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Ectopic Overexpression of the Hot Pepper *Cat1* in *Arabidopsis* Enhances Resistance to Paraquat, but not to Wounding

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We constructed transgenic *Arabidopsis* plants that over-express hot pepper *CaCat1* to gain more insight into that gene's functional roles in response to abiotic stresses. Although all the homozygous transgenic lines accumulated *CaCat1* transcripts, their degree of catalase activity varied, from 1.1-fold to 1.9-fold. The germination rates for transgenic lines on MS plates containing >0.5 μM paraquat were higher than those recorded for the wild type, while no difference in germinability was noted between the wild type and those exposed to 0.2 μM paraquat. However, fresh weights for the latter were greater than those of the wild type. Moreover, one transgenic line showed lower H_2O_2 production than did the wild-type plants in response to paraquat treatment. These results revealed that *CaCat1* overexpression enhances paraquat resistance in transgenic *Arabidopsis*. In response to wounding, however, our examination of *AtVSP1* expression, as defined by H_2O_2 levels, showed no difference between the WT and transgenic plants. Furthermore, catalase activities in our two transgenic lines decreased 2 h after this stress was applied, in both wounded and untreated leaves, further declining to levels similar to the wild type after 6 h. These results suggest that wounding may down-regulate *CaCat1* expression at the post-transcriptional level in transgenic *Arabidopsis* in order to maintain the wound-signaling process in transgenic lines.

Keywords: Arabidopsis, CaCat1, overexpression, paraquat, wounding

Catalase is a potent antioxidant enzyme that catalyzes the conversion of H_2O_2 to oxygen and water. Unlike with animal catalases, those from plants exist in numerous isoforms encoded by multiple genes (Scandalios et al., 1997). These multiple isoforms are thought to play several roles in a variety of plant tissues and environmental conditions (Willekens et al., 1994a, b; Esaka et al., 1997; McClung, 1997; Scandalios et al., 1997).

To elucidate the overall mechanism of plant catalases, studies on specific functions of individual genes and isoforms are indispensable. Among the species already examined, transgenic tobacco that expresses sense and antisense constructs of catalases from tobacco and cotton has shown altered levels of photosynthesis (Brisson et al., 1998). Suppression of the *Nicotiana plumbaginifolia cat1* isozyme in tobacco can reduce catalase activity, cause the development of necrotic lesions, or activate disease resistance under high-light conditions (Chamnongpol et al., 1996; Takahashi et al., 1997). Furthermore, Willekens et al. (1997) have proposed that tobacco catalase is a cellular sink for H_2O_2. These studies demonstrate that specific catalase isoforms are significant for eliminating photorespiratory H_2O_2, and can be correlated with plant defense responses to stress.

Interestingly, most of the catalase genes used in these previous studies have been structurally related, such as those exhibiting leaf-preferential expression, e.g., hot pepper *CaCat2* (Lee and An, 2005). To our knowledge, transgenic research with other types of C_3 plant catalases have not been reported except for *N. plumbaginifolia cat2*-deficient plants, which do not manifest any unique phenotype when exposed to high light intensities (Willekens et al., 1997).

Accordingly, we have constructed transgenic *Arabidopsis* plants that over-express *CaCat1*, a catalase isoform with stem-preferential expression that is enhanced by paraquat treatment (Kwon and An, 2001; Lee and An, 2005). Here, we have examined their responses to abiotic stresses such as paraquat exposure and wounding.

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MATERIALS AND METHODS

Construction of *Arabidopsis* Over-Expressing *CaCat1*

A cDNA fragment containing the coding region of *CaCat1* was subcloned between the CAMV35S promoter and ocs3' of pART7 (Gleave, 1992). A *Not*I fragment containing the overexpression cassette from pART7 was subcloned into binary vector pART27 (Gleave, 1992). The overexpression cassette in pART27 was then transformed into *Agrobacterium* GV3101 by electroporation, and introduced into *Arabidopsis* (Columbia ecotype) by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MS plates containing kanamycin (40 μg mL^{-1}). Primary transgenics (T_1) were then self-fertilized to produce T_2 seeds, and T_3 homozygous lines were established from T_2 plants that showed a Mendelian segregation ratio.

