The Arabidopsis Cytosolic Thioredoxin *h*5 Gene Induction by Oxidative Stress and Its W-Box-Mediated Response to Pathogen Elicitor[1]

Christophe Laloi[2], Dominique Mestres-Ortega, Yves Marco, Yves Meyer, and Jean-Philippe Reichheld*

Laboratoire Génome et Développement des Plantes, Université de Perpignan, Unité Mixte Recherche Centre National de la Recherche Scientifique 5096, 66860 Perpignan, France (C.L., D.M.-O., Y.Me., J.-P.R.); Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, Centre National de la Recherche Scientifique-Institut National de la Recherche Agronomique, Auzeville, Castanet-Tolosan, France (Y.Ma.)

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The AtTRXh5 protein belongs to the cytosolic thioredoxins *h* family that, in Arabidopsis, contains eight members showing very distinct patterns and levels of expression. Here, we show that the *AtTRXh5* gene is up-regulated during wounding, abscission, and senescence, as well as during incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. By electrophoretic mobility shift assays, a binding activity on a W-box in the *AtTRXh5* promoter region was found induced by treatments with the *P. syringae*-derived elicitor peptide flg22, suggesting that a WRKY transcription factor controls *AtTRXh5* induction upon elicitor treatment. Remarkably, *AtTRXh5* was up-regulated in plants overexpressing WRKY6. More generally, *AtTRXh5* is induced in response to oxidative stress conditions. Collectively, our data indicate a possible implication of the cytosolic thioredoxin AtTRXh5 in response to pathogens and to oxidative stresses. In addition, this regulation is unique to *AtTRXh5* among the thioredoxin *h* family, arguing in favor of a speciation rather than to a redundancy of the members of this multigenic family.

Thioredoxins are small thiol:disulphide oxidoreductases characterized by the canonical active site WCG/PPC of which the low redox potential confers strong reductive properties. In their reduced state, thioredoxins are able to reduce disulphide bridges of numerous target proteins. Subsequently, the oxidized thioredoxins are reduced by the flavoenzyme thioredoxin reductase, forming together the so-called thioredoxin system.

Since the discovery of the first *Escherichia coli* thioredoxin, acting as a potent hydrogen donor for ribonucleotide reductase (Laurent et al., 1964), these enzymes have been found in all prokaryotes and eukaryotes, including fungi, invertebrates, vertebrates, and plants. Plants are distinguishable from other organisms by their complex thioredoxin systems as revealed by plant genome sequencing projects, such as that of Arabidopsis (Arabidopsis Genome Initiative, 2000). The genome of this crucifer encodes 19 thioredoxins localized in the cytosol, the chloroplast, or the mitochondria, as well as a dozen of proteins closely related to thioredoxins (Meyer et al., 2002). The Arabidopsis cytosolic system consists of eight different thioredoxins *h* and two homodimeric NADPH-dependent thioredoxin reductases (Laloi et al., 2001; Meyer et al., 2002). In plants, thioredoxin *h* play a role during germination and early seedling development (Kobrehel et al., 1992; Besse et al., 1996; Wong et al., 2002; Marx et al., 2003), and might be involved in sulfate assimilation (Mouaheb et al., 1998). Their abundance in the phloem sap of rice (*Oryza satia* L. var Kantou) and a range of monocot and dicot species suggests that they could act as messenger proteins (Ishiwatari et al., 1995; Schobert et al., 1998).

The chloroplastic thioredoxin system of Arabidopsis is even more complex than the cytosolic one. It includes two major types of chloroplastic thioredoxins, *m* and *f*, as well as the two recently identified types *x* and *y* (Mestres-Ortega and Meyer, 1999; Meyer et al., 2002; Lemaire et al., 2003), and a heterodimeric ferredoxin-dependent thioredoxin reductase. Thioredoxins *f* and *m* regulate the activity of certain enzymes involved in photosynthetic carbon metabolism, such as Fru-1,6-bisphosphatase and NADP malate dehydrogenase (Ruelland and Miginiac-Maslow, 1999). Recently, a plant mitochondrial thioredoxin system, consisting of a thioredoxin *o* and an NADPH-dependent thioredoxin reductase, was also identified (Laloi et al., 2001).

In contrast to mammals, yeast, and bacteria, in which cytosolic and mitochondrial thioredoxins are clearly involved in response to oxidative stress (Carmel-Harel and Storz, 2000; Nordberg and Arner,

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[2] Present address: Institute of Plant Sciences, Plant Genetics, Swiss Federal Institute of Technology (ETH), Universitätsstrasse 2, CH–8092 Zürich, Switzerland.

* Corresponding author; e-mail jpr@univ-perp.fr; fax 33–4–68–66–84–99.

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2001), the putative role of these proteins in oxidative stress responses in plants remained, for a long time, elusive. However, in heterologous systems, Arabidopsis cytosolic thioredoxin *h3* and chloroplastic thioredoxin *m* partially complement the hydrogen peroxide hypersensitivity of a yeast thioredoxin-deficient mutant (Mouaheb et al., 1998; Issakidis-Bourguet et al., 2001). Moreover, an active-site mutant of Arabidopsis thioredoxin *h3* was found to interact in vivo with a yeast peroxiredoxin (Verdoucq et al., 1999). In planta, an Arabidopsis stromal protein, CDSP32, which contains two thioredoxin domains, has been recently shown to play a critical role in plastid defense against oxidative damage, related to its function as a physiological electron donor to the BAS1 peroxiredoxin (Broin et al., 2002; Broin and Rey, 2003). The cosuppressed Arabidopsis lines lacking CDSP32 exhibit an increased sensitivity to photooxidative stress conditions, demonstrating the existence of a thioredoxin-dependent defense system preventing oxidative damage in plant chloroplasts. In barley (*Hordeum vulgare* var. Gerbel), a chloroplastic 2-Cys peroxiredoxin that can be reduced by thioredoxin has also been proposed to catalyze peroxide detoxification in the dark (Konig et al., 2002). These studies indicate that plant chloroplastic thioredoxins are involved in the response to oxidative stress. On the other hand, the functions of thioredoxin *h* in response to oxidative stress are not yet established in planta. During desiccation and germination of cereal seeds, the localization of thioredoxin *h* to the nucleus of aleurone and scutellum cells coincides with tissues undergoing oxidative stress (Serrato et al., 2001; Serrato and Cejudo, 2003). However, the exposure of wheat (*Triticum aestivum* L. cv Chinese Spring) seeds to oxidative stress had no effect on the accumulation of thioredoxin *h* transcripts or proteins (Serrato and Cejudo, 2003).

