significantly in the presence of ethylene in the wounded leaf tissues (2). The wound-induced accumulation of repressor-type ERFs may be sufficient to suppress the GCC box-mediated transcription activated by activator-type ERFs and to retain the expression of defense genes at normal levels if cells do not perceive any additional signals. When tobacco cells perceive a substantial level of ethylene produced in response to serious damage by wounding and subsequent pathogen infection, expression of defense genes might consequently be induced.

The present study suggests that the wound-inducible expression of tobacco \( \text{ERF3} \) gene was autorepressed by its own gene products. Interestingly, autorepression of a stress-inducible gene for a transcriptional repressor, \( \text{ATF3} \) (activating transcription factor-3), has also been reported in human cells (30). A variety of extracellular stress signals, including wounding and protein synthesis inhibitors such as cycloheximide, induce \( \text{ATF3} \) gene expression (31). In most cases of \( \text{ATF3} \) induction under stressed conditions, the \( \text{ATF3} \) expression is immediate and transient (for a review, see Ref. 32). This feature of the human \( \text{ATF3} \) gene closely resembles that of the tobacco \( \text{ERF3} \) gene (2, 6, 7, 9). Autorepression of the \( \text{ATF3} \) promoter by its...
own gene product is shown to be involved in the transient expression pattern of \textit{ATF3} gene upon stress induction (30). Therefore, such autorepressions of stress-inducible immediate early genes encoding transcriptional repressors may be conserved strategies to achieve transient expression of inducible genes in animals and plants. It was also suggested that autorepression plays an important role in cellular homeostasis under stress conditions by prevention of sustained \textit{ATF3} expression (33). Similarly, autorepression of the \textit{ERF3} gene may also play an important role in maintaining cellular homeostasis during wound response by preventing its sustained expression.

As described above, we demonstrated the possible involvement of two different \textit{cis}-acting elements and corresponding transcription factors in positive and negative regulation, respectively, of the transcription of genes in the immediate early wound response. The results demonstrated here are important with regard to our understanding of the regulation of wound-induced expression of immediate early response genes in plants. However, further work is needed to elucidate the molecular details involved in the regulatory network of gene expression in wound stress in plants.

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