RNA Isolation, Northern Hybridization, and Slot Blot Analyses

Total RNA was isolated from plant tissues using Tri-Reagent (Sigma, USA) according to the manufacturer's recommendations. For the northern blot analyses, 10 μg of total RNA was separated on an 1% formaldehyde-agarose gel in 3-(N-morpholino)propanesulphonic acid (MOPS) buffer, then transferred to Hybond N^+ membranes (Amersham, UK). The RNA blots were hybridized with ^{32}P-labelled gene-specific probes for *CaCat1* in Church buffer at

65°C. They were also probed with *18S* rRNA to check for equal loading. The hybridized blots were washed at 65°C while gradually decreasing the salt concentration to 0.5x SSC/0.1% SDS, then exposed to X-ray film.

Protein Extraction

Two-gram samples of whole plants or rosette leaves were homogenized in 100 mM Tris-HCl (pH 8.0), 20% glycerol, and 30 mM dithiothreitol (DTT) at 4°C (Orendi et al., 2001). The homogenate was centrifuged at 15,000g for 30 min and the supernatant was filtered through cheesecloth. Filtered protein extracts were quantified according to the method of Bradford (1976), with BSA as a standard, and stored at -80°C. These extracts were used within 1 week after their preparation.

Measurement of Catalase Activity

Total catalase activity was determined by monitoring the consumption of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm for 2 min, as described by Rao et al. (1996). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0) and 20 μg mL^{-1} of protein extracts in 3 mL of solution. This reaction was initiated by adding 10 μL of 30% (w/v) H_2O_2.

Determination of Paraquat Resistance

Seeds of wild-type and transgenic *Arabidopsis* lines were surface-sterilized and plated on MS plates containing 0, 0.2, 0.5, or 1.0 μM paraquat. Plates were stored for 4 d under darkness at 4°C, then placed in a growth chamber at 23°C under a 16-h photoperiod (100 μmol s^{-1} m^{-2}). After 1 week, we determined their germination rates and fresh weights.

Paraquat and Wounding Treatments

For our stress treatments, seeds from wild-type and transgenic *Arabidopsis* lines were surface-sterilized and sowed into pots, which were then stored for 4 d under darkness at 4°C before being placed in a growth chamber at 23°C under a 16-h photoperiod (100 μmol s^{-1} m^{-2}). After 2 weeks, treatments were applied for 4 h after illumination. Halves of rosette leaves were either exposed to 50 μM of paraquat or were crushed twice with a hemostat, perpendicular to the main vein.

H_2O_2 Detection

H_2O_2 was detected by *in situ* histochemical staining, using 3,3′-diaminobenzidine (DAB) as described by Ren et al. (2002). Leaves were detached and placed in a solution containing 1 mg mL^{-1} DAB (pH 5.5) for 1 h. To remove chlorophylls, the leaves were boiled in 95% ethanol for 10 min and then stored in 95% ethanol. H_2O_2 production was visualized by the development of a reddish-brown color.

RT-PCR Analysis

RT-PCR was used to analyze the expression patterns of *AtVSP1* in wounded *Arabidopsis*.

The PCR primers (5′-AAACGGATCGAAGTTGACGCAA-3′ and 5′-GATATGAAATGGATACAAGGGGAC-3′) were designed specifically to amplify most of the 3′ UTR of *AtVSP1* cDNA. Total RNA (2 μg) from samples harvested 0, 2, 4, or 6 h after wounding was used as template for reverse transcription after treatment with RNase-free DNase (Promega, USA). PCR cycling conditions included an initial denaturation at 95°C for 5 min; followed by 95°C for 1 min, 51°C for 1 min, and 72°C for 1 min (total 25 cycles); with 10 min of final extension at 72°C. Amplified PCR products were electrophoresed on an 1% agarose gel.