In the present study, we show that expression of the Arabidopsis cytosolic thioredoxin *h5* gene, *AtTRXh5*, is closely related to wounding, abscission, senescence, and pathogen attack, as well as several different oxidative stress conditions. Moreover, a nuclear factor recognizes and binds to a W-box in its proximal promoter during bacterial elicitor treatment, suggesting that the response of *AtTRXh5* to pathogen attack could be driven by a WRKY transcription factor. In addition, *AtTRX-h5* is up-regulated in plants overexpressing WRKY6, suggesting that WRKY6 could be implicated in the transcriptional regulation of *AtTRXh5*. As in Arabidopsis chloroplasts or the cytosol of mammals, yeast, and bacteria, the plant cytosolic thioredoxin AtTRXh5 seems to be involved in oxidative stress response. We discuss this role that it is not apparently shared by other thioredoxin *h* in Arabidopsis, suggesting different and specific functions for these other proteins that, in contrast, might be unrelated to oxidative stress response.

RESULTS

Specific Expression of *AtTRXh5* Promoter after Wounding

We have previously reported the expression pattern of the *AtTRXh5* gene that was studied by semiquantitative reverse transcriptase (RT)-PCR using total RNA extracted from several organs and from cell suspensions in stationary or exponential phase (Reichheld et al., 2002). In addition, to visualize more precisely the spatial and temporal patterns of expression of the *AtTRXh5* promoter, transgenic Arabidopsis plants expressing the β-glucuronidase (GUS) reporter gene (*uidA* from *E. coli*) under the control of a 1.5-kb long *AtTRXh5* promoter, including the 5′-untranslated region were generated. Pr*AtTRXh5::GUS* transformants revealed GUS staining patterns that were overlapping but clearly distinct from those previously described for other *AtTRXh* genes (Reichheld et al., 2002). We further analyzed the response of *AtTRXh* genes to wounding; a rapid increase in GUS activity was observed in vascular tissues surrounding the wounding zone of green leaves or stems in Pr*AtTRXh5::GUS* plants (Fig. 1, A and B), but not in transgenic plants expressing *GUS* under the control of *AtTRXh1*, *h2*, *h3*, or *h4* promoters (data not shown).

AtTRX-h5 Promoter Expression Is Enhanced during Abscission and Senescence

No GUS staining was detected in the early stages of flower development, but was detected in pollen and sepals of mature flowers (Reichheld et al., 2002). At later stages, staining was visualized in anthers, primarily caused by a pollen-specific GUS activity, in siliques in residual stigmatic papillae, and in the floral organ abscission zone as well as in early senescent leaves. To further analyze the GUS activity associated with senescence, we studied the development of flower and rosette leaf in soil-grown transgenic plants. GUS activity increased in sepal abscission zone during abscission (Fig. 1C). Simultaneously, it became higher in vascular tissues of senescent sepals. The *AtTRXh5* promoter-driven *GUS* expression was similarly induced during the senescence of rosette leaves (Fig. 1D), reaching approximately a level five times higher in yellowing leaves than in green leaves (Fig. 1E). This senescence-induced expression of *AtTRXh5* was compared with that of *SAG12*, a senescent marker gene (Weaver et al., 1998), and of *PR1*, a defense-related gene previously shown to be also induced in senescent leaves (Robatzek and Somssich, 2001). The three genes were expressed at a low level in green leaves, and expression increased in senescent leaves (Fig. 1F). Interestingly, the expression of the *AtTRXh3* gene, the paralog of *AtTRXh5* (Reichheld et al., 2002), was not induced in senescent leaves, indicating that the regulation and consequently the functions of these two

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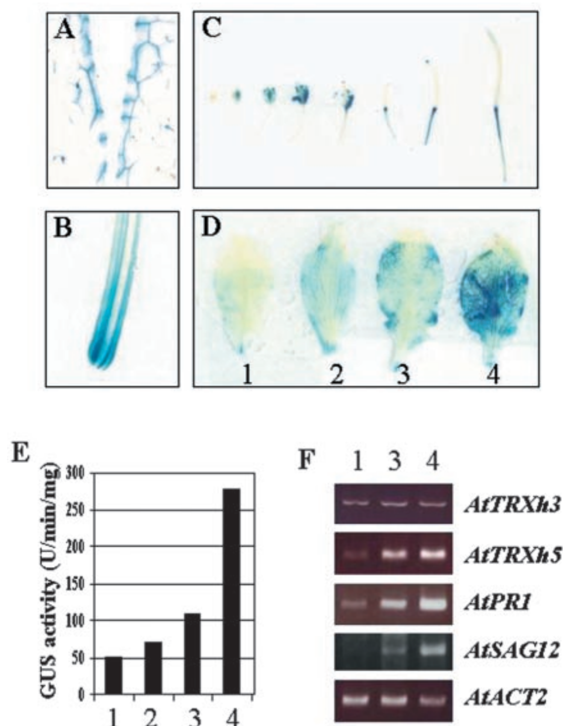

Figure 1. Expression of *AtTRXh5* after wounding and during abscission and senescence. A and B, Histochemical localization of GUS activity surrounding the incision wounding site in green leaves and stem, respectively, in Pr*AtTRXh5::GUS* transgenic lines. C, Time course and histochemical localization of GUS activity in Pr*AtTRXh5::GUS* transgenic lines in pistil, sepal, and sepal abscission zone. D, Time course and histochemical localization of GUS activity in Pr*AtTRXh5::GUS* transgenic lines during senescence of rosette leaves; green leaf (1), up to 25% of a leaf shows yellowing (2), about 50% of a leaf shows yellowing (3), and more than 50% of a leaf shows yellowing (4). E, GUS activity measurement at the same stages as in D. F, Semiquantitative RT-PCR analysis of the expression of *AtTRXh5*, *AtTRXh3*, the senescence-specific marker gene *AtSAG12*, the pathogen-related *AtPR1*, and the reference gene *AtACT2* during leaf senescence at the same stages as shown in D.

closely related genes have diverged, at least during senescence.

