Overexpression of a Gene Encoding Hydrogen Peroxide-Generating Oxalate Oxidase Evokes Defense Responses in Sunflower[1]

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Oxalate oxidase (OXO) converts oxalic acid (OA) and O_2 to CO_2 and hydrogen peroxide (H_2O_2), and acts as a source of H_2O_2 in certain plant-pathogen interactions. To determine if the H_2O_2 produced by OXO can function as a messenger for activation of defense genes and if OXO can confer resistance against an OA-producing pathogen, we analyzed transgenic sunflower (*Helianthus annuus* cv SMF3) plants constitutively expressing a wheat (*Triticum aestivum*) *OXO* gene. The transgenic leaf tissues could degrade exogenous OA and generate H_2O_2. Hypersensitive response-like lesion mimicry was observed in the transgenic leaves expressing a high level of OXO, and lesion development was closely associated with elevated levels of H_2O_2, salicylic acid, and defense gene expression. Activation of defense genes was also observed in the transgenic leaves that had a low level of *OXO* expression and had no visible lesions, indicating that defense gene activation may not be dependent on hypersensitive response-like cell death. To further understand the pathways that were associated with defense activation, we used GeneCalling, an RNA-profiling technology, to analyze the alteration of gene expression in the transgenic plants. Among the differentially expressed genes, full-length cDNAs encoding homologs of a PR5, a sunflower carbohydrate oxidase, and a defensin were isolated. RNA-blot analysis confirmed that expression of these three genes was significantly induced in the *OXO* transgenic sunflower leaves. Furthermore, treatment of untransformed sunflower leaves with jasmonic acid, salicylic acid, or H_2O_2 increased the steady-state levels of these mRNAs. Notably, the transgenic sunflower plants exhibited enhanced resistance against the OA-generating fungus *Sclerotinia sclerotiorum*.

Oxidative burst, including hydrogen peroxide (H_2O_2) production, is one of the early events that are associated with a hypersensitive response (HR) in many plant-pathogen interactions (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997). Several defensive roles for H_2O_2 have been proposed (Lamb and Dixon, 1997). For example, H_2O_2 in plant tissues may reach levels that are directly toxic to microbes (Peng and Kúc, 1992). H_2O_2 may contribute to the structural reinforcement of plant cell walls (Bolwell et al., 1995) and trigger lipid peroxide and salicylic acid (SA) synthesis (Leôn et al., 1995). Moreover, H_2O_2 appears to have roles in signal transduction cascades that coordinate various defense responses, such as induction of HR and synthesis of pathogenesis-related (PR) proteins and phytoalexins (Greenberg et al., 1994; Hammond-Kosack and Jones, 1996). These important roles of H_2O_2 have attracted molecular pathologists' interest in manipulating the H_2O_2 level by overexpressing an H_2O_2-generating enzyme, such as Glc oxidase (Wu et al., 1995; Kazan et al., 1998), to combat diseases in plants.

Oxalate oxidase (OXO; EC 1.2.3.4) is one of the enzymes that can produce H_2O_2 in plants. It releases CO_2 and H_2O_2 from O_2 and oxalic acid (OA) that is generally present at low levels in plants. This enzyme was first isolated and characterized from barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*; Lane et al., 1993; Kotsira and Clonis, 1997). Wheat OXO, also known as germin, is the best characterized member of the cupin family (Dunwell et al., 2000; Lane, 2000). Wheat germin is an apoplastic, multimeric, and glycosylated enzyme with extreme resistance to heat and chemical degradation by protease or H_2O_2 (Lane, 2000). Germin-like proteins (GLPs) have been isolated from many higher plants, including both dicotyledonous and monocotyledonous species. These reported GLPs have high sequence identity to the wheat germin (Dunwell et al., 2000). However, only wheat, barley, maize (*Zea mays*), oat (*Avena sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), and pine (*Pinus sylvestris*) germins have been shown to have OXO activity (Dunwell et al., 2000; Lane, 2000). Recently, it was reported that barley germin and, by inference, the related GLPs, represent a new group of extracellular manganese-containing enzymes with both OXO and superoxide dismutase activities (Woo et al.,

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2000). Superoxide dismutase activity can also lead to H_2O_2 production. Various studies on the highly conserved family of GLPs have revealed that GLPs may carry important roles in plant development and responsiveness to abiotic and biotic stresses (Dunwell et al., 2000). For example, germins are highly expressed during seed germination of wheat and barley and in the response of mature leaves to pathogen attack (Zhang et al., 1995; Berna and Bernier, 1999). It has been shown that a specific pathogen-responsive *OXO* transcript is found in the wall of barley mesophyll cells 6 h after inoculation with powdery mildew (*Blumeria graminis*; Zhou et al., 1998). The wheat OXO enzyme expressed in soybean (*Glycine max*; Donaldson et al., 2001) and hybrid poplar (*Populus euramericana*; Liang et al., 2001) is able to break down OA and conferred resistance against OA-generating pathogens. Of further interest is that germin-overexpressing maize exhibited enhanced insect resistance (Ramputh et al., 2002). Although several hypotheses have already been put forward for the potential roles of germin-like OXO in pathogen resistance (Lane, 1994; Lane, 2002), the exact biological significance of the H_2O_2 production by OXO in plants remains unknown.

One of the most important OA-generating pathogens is *Sclerotinia sclerotiorum*. This fungal pathogen is worldwide in distribution and is pathogenic to more than 400 plant species at all developmental stages (Purdy, 1979). It causes significant yield losses of crops including sunflower (*Helianthus annuus* cv SMF3), oilseed rape (*Brassica napus*), and soybean (Purdy, 1979). *S. sclerotiorum* synthesizes and secretes large amounts of OA into infected host tissues. This acid, acting as a mobile toxin, causes a wilting syndrome in infected sunflower (Noyes and Hancock, 1981). OA not only acidifies the plant tissue but also chelates Ca^{2+} from the cell wall, rendering the stressed tissue susceptible to a battery of fungal degradative enzymes (Lumsden, 1979). The acid also inhibits the activity of an *0*-diphenol oxidase (Ferrar and Walker, 1993), suppresses the oxidative burst (Cessna et al., 2000), and is a pathogenicity factor in host cells (Marciano et al., 1983; Godoy et al., 1990; Cessna et al., 2000). Few genetic sources of resistance to the pathogen are available to the breeder. Presently, control of this disease by fungicide application to the plant is expensive and not always effective. The significance of OXO is potentially 2-fold for combating this disease: degrading the *S. sclerotiorum* toxin OA and producing the defense-inducing molecule H_2O_2. Sunflower has very low germin-like OXO activity and is a host of the OA-generating *S. sclerotiorum* pathogen (Purdy, 1979); therefore, *OXO* transgenic sunflower is suitable for studying the biological significance of OXO enzyme in plant-pathogen interactions.

In this study, we report that the leaves of *OXO* transgenic sunflower showed elevated levels of OXO activity, H_2O_2, SA, and defense gene expression in the absence of pathogen inoculation. These transgenics exhibited enhanced resistance to *S. sclerotiorum* infection. Our results show that the H_2O_2-generating OXO can evoke defense responses and confer disease resistance in sunflower.