RESULTS AND DISCUSSION

Catalase Activity in Transgenic *Arabidopsis*

To better understand the functional roles of *CaCat1*, we produced transgenic *Arabidopsis* plants over-expressing that gene. Twenty primary transformed kanamycin-resistant lines were obtained, which were then self-fertilized to produce T_2 seeds. The segregating ratio of the T_2 plants was examined on MS plates containing kanamycin. In all, seven transgenic lines possessing one copy of the transgene were selected, and their homozygous transgenic T_3 lines were then obtained. Expression of *CaCat1* in these lines was confirmed by northern hybridization, using a *CaCat1*-specific probe (Fig. 1A). All the transgenic lines accumulated *CaCat1* transcripts, with the highest level being detected in Line #2. No transcript was detected in the untransformed wild type, indicating no cross-hybridization of *CaCat1* cDNA with the *Arabidopsis* catalase transcripts. Variable levels of catalase activity were present in the transgenic lines compared with

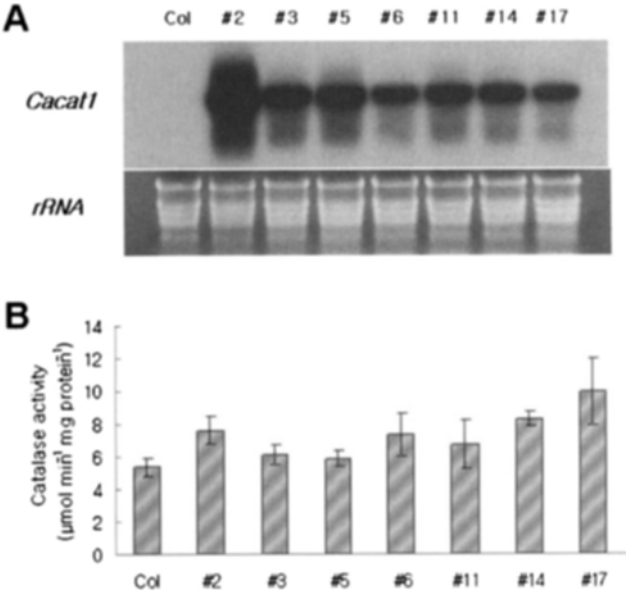

Figure 1. *CaCat1* mRNA levels (**A**) and total catalase activity (**B**) in transgenic *Arabidopsis* over-expressing *CaCat1*. Whole plants grown for 10 d were harvested at 4 h after beginning of illumination. (**A**) Total RNAs (10 μg) were hybridized with ^{32}P-labeled *CaCat1*-specific probe. rRNA was shown to ensure equal loading. (**B**) Bars represent SE of 5 experiments (n=5). Col, untransformed wild-type (Columbia ecotype); #2-#17, transgenic lines.

the control plants, ranging from a 1.1-fold increase in Line #5 to a 1.9-fold greater amount in Line #17 (Fig. 1B). These results, however, also revealed that catalase activity was not correlated with the steady-state level of transcripts in the transgenic plants. For example, although Line #2 had the highest degree of accumulation, its catalase activity was much lower than that in Line #17. This observation is consistent with previous reports (Ni and Trelease, 1991; Brisson et al., 1998). Therefore, from these results, it is clear that post-transcriptional factors play an important role in regulating plant catalase activity.

Based on their relatively greater levels of catalase activity compared with the untransformed WT, Lines #2 (1.4-fold higher), #14 (1.5-fold higher), and #17 (1.9-fold higher) were selected for further experiments.

Paraquat Resistance in Transgenic *Arabidopsis*

We previously reported that *CaCat1* expression and total catalase activity in hot pepper is enhanced at 4 to 12 h after paraquat treatment (Lee and An, 2005). Those observations suggested that the isozyme encoded by *CaCat1* may be an important scavenger of the H_2O_2 produced by paraquat.