AtTRXh5 Is Induced during an Incompatible Interaction with the Bacterial Pathogen *Pseudomonas syringae*

The *PrAtTRXh5::GUS* reporter line was tested for its response to pathogen attack. Toward this goal, green leaves of Pr*AtTRXh5::GUS* plants were inoculated with the virulent DC3000 *Pseudomonas syringae* pv. *tomato* strain carrying or not the *AvrRpm1* or the *AvrRpt2* avirulence genes (Dong et al., 1991). As shown in Figure 2A, a weak and transient expression was observed in all cases at the infiltration sites after 2 h and was probably caused by the wounding induced by the inoculation. However, inoculation of leaves with the avirulent Ps strains (carrying the *avrRpm1* or *avrRpt2* genes) resulted in a higher level of GUS activity that was detectable within 4 h after infiltration at the inoculation sites, which contained plant cells undergoing hypersensitive cell death. Inoculation with the virulent DC3000 *P. syringae* strain induced a weaker and more diffuse increase of the GUS activity, visible after 12 h and that was comparable with the one detected in mock-infected leaves infiltrated with $MgCl_2$. This activity was confirmed by studying the expression of the *AtTRXh5* gene by semiquantitative RT-PCR. Six hours after infiltration, *AtTRXh5* mRNA steady-state levels were higher in leaves inoculated with the avirulent strains than in mock-infiltrated leaves or leaves infiltrated with the virulent strain DC3000 (Fig. 2B). A similar expression profile was observed for the *AtPR1* gene.

Electrophoretic Mobility Shift Assays (EMSAs) Reveal a Functional W-Box in *AtTRXh5* Promoter Region

Because *AtTRXh5* is induced during senescence, wounding, elicitor treatment (Reichheld et al., 2002),

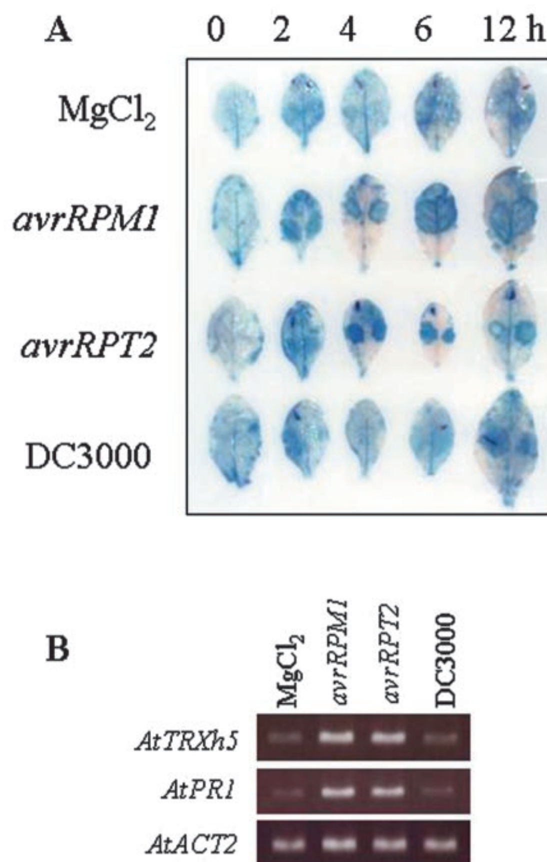

Figure 2. Expression of *AtTRXh5* during pathogen attack. A, Time course and histochemical localization of GUS activity in Pr*AtTRXh5::GUS* transgenic lines after infiltration of rosette leaves with avirulent *P. syringae* strains (avrRPM1 and avrRPT2), virulent strain (DC3000), or $MgCl_2$ as a control of infiltration-induced wounding. B, Semiquantitative RT-PCR analysis of the expression of *AtTRXh5*, the pathogen-related *AtPR1*, and the reference gene *AtACT2*, 6 h postinfiltration under the same conditions as in A.

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and pathogen attack, *cis*-acting regulatory DNA elements implicated in these processes were searched in the promoter of this gene. No senescence-responsive element could be found in the 1-kb promoter region (Noh and Amasino, 1999). However, seven copies of the W-box (TTGACC/T) were identified in a 1-kb region upstream of the translation initiation site. Two of them are situated within a 200-bp proximal region upstream from the putative transcription initiation site, which suggests that they could be functional (Fig. 3A). W-boxes are *cis*-elements known as binding sites for the WRKY plant-specific transcriptional regulators that control several types of plant stress responses, such as pathogen defense, wound response, and senescence (Eulgem et al., 2000). To analyze the capacity of the W-box localized at −185 to bind nuclear proteins, EMSAs were performed using as a radioactive probe, a 26-mer oligonucleotide containing the copy of this W-box localized (Fig. 3B). Nuclear extracts were prepared from Arabidopsis cell suspensions treated with flg22 for 8 h. We have previously reported that flg22, a synthetic peptide derived from the *P. syringae* flagellin (Felix et al., 1999), highly induces *AtTRXh5* under these conditions (Reichheld et al., 2002). One retarded band was observed (Fig. 3C). The specificity of binding was demonstrated by competition experiments using the oligonucleotides presented in Figure 3B. The protein binding is markedly competed by an excess of unlabeled wild-type W-box oligonucleotide, but is not by the same oligonucleotide in which the W-box has been mutated (Fig. 3C). In addition, binding experiments that have been performed with the same concentration of nuclear extracts prepared from cell sus-