RESULTS

Overexpression of Wheat *gf*-2.8 *OXO* Transgene in Sunflower

Transgenic sunflower plants expressing the wheat *gf*-2.8 *OXO* gene were generated to control *S. sclerotiorum* disease by metabolizing the pathogenicity factor OA to CO_2 and H_2O_2. To obtain transgenic lines that have a range of levels of OXO, we transformed the wheat *gf*-2.8 gene into sunflower under the control of two different constitutive promoters, SCP1 (Lu et al., 2000; Bowen et al., 2003) and SuperMas (Ni et al., 1995). The SCP1 promoter is generally about twice as strong as SuperMAS in various sunflower tissues (data not shown). In line 610255, which showed the highest level of OXO, the SCP1 promoter drove *OXO*, whereas in lines 539145 and 539149, the SuperMas promoter controlled expression of this gene (Fig. 1A). There was very low OXO activity in

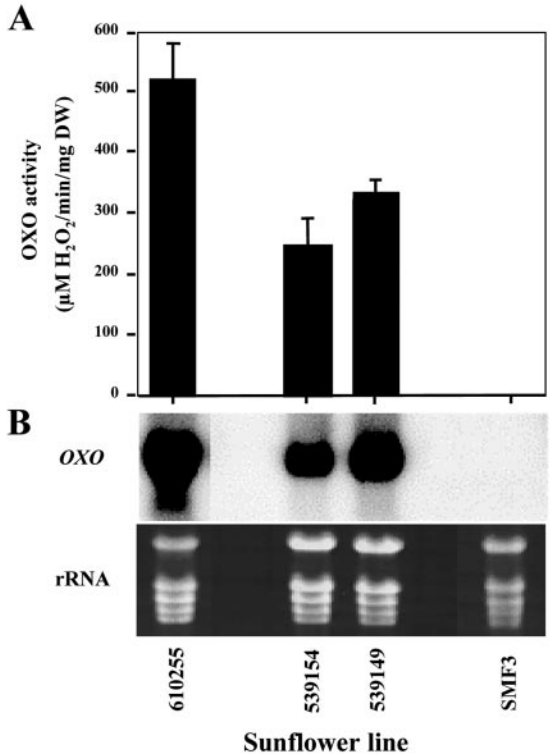

Figure 1. Expression of wheat *OXO* transgene in different sunflower lines. OXO activity (A) and steady-state level of *OXO* mRNA (B) in leaf tissues from 6-week-old transgenic lines (610255, 539154, and 539149) and untransformed control (SMF3). DW, Dry weight; rRNA, ribosomal RNAs that were stained with ethidium bromide in agrose gel. Error bars = SD (n = 6).

the untransformed SMF3 leaves, and the OXO activity in the transgenic lines correlated with mRNA levels of the *OXO* transgene (Fig. 1B). These three transgenic lines have one to three copies of the *OXO* transgene based on Southern-blot analysis. The OXO activity was associated with the oligomeric form of OXO (data not shown), which also was observed in wheat and transgenic tobacco (*Nicotiana tabacum*; Lane et al., 1993; Berna and Bernier, 1997). These results indicate that OXO proteins were processed and assembled into an active form capable of generating H_2O_2 in the *OXO* transgenic sunflower tissues. The transgenic leaves could effectively degrade the exogenous OA; therefore, OXO might protect sunflower leaf tissues from OA's toxic effect.

High Levels of OXO Activity Induce HR-Like Lesions in Sunflower Leaves

All of the *OXO* transgenic lines were phenotypically similar to the untransformed sunflower plants during the growth and development. However, lesion mimicry was observed in the mature leaves of uninoculated 6-week-old *OXO* transgenic plants of line 610255 (Fig. 2A). Four weeks after planting, these lesions started to develop from tiny yellow spots into large areas of chlorosis and necrosis and to appear progressively from lower leaves to the upper leaves and from leaf base to leaf tip. Lesions were not observed in stem, floral parts, or other tissues. Transgenic lines such as 610255 with high levels of OXO developed severe lesion symptoms, whereas lines 539149 and 539154 with moderate level of OXO expression had no visible lesions on the leaves before the initiation of leaf senescence. A trypan blue staining assay indicated that there were no microscopic necrotic lesions in the leaves of lines 539149 and 539154.

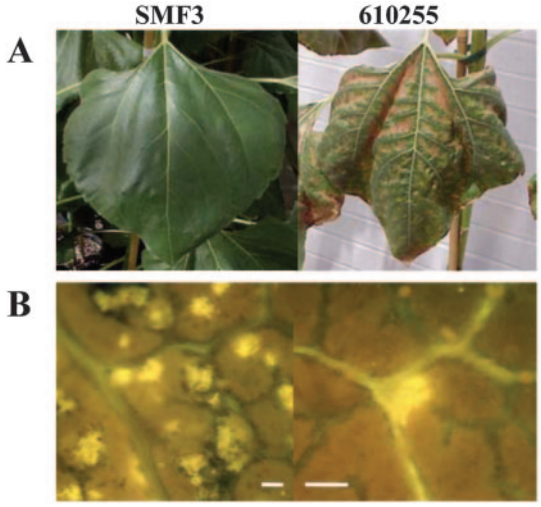

Figure 2. Phenotype of HR-like lesions and accumulation of autofluorescent compounds in *OXO* transgenic leaves. A, Phenotype of the lesions on *OXO* transgenic leaves (610255) was compared with that of untransformed control leaves (SMF3) at the 6-week-old stage. B, UV-stimulated autoflurescence of *OXO* transgenic leaves was detected in the early development of HR-like lesions in *OXO* leaves from 6-week-old plants. White scale bar = 0.05 mm.

The accumulation of autofluorescent materials in and around lesions is a histochemical marker of HR-like lesions (Hammond-Kosack and Jones, 1996). Consistent with an HR-like phenotype, we observed bright-yellow fluorescent substances in the leaves of 6-week-old *OXO* transgenic plants (Fig. 2B) but not in the leaves of control plants (data not shown). The appearance of autofluorescence was observed around lesions and in the cell walls of collapsed cells within lesions in the *OXO* transgenic leaves (Fig. 2B). The data suggest that a high level of OXO activity can induce an HR-like response in plant tissue in the absence of pathogen inoculation.