Here, we studied the germinability of seeds placed on paraquat-containing MS plates to investigate whether paraquat tolerance could be enhanced in transgenic lines (Fig. 2A). As expected, germination rates for the transgenics were higher than for the wild type at higher doses of paraquat (0.5 and 1.0 μM; Fig. 2B). Although rates were similar between the WT and the transgenic lines at a relatively lower dose (0.2 μM paraquat), fresh weights from the germinated transgenic lines were >1.6-fold higher than that from the WT plants (Fig. 2C). Furthermore, during 6 h of treatment with 50 μM paraquat, H_2O_2 levels in the transgenic plants from Line #14 were much lower than in the wild type, for both treated and systemic leaves (Fig. 3A). All of these results demonstrate that transgenic *Arabidopsis* over-expressing *CaCat1* has a high degree of paraquat tolerance.

Several earlier studies reported that altered catalase levels in transgenic plants are related to enhanced paraquat resistance, as was demonstrated with tobacco that expressed *E. coli katE* catalase (Shikanai et al., 1998). Paraquat treatment also increased ion leakage in leaf disks from *Cat1*-deficient transgenic tobacco plants (Willekens et al., 1997). Furthermore, Polidoros et al. (2001) showed that one transgenic tobacco plant expressing the maize *Cat2* gene suffered significantly less paraquat damage than untransformed, wild-type plants. We previously confirmed that *CaCat1*, exhibiting stem-preferential expression, belongs to a type of plant catalase different from those used in other transgenic research (Lee and An, 2005). Accordingly, our results suggest that plant catalases of the *CaCat1* type may also contribute to paraquat resistance in C_3 plants together with *N. plumbaginifolia Cat1*, which shows leaf-preferential expression.

Effects of Wounding on Transgenic *Arabidopsis*

To begin investigating the response of transgenic plants to stress, we first measured H_2O_2 production in wounded and systemic leaves of transgenic Line #14. No differences in