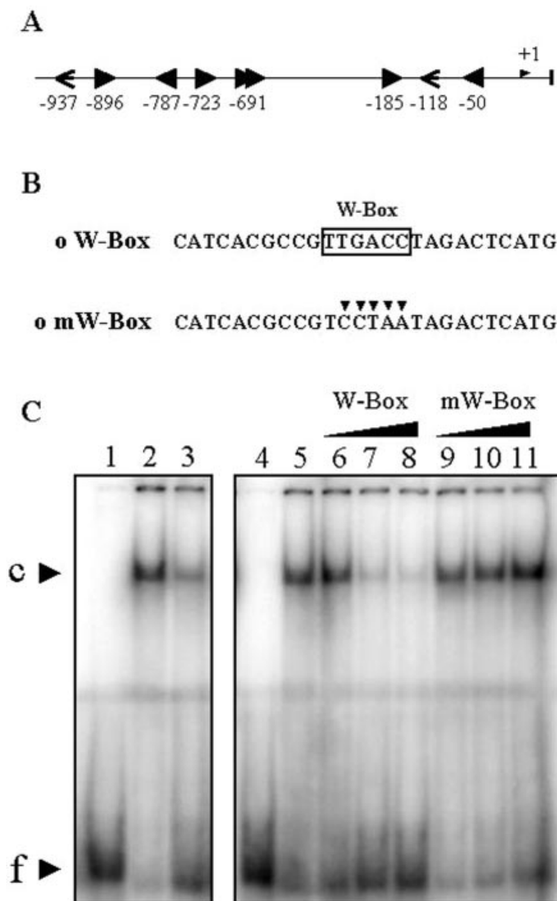

Figure 3. EMSA for binding of transcription factors to a W-box *cis*-element. A, *AtTRXh5* promoter. Black arrows represent W-boxes. White arrows represent as-1 elements. B, Sequence for wild-type −185 W-box (o W-Box) and mutant (o mW-Box) oligonucleotides used as DNA-binding probes or competitors. The W-box motif is marked by a white box and the base substitutions are indicated by black triangles on top of the mutated sequence. C, Binding reactions were performed with nuclear extracts prepared from Arabidopsis cell suspensions treated with flg22 for 8 h (lanes 2 and 5–11) or with water as a negative control (lane 3). Lanes 1 and 4 do not contain nuclear extract. Competition experiments were performed with 2-fold (lanes 6 and 9), 20-fold (lanes 7 and 10), and 50-fold (lanes 8 and 11) excess of unlabeled wild-type (lanes 6–8) or mutant (lanes 9–11) oligonucleotide or without unlabeled DNA added (lane 5). c, Complex; f, free probe.

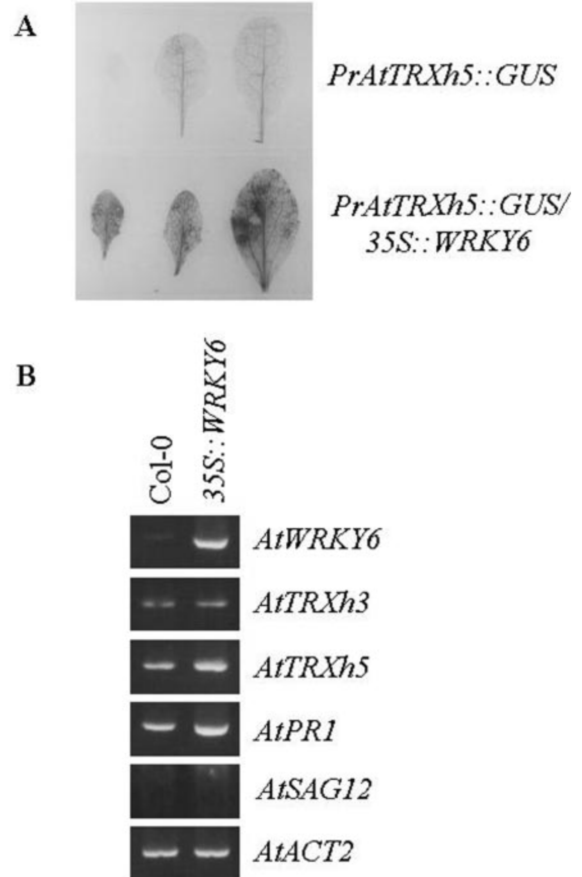

Figure 4. *AtTRXh5* expression in transgenic *35S-WRKY6* Arabidopsis. A, Histochemical localization of GUS activity in green rosette leaves of *PrAtTRXh5::GUS/35S::WRKY6* plants. B. Semiquantitative RT-PCR analysis of the expression of *AtWRKY6*, *AtTRXh5*, *AtTRXh3*, the senescence-specific marker gene *AtSAG12*, the pathogen-related *AtPR1*, and the reference gene *AtACT2*, in green rosette leaves of wild-type (Col-0) and *35S::WRKY6* plants.

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pensions untreated by flg22 showed reproducibly a lower intensity of the retarded band, suggesting that this binding is inducible by the flg22 treatment.

AtTRXh5 Gene Expression Is Up-Regulated in Plants Overexpressing the WRKY6 Factor

The expression profile and the regulation of the *AtWRKY6* gene are highly correlated with those of *AtTRXh5* (i.e. senescent tissues, pathogenesis infection, elicitor response, etc.; Robatzek and Somssich, 2001). To test the involvement of the WRKY6 factor in the regulation of *AtTRXh5*, we crossed the *PrAtTRXh5::GUS* plants with plants ectopically overexpressing the WRKY6 factor. In mature leaves of these plants, the GUS activity was found to be increased in comparison with *PrAtTRXh5::GUS* plants (Fig. 4A). An increased *AtTRXh5* mRNAs level was also observed in mature leaves of *PrAtTRXh5::GUS/35S-WRKY6* plants compared with control Colombia (Col-0) leaves. In contrast, the expression of the *AtTRXh3* gene is not increased in the *PrAtTRXh5::GUS/35S-WRKY6* plants. Interestingly, the promoter of this gene does not contain W-boxes. In addition, the *AtPR1* gene is overexpressed in the *35S-WRKY6* plants, confirming previous data of Robatzek and Somssich (2001). However, the senescent-specific gene *SAG12* shows no induction of expression in mature leaves of *PrAtTRXh5::GUS/35S-WRKY6* plants, indicating that the increased *AtTRXh5* gene induction is not due to an indirect aging effect (Fig. 4B).