HR-Like Lesions Are Associated with Elevated Levels of H_2O_2 and SA

To determine whether the lesion development in *OXO* transgenic lines is correlated with the increases of OXO activity and the levels of H_2O_2 and SA, we carried out time course experiments. The leaf samples were taken from the leaves that were located in the middle of the stem and not necrotic at 2-, 4-, and 6-week-old stages and showed visible lesions at the 8-week-old stage. As shown in Figure 3A, OXO activity increased significantly with plant development. The OXO activity was much higher in line 610255 than in lines 539149 and 539154. The OXO activity was more than 20-fold higher in the transgenic leaves than in untransformed leaves of 8-week-old plants. Leaf discs of 6-week-old line 610255 plants were stained for detection of H_2O_2 accumulation as described in "Materials and Methods." Compared with discs from untransformed SMF3 plants, the whole transgenic leaf disc, especially the veins, stained strongly purple in the absence of exogenously supplied OA (Fig. 3C). Progressive increases in total (free plus conjugated) SA were observed in both transgenic and control lines as the plants matured (Fig. 3B). However, total SA levels of 6- and 8-week-old leaves increased more dramatically in the transgenic lines than in the untransformed controls. In leaves of 8-week-old lines 610255, 539149, and 539154 plants, total SA levels were 25, 5, and 3 times, respectively, more than that in similar leaves of the controls (Fig. 3B). Free SA was also significantly increased in the leaves of 6- and 8-week-old *OXO*- plants (data not shown). Notably, the significant increase in the levels of total SA (Fig. 3B) occurred between the 4- and 6-week-old stages, a period in which the HR-like lesions developed in the leaves of line 610255.

To understand if the OXO-generated H_2O_2 stimulated the SA accumulation, we treated untransformed SMF3 leaves of 6-week-old plants by spray-

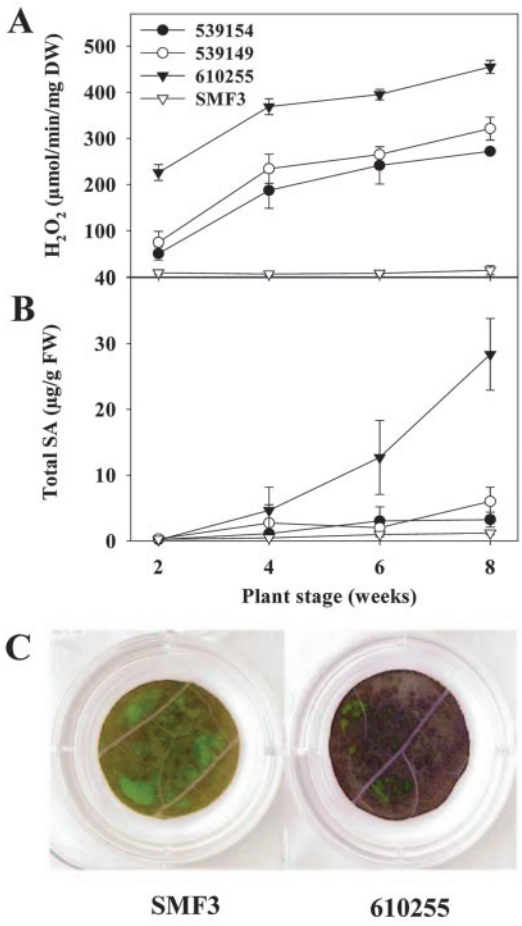

Figure 3. Changes of OXO activity, H_2O_2, and SA during the first 8-week growth period of *OXO* transgenic (610255, 539149, and 539154) and untransformed control (SMF3) plants. A, OXO activity. Values are the means of three samples; error bars = SD of the mean ($n = 3$). B, Endogenous levels of total SA. Values are the means of five samples; error bars = SD of the mean ($n = 5$). C, Accumulation of H_2O_2 in untransformed (SMF3) and transgenic (610255) leaves of 6-week-old plants. The purple color indicates the presence of H_2O_2. FW, Fresh weight; DW, dry weight.

ing a 5 mM H_2O_2 solution. There were more than 2-fold higher levels of SA in the H_2O_2-treated leaves (3.21 ± 1.13 μg SA g fresh weight^{-1}) compared with water-treated leaves (1.14 ± 0.32 μg SA g fresh weight^{-1}) 4 d after treatment. This indicated that H_2O_2 could stimulate SA accumulation in sunflower leaves.

Identification of Three Sunflower Defense-Related Genes by RNA Profiling Analysis

The close association of OXO activity with increased accumulation of H_2O_2 and SA and the appearance of HR-like lesions suggested that OXO might trigger endogenous defense pathways in the absence of pathogen challenge. To elaborate the molecular mechanisms of defense activation triggered by *OXO* expression, we compared transcript profiles in tissues from *OXO* transgenic (line 610255) and untransformed SMF3 control plants using an open architecture mRNA profiling method (Shimkets et al., 1999; Bruce et al., 2000). Three sets of tissues, including stems of 75 d after planting (DAP), leaves of 75 DAP, and mixed leaf and stem tissues of 48 DAP, were used for this study. The modulation of about 13,000 cDNA fragments was measured for each of the sets as indicated in Table I and Figure 4.

Three pair-wise comparisons of *OXO* transgenic and untransformed control plants were performed *in silico* to identify differentially modulated cDNA fragments. Using a threshold of a minimum 2-fold difference and statistical significance of $P < 0.1$, 4.0% and 5.4% differentially expressed bands were identified in leaf and stem samples, respectively, at 75 DAP. About 1.7% of total detected bands were differentially expressed in mixed stem and leaf tissues at 48 DAP (Table I). Relatively fewer differentially expressed genes were identified at the early stage as compared with the late stage. The differentially modulated fragments were used to query the known sunflower sequences and the sequence of the wheat *OXO* transgene by using the length of the fragments (in base pairs) and the 6-bp restriction site nucleotide sequence on either side of each of the fragments. A total of 1,198 fragments were differentially expressed among the three pair-wise comparisons. The sequence identity of the specific fragments among several sequenced clones was confirmed by using the competitive PCR method as described by Shimkets et al. (1999) and Bruce et al. (2000). BLAST analysis revealed that most of the differentially expressed

Table I. *Generation of differentially expressed bands in three sets of sunflower samples*

Sunflower leaf and stem samples were collected from *OXO* transgenic (line 610255) or untransformed SMF3 plants at 48 and 75 d after planting. A total of 1,198 differentially expressed bands were identified among the three pair-wise comparisons.

Samples	Set	Total Assayed	Differentially Expressed	Induced	Suppressed
			Bands		
OXO leaf (75 d) versus SMF3 leaf (75 d)	A	13,606	546 (4.0%)	257	289
OXO stem (75 d) versus SMF3 stem (75 d)	B	12,951	705 (5.4%)	510	195
OXO leaf and stem (48 d) versus SMF3 leaf and stem (48 d)	C	13,549	233 (1.7%)	138	95