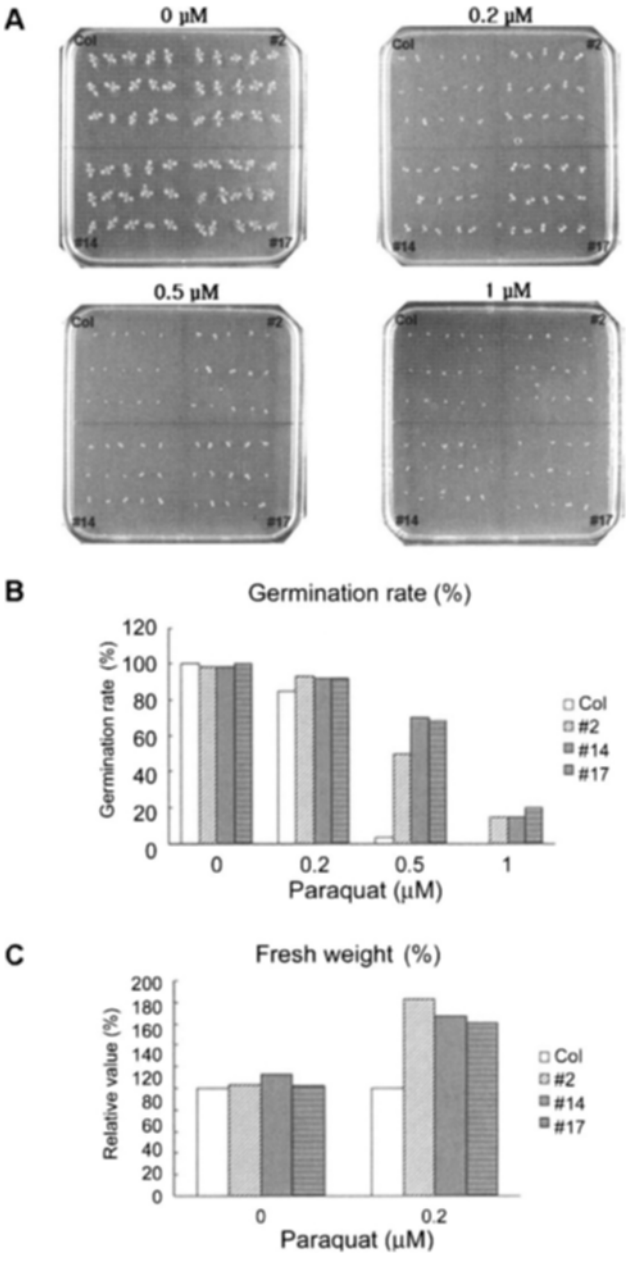

Figure 2. Tolerance of transgenic lines to paraquat. Surface-sterilized seeds were plated on MS plates containing 0, 0.2, 0.5, or 1.0 μM paraquat, then stored for 4 d in darkness at 4 before placement in growth chamber for 1 week (**A**). Germination rates were measured for 60 seedlings (**B**). Fresh weights for 50 seedlings grown on MS plates containing 0.2 μM of paraquat (**C**). Col, untransformed wild-type (Columbia ecotype); #2-#17, transgenic lines of *Arabidopsis* over-expressing *CaCat1*.

H_2O_2 levels appeared between the wild-type and transgenic plants (Fig. 3B), suggesting that the overexpression of *CaCat1* did not affect wounding-induced generation of H_2O_2. Thus, we also conducted RT-PCR to examine whether such over-expression would affect the wounding response in *Arabidopsis*. The mRNA accumulation of *AtVSP1* (Rojo et al., 1999; Wang et al., 2000) a well-known wound-responsive gene, was determined at 0, 2, 4 and 6 h after wounding in both wild-type plants and those from transgenic Line #14.

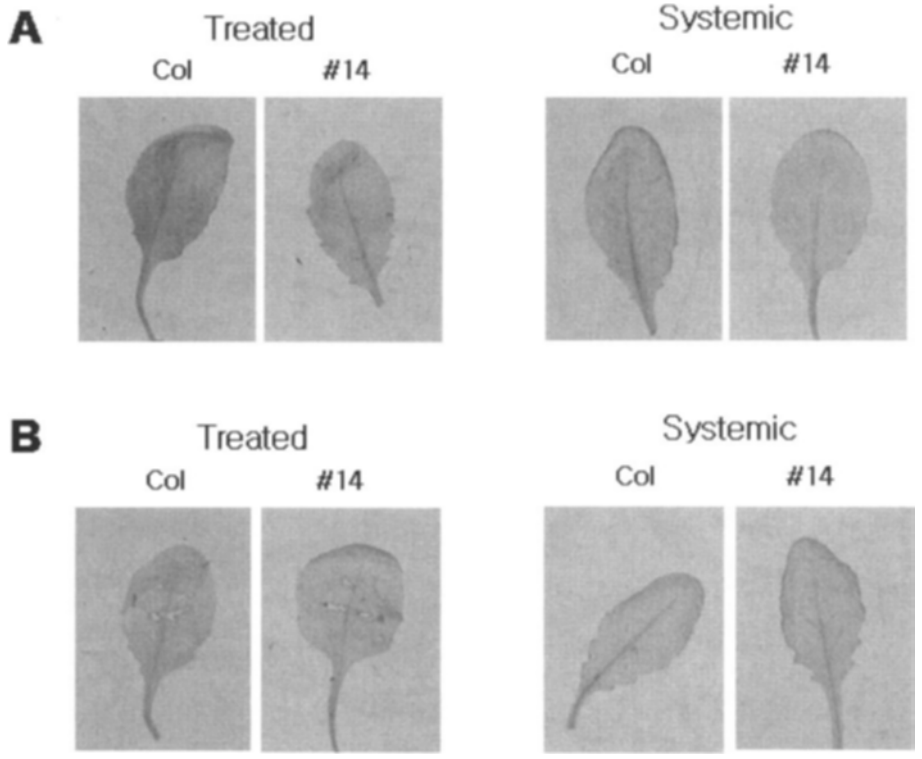

Figure 3. Generation of H_2O_2 in treated and systemic leaves of *Arabidopsis* after paraquat (50 μM) treatment (**A**) or wounding (**B**). Leaves were detached at 6 h after stress was induced, then stained with DAB for 1 h. H_2O_2 production was visualized as reddish-brown color by DAB staining. Col, untransformed wild-type (Columbia ecotype); #14, transgenic line over-expressing *CaCat1*.