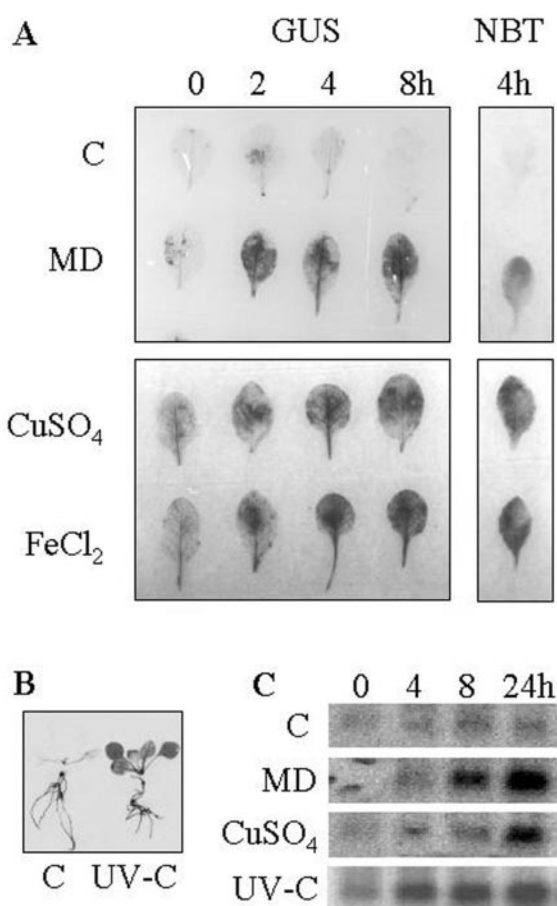

Figure 5. Time course and histochemical localization of GUS activity in Pr*AtTRXh5::GUS* transgenic lines under oxidative stress conditions. A, GUS activity after infiltration of rosette leaves with 50 μM menadione (MD), 100 μM $CuSO_4$, 100 μM $FeCl_2$, or water as a control. Four hours after infiltration, the leaf samples were also stained for superoxide accumulation with the nitroblue tetrazolium (NBT) staining. B, GUS activity after irradiation of in vitro Pr*AtTRXh5::GUS* seedlings with 5 kJ m^2 UV-C light. C, RNA gel blot from total RNAs extracted 0, 4, 8, and 24 h after menadione, copper treatments, and UV-C irradiation was hybridized with a 3′-specific probe of *AtTRXh5*.

AtTRXh5 Promoter Activity Is Induced in Response to Oxidative Stress Conditions

Induction of an oxidative burst is tightly correlated with senescence, pathogen attack, and elicitor treatment. To address the question of the relationship between the induction of *AtTRXh5* and oxidative stress conditions in plants, homozygous Pr*AtTRXh5::GUS* lines were submitted to various treatments, including menadione, hormonal treatments, and chemical inducers at concentrations known to induce strongly a variety of plant genes and trigger production of reactive oxygen species (ROS).

Menadione, a redox active quinone that is partially reduced in vivo to yield a semiquinone radical that reduces molecular oxygen into a superoxide anion (Thor et al., 1982), rapidly and strongly induced GUS activity in green rosette leaves, showing that *AtTRXh5* is induced by oxidative stress conditions (Fig. 5A). In addition, GUS activity was also increased by treatments of Arabidopsis rosette leaves with copper and iron (Fig. 5A), two transition metals that trigger production of superoxides by autooxidation of divalent cations to the trivalent form or to hydroxyl radicals by the Fenton reaction (Schutzendubel and Polle, 2002). To check if these treatments induced the generation of ROS in leaves, we performed NBT staining. Purple formazan deposits, which result from the reaction of NBT with superoxide, identify formation of superoxide in a leaf. Intense purple staining was observed in copper and iron treatment (Fig. 5A). As shown in Figure 5B, Pr*AtTRXh5::GUS* was also strongly activated by irradiation with a nonlethal dose of UV-C light (5 kJ m^2; Danon and Gallois, 1998). Moreover, GUS staining as well as *AtTRXh5* mRNAs also accumulated after treatment with two stress hormones, abscisic acid (ABA) and salicylic acid (SA; Fig. 6A). Formazan deposits were also observed after ABA and SA treatments, showing superoxide formation upon these two hormonal treatments (Fig. 6A). In contrast, GUS activity was not induced by auxin, cytokinin, or ethylene treatments, and only slightly (less than a 2-fold rate) by jasmonic acid treatments (data not shown). In all cases, the similar induction patterns observed

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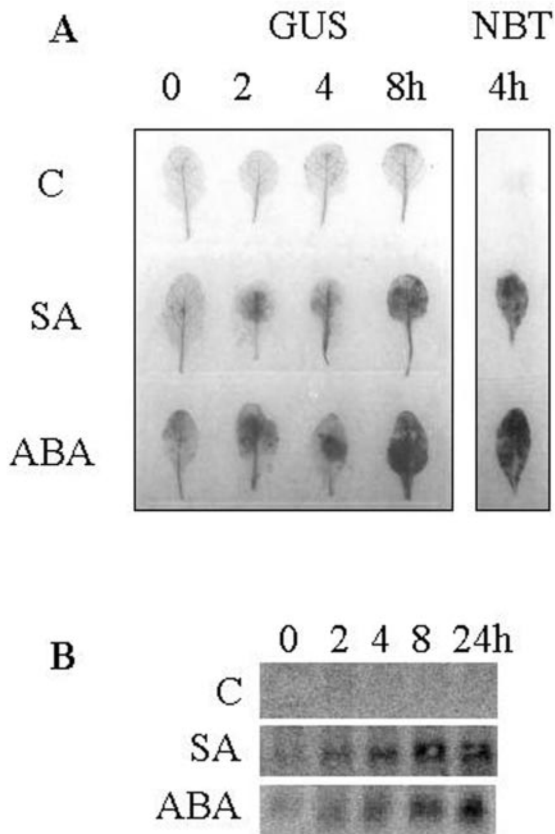

Figure 6. Time course of *AtTRXh5* expression under hormone treatments. A, GUS activity after infiltration of rosette leaves with 100 μM SA, 100 μM ABA, or water (C); 4 h after infiltration, the leaf samples were also stained for superoxide accumulation with the NBT staining. B, RNA gel blot from total RNAs extracted 0, 2, 4, 8, and 24 h after hormone treatment was hybridized with a 3′-specific probe of *AtTRXh5*.

by northern blot (Figs. 5C and 6B) indicate that the main *cis*-acting elements that are involved in the response of *AtTRXh5* to the stress conditions tested are present in the 1.5-kb-long 5′-regulating region fused to the *GUS* reporter gene. In addition, these results indicate that the expression of *AtTRXh5* in response to these treatments is regulated at the transcriptional level.