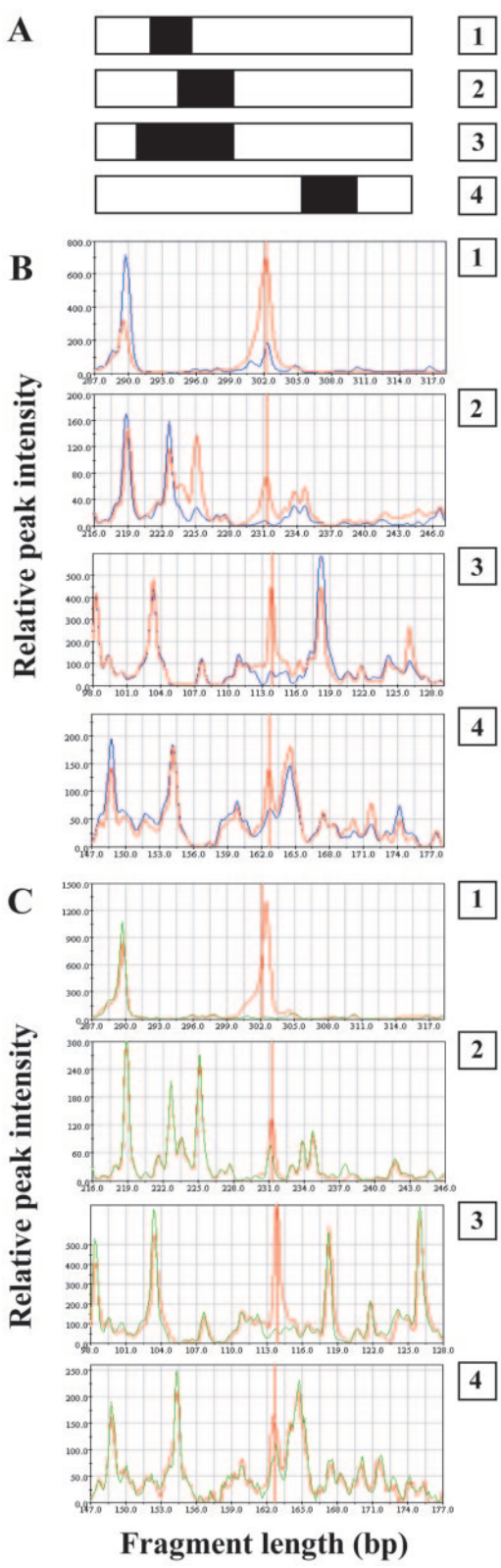

Figure 4. Differential expression profiling of genes in the *OXO* transgenic (line 610255) and untransformed leaves and detection of cDNA-fragments associated with four genes. The leaves were harvested from 6-week-old plants. 1, *OXO* transgene (fragment ID: h0a0-302.2); 2, *PR5-1* (fragment ID: h0a0-231.3); 3, *Defensin* (fragment ID: d0l0-113.9); 4, *SCO* (fragment ID: n0s0-162.7). A, Schematic diagrams of the four full-length genes showing the relative positions of detected fragments (shaded area). The lengths of the boxes are not proportional to the gene lengths. B, Detection of the cDNA fragments of the four differentially modulated genes in *OXO* transgenic (red trace) compared with untransformed (blue trace) leaf tissues. Vertical red lines show the specific fragments of the genes. C, Confirmation of the cDNA fragments associated with the four genes after competitive PCR with (green trace) or without (red trace) an unlabeled oligonucleotide specific to the transgene (for details, see "Materials and Methods").

genes encoded peptides that have homology with transcription factors, protein kinases, and defense proteins. Three of the defensive genes encode homologs of the antimicrobial PR5-1, defensin, and sunflower carbohydrate oxidase (SCO) proteins. In the *OXO* transgenics, they were induced 9.0, 2.1, and 6.9-fold, respectively (Fig. 4).

To further understand the impact of OXO-generated H_2O_2 on defense gene expression, the full-length cDNA clones were isolated (for details, see "Materials and Methods"). One of the full-length cDNAs had an open reading frame encoding a protein of 222 amino acids that we designated as PR5-1. Sequence analysis revealed that PR5-1 has significant sequence similarity with previously reported PR5 proteins from other plant species. For example, sunflower PR5-1 has 77% and 73% identity at the amino acid sequence level with soybean P21 protein (accession no. AF005655; Graham et al., 1992) and grape (*Vitis vinifera*) osmotin-like protein (accession no. Y10992; Loulakakis, 1997), respectively. The sunflower *defensin* cDNA encoded a protein of 108 amino acid residues with a putative signal peptide at the amino-terminal end. The sunflower defensin contained the eight conserved and defensin-specific Cys residues and showed high homology to defensins from other plants (accession nos. U18556 and U18557; Chiang and Hadwiger, 1991; Terras et al., 1993, 1995). However, this *defensin* gene has no significant sequence homology with the sunflower defensin gene isolated by Urdangarín et al. (2000). The full-length cDNA of *SCO* had an open reading frame coding for a protein of 546 amino acid residues. Forty percent of the amino acid residues of this SCO are identical to two recently described sunflower genes that encode carbohydrate oxidases (Stuiver et al., 2000). It has also high homology (more than 35% identity) with berberine bridge enzyme from opium poppy (*Papaver somniferum*) and California poppy (*Eschscholzia californica*; accession nos. U59232 and U59233; Facchini et al., 1996; Hauschild et al., 1998).

Expression of the Defense Genes Is Induced in *OXO* Transgenic Leaves and by *S. sclerotiorum* Infection

To understand the regulation of these defense-related genes by *OXO* expression and *S. sclerotiorum*

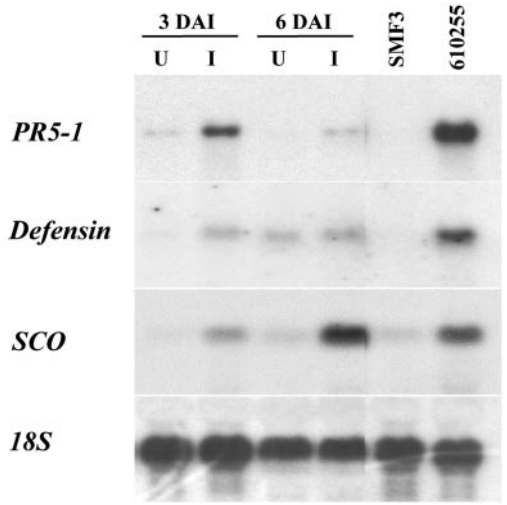

Figure 5. Expression of *PR5-1*, *Defensin*, and *SCO* in *S. sclerotiorum*-infected SMF3 and *OXO* transgenic sunflower (line 610255) leaves of 6-week-old plants. The leaves of SMF3 and line 610255 plants were also used to generate the mRNA profiling data shown in Figure 4. DAI, Days after inoculation; I, infected plants; U, uninfected plants; 18S, 18S ribosomal RNA.

infection, we carried out RNA blot analysis. The steady-state levels of *PR5-1*, *defensin*, and *SCO* transcripts were significantly induced in the leaves of 6-week-old *OXO* transgenic plants (Fig. 5), whereas the expression of these three genes was very low or at undetectable levels in the untransformed SMF3 leaves (Fig. 5). These results confirmed the quantitative expression analysis of the RNA profiling experiment. The relative intensities of the corresponding bands (h0a0-231.3, d0l0-113.9, and n0s0-162.7) for these three genes were much stronger in transgenic than in SMF3 control leaves (Fig. 4).

Gene expression of untransformed sunflower in response to *S. sclerotiorum* infection was also analyzed. Total RNAs were isolated from infected and uninfected sunflower leaves 3 and 6 d post inoculation. The steady-state levels of *PR5-1*, *defensin*, and *SCO* mRNAs were very low in the uninfected leaves (Fig. 5). However, expression of these three genes was significantly induced in *S. sclerotiorum*-infected sunflower leaves (Fig. 5).