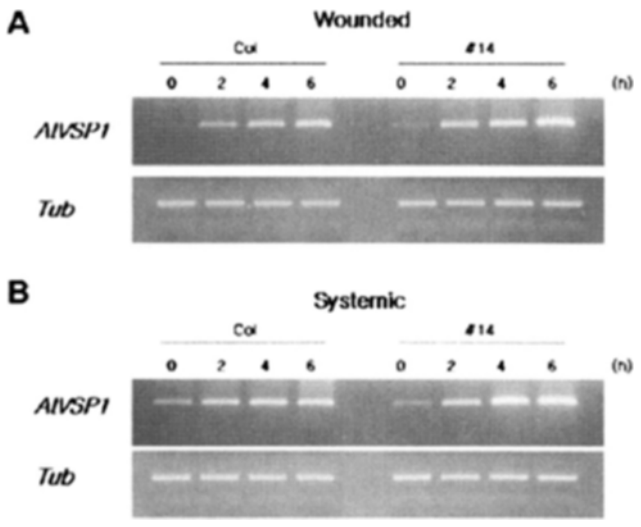

Figure 4. RT-PCR analysis of *AtVSP1* expression in wounded (**A**) and systemic (**B**) leaves of wild-type and #14 transgenic line. Total RNA (2 μg) from samples harvested 0, 2, 4, and 6 h after wounding was used as template for reverse transcription. Amplified PCR products were electrophoresed on an 1% agarose gel. Tubulin was used as control to ensure equal loading.

Here, *AtVSP1* expression did not differ significantly between the two phenotypes (Fig. 4). Therefore, we can conclude that *CaCat1* overexpression hardly affects the wounding response in *Arabidopsis*.

We can suggest two possible explanations for this response. First, the catalase isozyme encoded by *CaCat1* may have no connection with the plant reaction to wounding. Second, the effect of *CaCat1* overexpression in transgenic plants may disappear under wounding conditions. Based on those possibilities, we investigated any induced changes in total catalase activity within wild-type and transgenic plants, and found that such stress had no effect in either the wounded or systemic leaves of the former type (Fig. 5A). This finding agreed with our previous discovery that wounding does not influence the degree of catalase activity in hot pepper (Lee and An, 2005). However, for the two transgenic lines, catalase activities declined 2 h after treatment in both wounded and systemic leaves, dropping after 6 h to amounts similar to those measured in the WT (Fig. 5B, C). Although the *CaCat1* transcripts level had been barely affected by wounding in hot pepper (Lee and An, 2005), the continuous accumulation of *CaCat1* mRNA, using the CaMV 35S promoter system, had been thought to increase the ability of plants to scavenge H_2O_2 generated locally and systemically by wounding in transgenic lines. However, our results revealed that those lines showed no such elevated tolerance to wounding, perhaps because wound-signaling down-regulated this CAT activity.