DISCUSSION

AtTRXh5 Regulation during Senescence, Not during Cell Cycle

In the present study, we examined the regulation of the *AtTRXh5* gene to get some clues about its function. In animals, a high level of thioredoxin is frequently associated with active cell division. Thioredoxins are also known to play an inhibitory role in programmed cell death pathways (Saitoh et al., 1998). In particular, increased levels of thioredoxin are observed in many human primary cancers compared with normal tissues (Powis et al., 2000). In planta, *AtTRXh5* is not expressed in young tissues (flower buds, young leaves, and shoot and root meristems), excluding a role in cell proliferation. In a recent study on cell cycle-regulated genes in Arabidopsis, Menges et al. (2002) found that the expression of three thioredoxin *h* genes fluctuated during the cell cycle: *AtTRXh1* and *AtTRXh2* showed a peak of expression in G2 phase, whereas *AtTRXh4* showed a peak of expression in M phase. These data suggest that these three Arabidopsis thioredoxin *h*, as well as the previously reported tobacco (*Nicotiana tabacum* cv Xanthi) growth-related thioredoxin *h*1 (Brugidou et al., 1993; Marty et al., 1993), could be involved in a redox control of cell proliferation (Reichheld et al., 1999).

In contrast and in agreement with our data, *AtTRXh5* expression, included in this transcriptomic analysis, shows no fluctuation during the cell cycle, suggesting that the gene is not involved in controlling cell division. Rather than being expressed in proliferating cells, we found that the expression of *AtTRXh5* is associated with senescent tissues such as old leaves, abscission of floral organs, and root cap. Senescence is the terminal stage of leaf development and is considered as a type of programmed cell death (Yen and Yang, 1998; Simeonova et al., 2000). This switch from anabolism to catabolism is characterized by severe morphological, physiological, biochemical, and molecular changes. This highly coordinated process includes not only down-regulation of genes, but also up-regulation of gene expression. The gene coding for the Cys protease SAG12 is a well-known marker of leaf senescence (Gan and Amasino, 1995). The fact that we found that it is coregulated with *AtTRXh5* in senescent leaves supports our hypothesis that this latter gene is senescence associated. This is not a general feature of *AtTRXh* genes because the expression of other *AtTRXh* genes is not affected by aging (data not shown). A number of defense-related genes like *AtPR1* are also induced during senescence (Robatzek and Somssich, 2002).

AtTRXh5 Induction during Pathogen Attack Is Mediated by a W-Box cis Element

AtTRXh5 is induced upon pathogen attack and its expression is associated with hypersensitive reaction (HR) during an incompatible interaction. However, *AtTRXh5* induction is not specific to an avirulent strain, as it is also observed after challenge with a virulent pathogen at later time points, corresponding to the appearance of disease symptoms. Nevertheless, the intensity is stronger and the timing of induction is much faster in response to avirulent strains, in agreement with other data coming from a recent large-scale transcriptomic analysis of responses during compatible and incompatible interactions of Arabidopsis plants with *P. syringae* (Tao et al., 2003). In agreement with our data, *AtTRXh5* is highly induced in responses to host (*Erysiphe cichoracearum*) and non-

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host (*Blumeria graminis*) pathogens that do not induce HR in plant (L. Zimmerli, personal communication). We found that treatments of cell suspensions or plants with the flagellin elicitor flg22 are able to induce the expression of *AtTRXh5* in a very similar manner. Recent works strongly indicate that FLS2, a leucine-rich repeat (LRR) receptor-like kinase, is involved in flagellin recognition and signaling in Arabidopsis (Gomez-Gomez and Boller, 2000; Bauer et al., 2001). Although at present there is no direct proof for the physical binding of flg22 to FLS2 (Gomez-Gomez and Boller, 2002), this signaling pathway may induce defense genes in response to elicitation by flg22. Whether *AtTRXh5* is a component of this signaling cascade remains an open question.

WRKY proteins are plant-specific transcription factors implicated in the response to bacterial elicitors and encoded by a multigene family comprising over 75 members (Eulgem et al., 2000). WRKY cognate binding sites are the W-box elements (TGACC/T). Clustered occurrences of W-boxes are common among promoters of WRKY-regulated genes (Maleck et al., 2000; Mahalingam et al., 2003). The presence of seven W-boxes in the 1-kb proximal region of the *AtTRXh5* promoter is in favor of a regulation by WRKY factor. The fact that we found an elicitor-induced binding activity on one copy of the W-box supports this hypothesis and suggests that such an interaction may direct the induction of the gene under elicitor treatment. It remains to be demonstrated whether this W-box is sufficient to direct the induction of the gene or if other *cis*-elements are also required. Mutational experiment performed on the W-boxes of the WRKY6-regulated gene *SIRK* shows that at least two copies of the W-box are necessary to perform the WRKY6-dependent gene activation (Robatzek and Somssich, 2002). Supporting this point, the *AtTRXh3* promoter that harbors a single W-box is not induced in response to elicitor treatment, suggesting that a single copy would not be sufficient.

WRKY6 May Direct *AtTRXh5* Expression

We also showed that *AtTRXh5* is overexpressed in the *35S::WRKY6* line. This suggests that WRKY6 acts as a positive regulator on *AtTRXh5* expression. The overexpression of *AtTRXh5* is correlated with the induction of the expression of the *AtPR1* gene that was previously shown to be induced in *35S::WRKY6* line (Robatzek and Somssich, 2001). However, the expression of the closely related *AtTRXh3* gene is not increased in this line, and neither is the senescence-specific gene *SAG12*, suggesting that this induction is not a general induction due to ectopic overexpression of *WRKY6* gene. Robatzek and Somssich (2001) have previously documented the expression profile of *AtWRKY6*. This expression is closely correlated with the expression profile of *AtTRXh5* (induction by wounding, senescence, pathogen attack, flg22, etc.). This coregulation of the two genes makes possible the fact that WRKY6 may direct *AtTRXh5* expression. Surprisingly, *AtTRXh5* has not been found by Robatzek and Somssich (2001) as differentially expressed gene in the transcript profiling performed on the *wrky6* knockout mutant and *35S::WRKY6* plants. The involvement of WRKY6 in *AtTRXh5* transcription will have to be studied more carefully by measuring the expression of *AtTRXh5* in the *wrky6* knockout mutant.