To evaluate whether the expression of the *PR5-1*, *defensin*, and *SCO* genes in *OXO* transgenic leaves is correlated with increased OXO activity and SA (Fig. 3), total RNA was isolated from leaf tissue of line 610255 and control plants at 4, 6, and 8 weeks after planting in the greenhouse. As shown in Figure 6A, the transcripts of *PR5-1*, *defensin*, and *SCO* in the leaves of *OXO* transgenic plants were significantly induced. The highest levels of induction were detected at the 8-week-old stage (Fig. 6A). The induced expression of *PR5-1* and *defensin* genes is correlated with the increases of OXO activity and SA level in the *OXO* leaf tissues (Figs. 3,A and B, and 6A).

RNA-blot analysis was also carried out to determine if these defense genes are induced in other events such as lines 539149 and 539154 that do not develop HR-like lesions. As indicated in Figure 6B, these three genes were significantly induced in the leaves of line 610255 and lines 539149 and 539154 at the 6-week-old stage. The induced expression of *PR5-1* and *defensin* genes paralleled the increase of *OXO* mRNA, whereas the regulation of *SCO* expression was more complex.

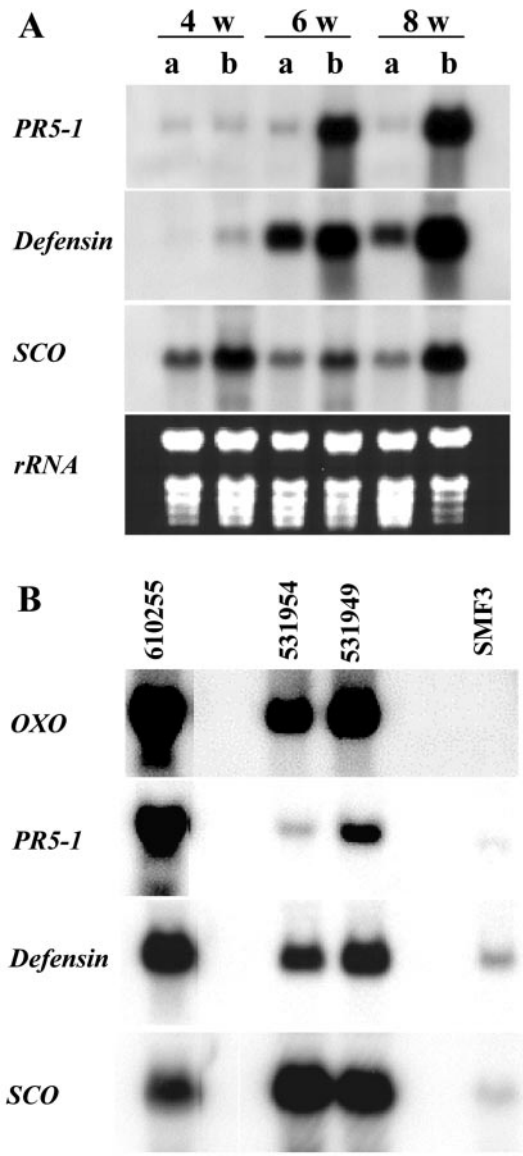

Figure 6. Accumulation of *OXO*, *PR5-1*, *Defensin*, and *SCO* transcripts in *OXO* transgenic sunflower leaves. A, Developmental regulation of the defense gene expression. Lane a, SMF3; lane b, 610255; w, week; rRNA, ribosomal RNAs that were stained with ethidium bromide in agrose gel. B, Expression of *OXO*, *PR5-1*, *defensin*, and *SCO* genes in the leaves of transgenic lines 610255, 539149, and 539154 and in the untransformed SMF3 at the 6-week-old stage.

Effects of Different Chemical Treatments on the Expression of *PR5-1*, *Defensin*, and *SCO* Genes

To further our understanding of the mechanisms of OXO-induced gene expression, we examined the effects of foliar application of 5 mM SA, 45 μM jasmonic acid (JA), or 5 mM H_2O_2 on the mRNA levels of *PR5-1*, *defensin*, and *SCO* genes in 6-week-old untransformed sunflower leaves. As shown in Figure 7, these treatments significantly altered the expression levels of the three genes. The accumulation of *PR5-1* reached maximum at 6 h after application of JA, 12 h after application of H_2O_2, and 24 h after application of SA. *SCO* expression was induced by H_2O_2, SA, or JA at early time points and declined to the control level 24 h after application. This early strong induction may have been caused by the spraying action. Sunflower *defensin* expression was significantly upregulated by both SA and H_2O_2 and slightly stimulated by JA (Fig. 7).

OXO Transgenic Plants Show Increased Resistance to *S. sclerotiorum*

S. sclerotiorum infection assays were conducted to examine if the *OXO* transgenic plants have enhanced resistance to *S. sclerotiorum*. As indicated in Figure 8A, the pathogen-induced lesions in transgenic leaves of lines 610255, 539149, and 539154 were significantly smaller than those in the control leaves. The lesion sizes in the transgenic leaves are inversely related to the endogenous levels of OXO activity (Fig. 3A), SA (Fig. 3B), and defensive proteins (Fig. 6). Petiole and stem tissues of line 610255 plants also had high levels of OXO activity (Lu et al., 2000). Lesion development on the inoculated petioles of transgenic and control plants was similar. Three days after inoculation, *S. sclerotiorum* infection spread into the stem of untransformed SMF3 plants, but the lesions were limited to the petioles of line 610255 plants. Ten days after inoculation, the lesion length in the *OXO* transgenic stems was about 6-fold smaller than that in the untransformed SMF3 controls (Fig. 8B).

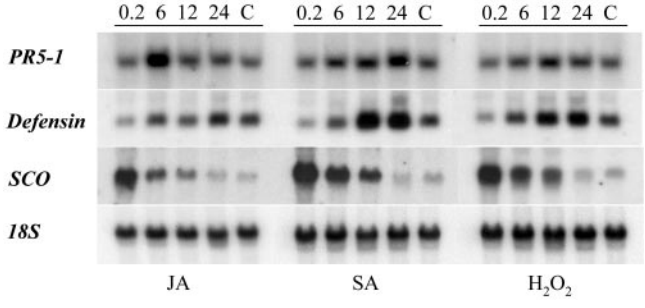

Figure 7. Effects of exogenous JA, SA, and H_2O_2 on the steady-state levels of *PR5-1*, *Defensin*, and *SCO* mRNAs in the leaves of 6-week-old untransformed sunflower plants. The numbers on the top of the figure indicate the time (hours) after spraying the chemicals. C, Untreated control; 18S, 18S ribosomal RNA.