This result contrasts with previous data that demonstrated enhanced resistance to paraquat stress in transgenic lines over-expressing *CaCat1* (Fig. 2, 3A). Although we did not conduct any experiments to estimate the change in catalase activity in *CaCat1* transgenic *Arabidopsis*, the enhanced tolerance to paraquat found in those transgenic lines (Fig. 2, 3A) clearly supports our conclusion that the elevation in

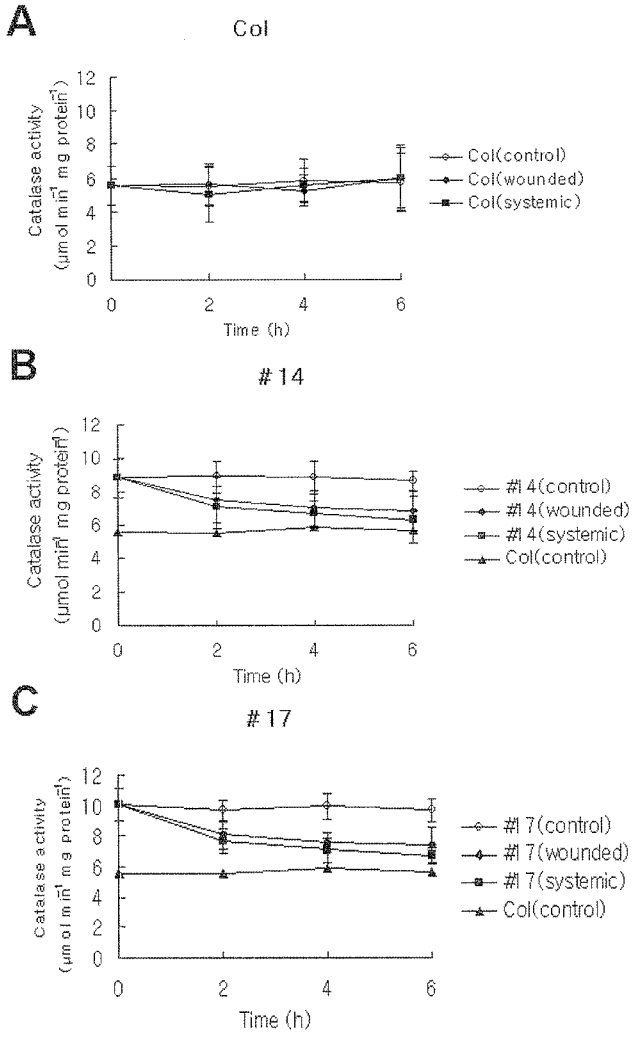

Figure 5. Total catalase activities in wounded and systemic leaves of wild type (**A**), #14 transgenic line (**B**), and #17 transgenic line (**C**). Bars represent SE of 5 experiments (n=5). Col, untransformed wild-type (Columbia ecotype); #14 and #17, #14 and #17 transgenic line over-expressing *CaCat1*, respectively. Control, unwounded leaves; wounded, wounded leaves; systemic, systemic leaves.

enzyme activity through the continuous accumulation of *CaCat1* transcripts was maintained during paraquat treatment. Thus, the machinery for regulating *CaCat1* expression in transgenic lines seems to have distinct sensitivities to paraquat and wounding treatments. Perhaps this activity is reduced at the post-transcriptional level only in response to wounding but not to paraquat. Previously, we suggested that *CaCat1* might play an important role in controlling the H_2O_2 level, as propagated systemically, because *CaCat1* transcripts were shown to accumulate mainly in the vascular bundles, e.g., as in the case of *N. plumbaginifolia cat2* (Willekens et al., 1994a; Lee and An, 2005). Because the propagation of H_2O_2 can be important to the systemic responses of a plant system, *CaCat1* transcript levels and/or catalase activity may be regulated by some particular regulatory mechanism distinct from those of other isoforms. In this respect, our results suggest the presence of a specific mechanism that down-regulates *CaCat1* expression at the post-transcriptional level in wounded *Arabidopsis*, possibly by maintaining this signaling process in transgenic lines. Further studies on the possible role of post-transcriptional regulators, such as salicylic acid and Ca^{2+}/calmodulin, would be helpful to extending our knowledge of this phenomenon.

In conclusion, we have now demonstrated that overexpression of *CaCat1* enhances catalase activity, which in turn elevates paraquat resistance in *Arabidopsis*. However, resistance to wounding is not affected in a similar manner, perhaps because catalase activity is diminished by wound-signaling.

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