AtTRXh5 Specific Response to Oxidative Stress

Oxidative burst is a common and early response to senescence, pathogen attack, and elicitor treatment. Our data indicate that *AtTRXh5* is induced under conditions that trigger oxidative stress. An increasing body of evidence indicates that ABA action is associated with oxidative stress in plant cells. First, treatment with high concentrations of ABA results in cellular oxidative damage (Bueno et al., 1998; Jiang and Zhang, 2001). Second, ABA also induces the expression of antioxidant genes and enhances the capacity of antioxidant defense systems (Bueno et al., 1998; Guan and Scandalios, 1998a, 1998b; Bellaire et al., 2000; Jiang and Zhang, 2001, 2002a, 2002b). Third, ROS play an important intermediary role in the ABA signal transduction pathway leading to the induction of antioxidant defense systems (Guan et al., 2000; Jiang and Zhang, 2002a). Finally, ABA directly causes increased generation of ROS such as $O_2^{\cdot -}$ and H_2O_2 that mediates the activation of calcium channels, probably by a mechanism involving the activation of a plasma membrane-bound NADPH oxidase (Pei et al., 2000; Murata et al., 2001; Zhang et al., 2001; Jiang and Zhang, 2003). Similarly, the activating effects of SA may also be mediated by oxidative species via the activation of the as-1 promoter element (Garreton et al., 2002). Interestingly, the presence of two copies of this element in the 1-kb proximal region of the promoter of *AtTRXh5* suggests that the activation of *AtTRXh5* by SA could be mediated by oxidative species and the activation of the as-1 element. It is also possible that the UV-C-induced activation of *AtTRXh5* results from the activation of the same signaling pathway because SA accumulates after exposure of tobacco to UV-C light (Yalpani et al., 1994; Nawrath et al., 2002). The activity of these as-1 elements under SA and UV-C light treatments is to be tested.

AtTRXh5 was also induced by heavy metals. In *Chlamydomonas reinhardtii*, Lemaire et al. (1999) have reported that the expression of the chloroplastic thioredoxin *m* and one cytosolic thioredoxin *h* is up-regulated by the heavy metals Cd and Hg. Because treatments with oxidants did not trigger any accumulation of *TRX* messengers, Lemaire et al. (1999) concluded that the induction of *TRX* expression by heavy metals was a direct response to the cations as

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suggested by the presence of *cis*-acting elements related to cadmium induction in the *TRX h* promoter. In contrast, in our case, the activation of the Arabidopsis *AtTRXh5* gene by oxidants such as menadione as well as the absence of *cis*-acting elements related to heavy metal induction in the *AtTRXh5* promoter are in favor of an activation of expression mediated by ROS. Supporting this point, the induction of *AtTRXh5* expression was also associated with accumulation of superoxides, as shown by NBT coloration. Moreover, *AtTRXh5*::GUS also accumulates under stress conditions where no cell death occurred, suggesting that the expression of the gene may not be directly associated with cell death (i.e. senescence and HR). In contrast to *AtTRXh5*, none of the other *AtTRXh* genes were found to be induced under oxidative stress conditions (data not shown). This suggests that among the thioredoxin *h* family, AtTRXh5 might be specifically involved in resistance against oxidative stress. In agreement with a specific response to ROS, *AtTRXh5* was found to be induced in knockout Arabidopsis plants mutated in the cytosolic H_2O_2-scavenging enzyme ascorbate peroxidase APX1 (Pnueli et al., 2003). The authors found that the disruption of the *APX1* gene result in an internal oxidative stress to plants, associated with higher level of ROS and overexpression of a number of genes previously shown to be associated with oxidative stresses (Pnueli et al., 2003). *AtTRXh5* was also recently found to be induced in response to infection by various viruses (Whitham et al., 2003). Viral infection might trigger a rapid induction of ROS (Allan et al., 2001). Again, none of the four other *AtTRXh* genes included in this study (*AtTRXh1-4*) was affected by those treatments, reinforcing the hypothesis of a specific involvement of *AtTRXh5* in oxidative stress response among the thioredoxin *h* family.

The recent discovery that the localization and the activity of NPR1, a key regulator of plant systemic acquired resistance, are influenced by the formation/reduction of intermolecular disulfide bonds (Mou et al., 2003), strongly suggests that disulfide reductases like thioredoxins might regulate the activity of such a transcription factor involved in defense responses in plants. The more recent discovery that, under SA treatment, the interaction between NPR1 and the basic domain/Leu zipper transcription factor TGA1 is correlated with a reduction of TGA1 Cys residues, and that NPR1 can only stimulate the DNA-binding activity of the reduced form of TGA1 (Despres et al., 2003), reinforces the hypothesis that thioredoxins might participate in the defense of plants against pathogens, not only by an antioxidant effect, e.g. as a reductant for peroxiredoxin (Dietz, 2003), but also by intervening in the fine regulation of specific transcription factors.

Induction of *AtTRXh5* under stress conditions is not sufficient per se to prove a potential role of corresponding protein in oxidative stress response. A T-DNA insertion mutant that inactivates this gene was isolated, and these plants do not show a particular visible phenotype under standard growth conditions or under stress conditions that triggered induction of the gene. Compensation by other members of the thioredoxin family, which we cannot definitely exclude, or other disulfide reductase enzymes, may account for this apparent lack of phenotype. To study this possible redundancy, analysis of multiple thioredoxin mutants and thioredoxin/glutaredoxin mutants is under way.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seedlings and plants from Arabidopsis ecotype Col-0 were used for the experiments. For in vitro seedlings, seeds were surface sterilized and plated on 0.5× Murashige and Skoog medium including Gamborg B5 vitamins (M0231; Duchefa, Haarlem, The Netherlands), 1% (w/v) Suc, and 0.8% (w/v) agar. For plants, seeds were sown in pots containing a mixture of soil and vermiculite (3:1, v/v), and were irrigated with water. Plants and seedlings were grown at 22°C under continuous light (4,500–6,000 lux). The promoter reporter Pr*AtTRXh5::GUS* line and the *cauliflower mosaic virus* 35S::*WRKY6*-overexpressing line have been previously described (Reichheld et al., 2002; Robatzek and Somssich, 2002).