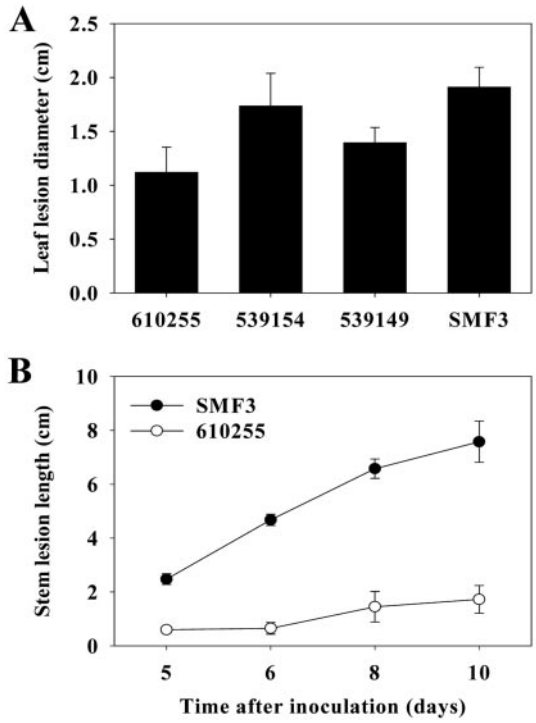

Figure 8. Inhibition of *S. sclerotiorum* infection in *OXO* transgenic sunflower leaf and stem tissues. A, *S. sclerotiorum* leaf rot assay. The length and width of the lesions in leaf segments were measured 24 h after inoculation. Leave lesion diameter represents the average of length and width of the lesion on the leaf segment. Data shown are the average of eight replicates ± SD (n = 8). B, *S. sclerotiorum* stalk rot assay. Plants were inoculated at the 6-week-old stage. Lesion size is presented as the vertical length of lesions on the stem. Data shown are the average of nine replicates ± SD (n = 9). SMF3, Untransformed control; line 610255, *OXO* transgenic line.

Whereas *S. sclerotiorum* spread to the head tissue of the control plants within 2 weeks, the lesions on the transgenic plants were primarily confined to the main stem. These results indicate that *OXO* can confer resistance against *S. sclerotiorum* to transgenic sunflower plants.

DISCUSSION

Wheat and barley germins likely have both OXO and superoxide dismutase activities that lead to production of the defense-inducing molecule H_2O_2 (Lane et al., 1993; Kotsira and Clonis, 1997; Woo et al., 2000). However, it is not clear if the H_2O_2 generated by these activities induces defense responses and plays a role in plant disease resistance. Wild-type sunflower tissues contain very low levels of germin-like OXO activity (Figs. 1A and 3A) and provide us with a suitable system for transgenic studies with the wheat gf-2.8 *OXO* gene. We observed that uninoculated sunflower leaves constitutively expressing this *OXO* gene show increased accumulation of H_2O_2, SA, and defense gene transcripts compared with untransformed controls, and this accumulation was

closely associated with progressive increase of OXO activity during the plant development. Furthermore, the *OXO* transgenic sunflower exhibited enhanced resistance against *S. sclerotiorum* disease.

The *OXO* transgenic sunflower plants can be divided into two groups: One group including line 610255 expressed higher OXO activity and formed lesions on the mature leaves in the absence of pathogen challenge, and the other group including lines 539149 and 539154 had lower OXO activity and did not show visible lesions on the leaves before the leaf senescence. Autofluorescence was observed around lesions and in the cell walls of collapsed cells within the lesions in mature leaves of line 610255 (Fig. 2B). Lesion formation was closely correlated with increased OXO activity, elevated levels of H_2O_2 and SA, and accumulation of defense gene transcripts (Figs. 2, 3, and 6). These characteristics of the HR-like lesions in the *OXO* leaves are similar to those of transgenic tobacco plants expressing Glc oxidase (Kazan et al., 1998), and lesions formed as a result of pathogen-induced HR and disease lesion mimic mutations (Greenberg et al., 1994). Therefore, the HR-like cell death may not be a consequence of direct killing of cells by the OXO-produced H_2O_2 but result from the activation of a programmed cell death pathway as observed in other transgenic plants (Mittler and Rizhsky, 2000). No visible lesions were observed in lines 539149 and 539154, which expressed lower levels of *OXO*, even though the expression of *PR5-1*, *defensin*, and *SCO* genes was associated with those lines (Fig. 6B). Low OXO activity induced a significant induction of *SCO* and *defensin* transcripts (Fig. 6, A and B). Activation of defense genes, but not deterioration of single cells, was observed in the tobacco leaves exposed to low levels of H_2O_2 (Chamnongpol et al., 1998). Therefore, defense gene activation and HR-like cell death may involve separate signaling pathways, or the thresholds of H_2O_2 needed for cell death and defense gene activation are different.

It has been reported that H_2O_2 stimulates SA biosynthesis and both H_2O_2 and SA could induce HR-like cell death in plants (Leôn et al., 1995; Wu et al., 1995; Alvarez, 2000). We observed that exogenous H_2O_2 treatment significantly increased the SA level in the untransformed sunflower leaves. Leaves of F_1 plants from a cross of homozygous line 610255 with untransformed SMF3 plants had about 60% of the OXO activity of leaves of homozygous line 610255 and did not develop HR-like lesions (data not shown). Therefore, it is plausible that relatively high levels of OXO-generated H_2O_2 in the leaves of line 610255, through SA, triggered the HR-like cell death. The elevated level of H_2O_2 might not only come from the OXO reaction but also from its potential superoxide dismutase activity and OXO-induced enzymes such as SCO. It is not clear yet if the HR-like lesions contribute to *S. sclerotiorum* resistance because HR-like lesion formation was closely associated with a higher level of OA-degrading activity, and the transgenic plants expressing a higher level of *OXO* generally exhibited a higher level of *S. sclerotiorum* resistance (Fig. 8A; C. Scelonge and D.L. Bidney, unpublished data).

We have identified a number of differentially regulated genes (Table I) in the *OXO* transgenic tissues. Relatively fewer differentially expressed genes were identified at 48 DAP as compared with 75 DAP (Table I). This may reflect the difference in the responsiveness of sunflower tissues at different stages to elevated levels of H_2O_2 and SA. On the other hand, the less differentially expressed genes at 48 DAP may have been caused by the dilution of transcripts with mixing stem and leaf RNA. A GenBank database search using the gene fragment sequences indicated that the *OXO*-induced genes include transcription factors, protein kinases, and defense genes. To understand the impact of OXO-generated H_2O_2 on the defense response, three up-regulated and antimicrobial protein gene fragments (*PR5-1*, *defensin*, and *SCO*; Fig. 4) were characterized in this study. Expression of these genes was dramatically up-regulated in the leaves of uninfected *OXO* transgenics (Figs. 5 and 6), which showed elevated levels of SA and H_2O_2 (Fig. 3, B and C). Their expression was also significantly induced in the untransformed leaves by treatment with SA, JA, or H_2O_2 (Fig. 7). These observations suggest that H_2O_2 generated by OXO, reacting with unknown endogenous substrates directly or possibly through SA or JA, can trigger the expression of these defense genes. The sunflower defensin has homology to other plant defensins, but its regulation appeared to be different from others. Defensin genes such as Arabidopsis PDF1.2 and radish (*Raphanus sativus*) *defensin* are induced via an SA-independent and JA-dependent pathway (Terras et al., 1995; Thomma et al., 1998). Our RNA gel blot results indicated that SA and H_2O_2 induced a rapid but transient up-regulation of the sunflower *defensin* expression. Only a slight increase was detected 24 h after the JA treatment (Fig. 7). To our knowledge, this is the only plant *defensin* gene that is impacted by SA. We do not know if the JA concentration was impacted by *OXO* expression, but JA treatment significantly up-regulated expression of *PR5-1* and *SCO*. Thus, JA may also be involved in the OXO-activated signal transduction pathways. Sunflower *PR5-1* shares significant sequence similarity with published antifungal osmotin and thaumatin members of the PR5 family (Graham et al., 1992; Loulakakis, 1997). It has been demonstrated that PR5-like proteins have in vitro antifungal activity against a variety of fungi, including *Phytophthora infestans*, *Candida albicans*, *Neurospora crassa*, and *Trichoderma reesei* (Vigers et al., 1991; Liu et al., 1994; Hu and Reddy, 1997). Plant defensins inhibit growth of a broad range of fungi at micromolar concentrations by inhibiting hyphal elongation or slowing down hyphal extension (Broekaert et al.,

1995; Terras et al., 1995). SCO has high homology to an SCO that has antifungal activity and confers resistance to fungal pathogens when expressed in transgenic Arabidopsis (Stuiver et al., 2000). SCO has also high homology to berberine bridge enzyme (Dittrich and Kutchan, 1991), a key enzyme in the benzophenanthridine alkaloids pathway that generates antifungal compounds in poppies (Dittrich and Kutchan, 1991; Blechert et al., 1995). Interestingly, both berberine bridge enzyme and carbohydrate oxidase can generate H_2O_2 (Dittrich and Kutchan, 1991; Stuiver et al., 2000), indicating that OXO-induced SCO may further elevate the H_2O_2 level in *OXO* transgenic leaves. Although the alkaloid biosynthetic pathway may not exist in sunflower plants, the potential antifungal and carbohydrate oxidase activities of SCO suggest its importance in the sunflower defense system.

The activation of defense genes by OXO encouraged us to further evaluate the resistance of transgenic plants against *S. sclerotiorum* because the *OXO* transgenic leaves degraded the pathogenicity factor OA (Figs. 1A and 3A). Our leaf disc and petiole inoculation assays showed that overexpression of the H_2O_2-generating *OXO* transgene significantly limits *S. sclerotiorum* growth and reduces the size of lesions in the transgenic leaf and stem tissues (Fig. 8). Extensive greenhouse and field evaluations have demonstrated that *OXO* transgenic sunflower plants including lines 610255, 539149, and 539154 exhibited enhanced *S. sclerotiorum* resistance and that the resistance is apparently correlated to the expression levels of OXO (C. Scelonge and D.L. Bidney, unpublished data). The efficacy of constitutively expressed OXO in enhancing the *S. sclerotiorum* resistance of sunflower, therefore, may be a consequence of multiple mechanisms. It may result from metabolism of endogenous substrate(s) to form H_2O_2, which in turn triggers increased defense gene expression and enhanced sensitivity to subsequent pathogen attack. Degradation of OA produced by *S. sclerotiorum* may reduce the damage that this pathogen causes in the plant tissues (Lumsden, 1979; Noyes and Hancock, 1981; Godoy et al., 1990), thereby slowing down the advance of this necrotrophic fungus. Metabolism of OA may allow also a more rapid or powerful modification of plant defenses by counteracting the OA-mediated suppression of the oxidative burst (Cessna et al., 2000). Cell wall reinforcement (Schweizer et al., 1999) and direct antifungal activity of H_2O_2 (Lane, 1994; Zhang et al., 1995) may also play a role. It is likely that constitutive expression of OXO may confer enhanced resistance to other OA-generating pathogens, such as *Cristulariella pyramidalis* (Kurian and Stelzig, 1979) and *Septoria musiva* (Liang et al., 2001). Our results suggest that H_2O_2-generating enzymes such as OXO have potential utility for engineering resistance to a spectrum of pathogens in plants.

MATERIALS AND METHODS

Plant and Fungal Materials

The *OXO* transgenic lines were generated by introducing the wheat (*Triticum aestivum*) *OXO* gene into sunflower (*Helianthus annuus* cv SMF3) plants using *Agrobacterium tumefaciens*-mediated transformation (Scelonge et al., 2000). The *OXO* gene was isolated from a wheat line (Pioneer 2548), which encodes a protein identical to the wheat germin *gf-2.8 OXO* (Lane et al., 1991). The constitutive promoters used to express the *OXO* gene were SCP1 (Lu et al., 2000; Bowen et al., 2003) for line 610255 and SuperMas (Ni et al., 1995) for lines 539149 and 539154. The transgenic sunflower plants were selfed, and T_4 homozygous seeds were used in this study. Sunflower seeds were planted weekly and grown in the greenhouse and/or a growth chamber with a controlled environment (22°C, 80% relative humidity, and 16-h photoperiod). Leaf samples were collected at selected times for biochemical and molecular analyses. The sunflower pathogen, *Sclerotinia sclerotiorum* (isolate 255 M^7), was maintained on potato dextrose agar (PDA) plates at 22°C in the dark.

S. sclerotiorum Infection Assays

For *S. sclerotiorum* infection experiments, infected carrot (*Daucus carota*) tissue was prepared by placing the carrot plugs (5 mm thick and 6–8-mm diameter) in front of the advancing *S. sclerotiorum* mycelium on PDA and incubated at 22°C in the dark for 20 to 24 h. All transgenic and untransformed sunflower plants were planted in 10- to 15-cm pots and grown in the greenhouse. Stalk rot trials were initiated by inoculating three petioles per plant (6 weeks old) with an *S. sclerotiorum*-infested carrot plug, which was placed on the petiole approximately 3 cm distal to the stem. The inoculated sites were wrapped with a piece of Parafilm (50 × 80 mm) to maintain contact and high humidity. Three plants per line were used for inoculation. The vertical length of stem lesions was measured and used as a parameter of the lesion size. For leaf disc infection, a mycelial plug (0.8 cm in diameter) was placed in the center of leaf segments that were excised from the top leaves of 6-week-old sunflower plants. There were no visible lesions on these transgenic leaves. Ten leaf segments were tested per transgenic line and untransformed SMF3. In parallel, control plants or leaf discs were mock inoculated with carrot or PDA plugs. On the 3rd and 6th d after inoculation, leaves and stems were harvested, frozen in liquid nitrogen, and stored at −80°C for RNA isolation. The experiments were repeated twice.

Analysis of SA Accumulation

Total (free plus conjugated) SA was extracted from 0.06-g leaf samples (five replicates) as previously described (Enyedi et al., 1992). Samples were analyzed with a liquid chromatography system (Waters, Milford, MA). Ten microliters of each extract was injected at a flow rate of 1 mL min^{-1} into a Waters Nova-Pak 4-μm C-18 column (3.9 × 0.75 cm). The column was maintained at 40°C and equilibrated in 22% (v/v) acetonitrile against 78% of 0.1% (v/v) acetic acid in water. SA was eluted isocratically under these conditions (retention time = 3.1 min) and quantified using a scanning fluorescence detector (model 474, Waters) using excitation and emission wavelengths of 300 and 405 nm, respectively. The identity of SA in sunflower extracts was confirmed by its co-elution with authentic standard and by analysis of its UV light absorption spectrum, as measured with a photodiode array detector (model 996, Waters).