Abiotic Stress Treatments

For the different abiotic stress treatments, rosette leaves were infiltrated with a syringe with 50 μM menadione, 100 μM $CuSO_4$, 100 μM $FeCl_2$, 100 μM SA, 100 μM ABA. UV-C irradiation was performed using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) fitted with 254-nm UV-C light bulbs. Three-week-old in vitro seedlings were irradiated in open petri dishes (9-cm diameter; Corning, Corning, NY). The UV-C energy delivered in each experiment was measured by a UV-C sensor fitted inside the Stratalinker irradiation chamber.

Pathogen Inoculations and Elicitor Treatments

Green rosette leaves of 6-week-old *PrAtTRXh5::GUS* transgenic Arabidopsis plants were inoculated with a syringe, as previously described (Pontier et al., 1994). Virulent (DC3000) *Pseudomonas syringae* pv. *tomato* strain cultures, containing or not one of the two avirulence genes (*avrRPM1* and *avrRPT2*), were inoculated at a concentration of 1.10^6 to 1.10^7 cfu mL^{-1}. Elicitor treatments were performed by adding 1 μM flg22 peptide to 3-d-old cell suspensions. Six-week-old green rosette leaves were infiltrated as described above with a 1-μM flg22 solution.

Histochemical GUS Staining and Fluorometric GUS Assays

GUS histochemical staining was performed according to Lagarde et al. (1996). Cross-sections of GUS-stained material were prepared using a microtome (LKB, Bromma, Sweden) from tissues embedded in hydroxyethyl methacrylate (Technovit 7100; Heraus-Kulter, Wehrheim, Germany) and were counterstained in purple with periodic acid Schiff reagents. Fluorometric GUS assays were carried out according to the procedure of Hull and Devic (1995) by measuring the kinetics of appearance of 4-methylumbelliferone, produced by cleavage of methylumbelliferyl-β-D-glucuronide, and the fluorescence was read with a Fluoroskan (Fluoroskan II; Labsystems, Chicago). GUS activity was expressed as picomoles of 4-methylumbelliferone produced per minute per milligram of proteins. Protein concentration was determined by using a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as standard.

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Histochemical Detection of Superoxide in Arabidopsis Leaves

For histochemical detection of superoxide, leaves were detached 4 h after infiltration and were vacuum infiltrated with 6 mM NBT in NaN_3 1 mM. When the pale yellow NBT reacts with superoxide, a dark blue insoluble formazan compound is produced within 20 min (Flohe and Otting, 1984; Beyer and Fridovich, 1987). Chlorophyll was removed from the leaves before imaging by boiling them in 95% (v/v) ethanol for 10 min.

Nuclear Extracts and EMSAs

Arabidopsis cell suspensions were harvested by centrifugation at 200*g* for 10 min and frozen in liquid nitrogen. Pelleted cells were ground in liquid nitrogen and were resuspended in 4 mL g^{-1} of buffer A containing 25 mM Tris-HCl, pH 6.5, 0.45 M Suc, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% (v/v) Triton X-100. Nuclear extracts were prepared as described by Shen and Gigot (1997) and were stored in aliquots at −80°C.

EMSAs were performed with these nuclear extracts, and the double-stranded oligonucleotides were end-labeled by filling in their 5′ overhangs with α-^{32}P dCTP and the Klenow fragment (Invitrogen, Carlsbad, CA). The probes were purified on nondenaturing 10% (w/v) polyacrylamide gels, and 20,000 cpm of the probes (0.1–0.5 ng) were added to 6 μg of nuclear proteins in a final volume of 30 μL of binding buffer (25 mM HEPES-KOH, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% [w/v] glycerol, 5 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride) in the presence of 0.05% (w/v) Nonidet P-40 and 1 μg of poly (dI-dC)-poly (dI-dC) (Pharmacia, Peapack, NJ) as a nonspecific competitor. For competition experiments or immunoassays, unlabeled double-stranded oligonucleotide was added to the reaction mix 5 min before adding the DNA probe. After 20 min on ice, the samples were analyzed on nondenaturing 6% (w/v) polyacrylamide gels in 0.5× TBE (1× TBE is 90 mM Tris-HCl, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.3) at 8 V cm^{-1} at 4°C. After electrophoresis, the gels were dried and autoradiographed.

RNA-Blot Analyses and Semiquantitative RT-PCR

Total RNA was extracted from frozen plant organs or cell suspension pellets using the Trizol reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer's protocol. For northern blots, hybridizations were carried out using total RNA (10 μg) and randomly labeled 3′-specific probes as previously described (Reichheld et al., 2002). For semiquantitative RT-PCR, 5 μg of DNase I-treated total RNA was used for first strand synthesis of cDNA by using oligo(dT) primer reverse transcription using the Moloney Murine Leucemy Virus Reverse Transcriptase as described by the manufacturer's protocol (First Strand RT-PCR kit, ProSTAR; Stratagene). Radioactive PCR (15 cycles) and nonradioactive PCR (25 cycles) were performed as described (Reichheld et al., 2002). The following primers were used for RT-PCR experiments: *AtTRXh3* forward primer, 5′-CCGAGAAGCTCAAAGCCGCC-3′, and reverse primer, 5′-CTTTCTTCTCAAGTTCGTCG-3′; *AtTRXh5* forward primer, 5′-AGTGATTGCTTGCCATACCC-3′, and reverse primer, 5′-GACCACCATGCTTCATCAGC-3′; *AtPR1* forward primer, 5′-TCGTCTTTGTAGCTCTTGTAGGTG-3′, and reverse primer, 5′-TAGATTCTCGTAATCTCAGCTCT-3′; *AtSAG12* forward primer, 5′-GTCAGAACAACAGCTTGTTGATTGC-3′, and reverse primer, 5′-GACATCAATCCCACACAAACATACAC-3′; *AtACT2* forward primer, 5′-GTTAGCAACTGGGATGATATGG-3′, and reverse primer, 5′-AGCACCAATCGTGATGACTTGCCC-3′; *AtWRKY6* forward primer, 5′-GATGATAAGCGATGGATGTCAATGG-3′, and reverse primer, 5′-TGGTGGTTCGTACCATTGATCATAG-3′.

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