Histochemistry and Microscopy

For determination of autofluorescent material, leaf tissues from 5-week-old plants were cleared by boiling in alcoholic lactophenol (95% [v/v] ethanol-lactophenol, 2:1) for 3 min, washed in 50% (v/v) ethanol, and finally rinsed with water. Autofluorescence of leaves was observed from the upper side of leaves using a DM RB epifluorescence microscope (excitation filter = 365 nm, dichoric filter = 510 nm, and barrier filter = 520 nm, Leica, Wetzlar, Germany).

Detection of OXO Activity and H_2O_2 Accumulation

Determination of OXO activity in sunflower tissues was performed as described by Sugiura et al. (1979) with some modifications. This colorimetric

assay is based on the quantification of H_2O_2 produced from OA. Lyophilized leaf tissues were ground in a GenoGrinder (Spex, Metuchen, NJ) and suspended in 100 mM sodium succinate (pH 3.5). The reaction was started by adding OA to a final concentration of 1 mM, incubating at 37°C for 5 min, and stopped by adjusting the pH to 7.0 using a 1 M Tris solution. An aliquot of the 4,300*g* supernatant from each sample was mixed with an H_2O_2-detecting reagent containing 200 mM Tris (pH 7.0), 400 μM of 4-aminoantipyrine, 20 μL of *N*,*N*-dimethylanaline and 2 units of horseradish peroxidase (Sigma, St. Louis). The absorbance of the solution was measured at 550 nm. H_2O_2 was quantitated by comparison with an H_2O_2 standard curve. The same H_2O_2-detecting reagent was used for analyzing H_2O_2 accumulation in *OXO* transgenic leaf discs (Fig. 3D). To detect the accumulation of H_2O_2 in leaves, detached leaf segments from 6-week-old plants were incubated in H_2O_2-detecting colorimetric reagent in the absence of exogenous OA for 24 h.

mRNA Expression Profiling

RNA profiling studies were conducted using an open architecture RNA profiling technology (Shimkets et al., 1999; Bruce et al., 2000). Leaf and stem tissues of the *OXO* transgenic and untransformed control plants (Table I) were sampled from the top parts of plants 48 and 75 DAP. At 48 DAP, the leaf and stem samples had no lesion mimic phenotype. To identify the differentially expressed genes in both stem and leaf tissues and to save cost, we combined the stem and leaf tissues at this stage. With plants getting mature, lesions developed on the leaves of line 610255. To identify the differentially expressed genes that are associated with triggering HR-like cell death, we collected the leaf tissues and stem tissues at 75 DAP. We used 48 pairs of restriction endonucleases to profile accumulation of mRNA species for each sample. Pair-wise comparisons between the transgenic and untransformed tissues were made for each tissue and developmental stage to identify cDNA fragments that were modulated at least 2-fold. To associate differentially modulated cDNA fragments with known genes, the data on fragment size (base pairs) and the restriction site-specific sequence for every cDNA fragment was used to query the EST sequence database consisting of sunflower sequences and the sequence of the wheat *OXO* gene. Confirmation that the cDNA fragment is part of the sequence was obtained when the unlabeled oligonucleotide successfully competed with the labeled primer and reduced the intensity of the band of interest, whereas the intensities of the remaining bands in the digital gel image were the same as those in the original gel image.

Cloning of Full-Length cDNA by PCR Amplification

The sequence information generated by RNA profiling studies was used for designing gene-specific primers for amplifying both 3′ and or 5′ end regions of target genes using a SMART RACE cDNA amplification kit (CLONTECH, Palo Alto, CA). Polyadenylated RNA from 6-week-old *OXO* transgenic sunflower leaf and stem tissues were used for cDNA library construction with Lambda ZAPII vector (Strategene, La Jolla, CA). The cDNA library mixture was used as a template for PCR amplification. To facilitate cloning, we designed a pair of 28-bp vector primers flanking cDNAs on both ends of the pBS vector (Strategene; pBS upper, GCGATTAAGTTGGGTAACGCCAGGGT; and pBS lower, TCCGGCTCGTATGTTGTGTGGAATTG). The amplification of either 5′ or 3′ end of cDNA was done using one vector primer and one gene-specific primer. For each of the three genes (*PR5-1*, *defensin*, and *SCO*), two gene-specific primers (5′ end RACE primer and 3′ end RACE primer) were designed based on the sequences of the cloned gene fragments of the genes (h0a0-231.3, d0l0-113.9, and n0s0-162.7 respectively) as follows: *PR5-1* (5′ end RACE, TCCGCAGTACATGAGATACCC; and 3′ end RACE, ACAATGACAACCTCCACCCTTCCCACTTT), *defensin* (5′ end RACE, GACCATGTCTGGCTTGCCTTCTCACA; and 3′ end RACE, GAGCTTGAGCTTAGTTCAGTAACTTAAAAATGGCC), and *SCO* (5′ end RACE, GGGAAGATGGAGGAGTACTCAGAT; and 3′ end RACE, CGGCACGAGTAACTCTCGTTCAGTGTTCC). PCR products were cloned into pCR vector (Invitrogen, Carlsbad, CA), and the inserts were sequenced using an Applied Biosystems 373A automated sequencer (PE-Applied Biosystems, Foster City, CA). The GenBank accession numbers for these three cDNA clones are AF364864 (*PR5-1*), AF364865 (*defensin*), and AF364866 (*SCO*).

Chemical Treatments

Six-week-old untransformed sunflower (SMF3) plants were treated with different chemicals in the greenhouse. SA and H_2O_2 were purchased from Sigma, and JA was obtained from Apex Organics Ltd. (Devon, UK). For chemical treatments, plants were sprayed until runoff with 0.1% (v/v) ethanol in the absence or presence of 5 mM SA, 5 mM H_2O_2, or 45 μM JA. Tissue samples were collected at the indicated time points, immediately frozen in liquid nitrogen, and stored at −80°C for RNA isolation.

RNA Analysis

Tissues were ground in liquid nitrogen, and total RNA was extracted using TriPure Reagent (Boehringer Mannheim/Roche, Indianapolis, IN) according to the manufacturer's protocol. Twenty micrograms of total RNA was separated in a 1% (w/v) agarose gel containing formaldehyde. Ethidium bromide was included in the gel to verify equal loading of RNA. After transfer onto a Hybond N^+ membrane (Amersham, Piscataway, NJ), the blots were hybridized with ^{32}P-labeled *PR5-1*, *defensin*, *SCO*, or wheat *OXO* cDNA probes. A duplicate blot was hybridized with 18S ribosomal RNA (Nairn and Ferl, 1988) as a control to allow normalization. Hybridization and washing conditions were performed according to Church and Gilbert (1984).

Distribution of Materials

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