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Tomato Plants Ectopically Expressing Arabidopsis CBF1 Show Enhanced Resistance to Water Deficit Stress[1]

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A DNA cassette containing an Arabidopsis C repeat/dehydration-responsive element binding factor 1 (*CBF1*) cDNA and a *nos* terminator, driven by a cauliflower mosaic virus 35S promoter, was transformed into the tomato (*Lycopersicon esculentum*) genome. These transgenic tomato plants were more resistant to water deficit stress than the wild-type plants. The transgenic plants exhibited growth retardation by showing dwarf phenotype, and the fruit and seed numbers and fresh weight of the transgenic tomato plants were apparently less than those of the wild-type plants. Exogenous gibberellic acid treatment reversed the growth retardation and enhanced growth of transgenic tomato plants, but did not affect the level of water deficit resistance. The stomata of the transgenic *CBF1* tomato plants closed more rapidly than the wild type after water deficit treatment with or without gibberellic acid pretreatment. The transgenic tomato plants contained higher levels of Pro than those of the wild-type plants under normal or water deficit conditions. Subtractive hybridization was used to isolate the responsive genes to heterologous *CBF1* in transgenic tomato plants and the *CAT1* (*CATALASE1*) was characterized. Catalase activity increased, and hydrogen peroxide concentration decreased in transgenic tomato plants compared with the wild-type plants with or without water deficit stress. These results indicated that the heterologous Arabidopsis *CBF1* can confer water deficit resistance in transgenic tomato plants.

Many environmental stresses, such as heat, salinity, low temperature, and drought, and developmental processes, such as seed maturation, cause water deficit in plants (Ingram and Bartels, 1996). To understand water deficit stress at the molecular level, many genes have been isolated, such as *rd* (responsive to dehydration), *erd* (early responsive to dehydration), and *Lea* (late embryogenesis abundant; Shinozaki and Yamaguchi-Shinozaki, 2000). The accumulation of LEA protein occurs during seed maturation, desiccation, and increases in vegetative tissue when plants are exposed to water deficit (Ingram and Bartels, 1996). Overexpression of a barley (*Hordeum vulgare*) group 3 LEA protein gene, *HVA1*, enhances tolerance of water deficit and salt stress in transgenic rice (*Oryza sativa*; Xu et al., 1996). Arabidopsis *RD29A* (*COR78*) responds to water deficit and low-temperature stresses (Horvath et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1993). Study of the promoter *RD29A* has lead to the characterization of a 9-bp element, TACCGACAT, referred to as dehydration-responsive element (DRE), that is also found in the promoter regions of many water deficit and cold responsive genes, such as *RD17*, *ERD10*, *KIN1*, *COR15a*, and *COR6.6* (Yamaguchi-Shinozaki and Shinozaki, 1994; Wang et al., 1995; Thomashow, 1999). The DRE element contains a 5-bp core sequence of CCGAC, also known as C repeat (CRT), that plays an important role in regulating gene expression in response to low temperature, water deficit, and high salinity (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). Proteins that bind to the DRE/CRT element and mediate transcription were isolated by the yeast (*Saccharomyces cerevisiae*) one-hybrid system and named DRE-binding proteins (DREBs)/CRT-binding factors (CBFs; Stockinger et al., 1997; Liu et al., 1998). DREBs/CBFs are encoded by multigene families. Among them, the *DREB1A* and *DREB2A* respond to low temperature and water deficit stresses, respectively (Liu et al., 1998).

CBF1 (DREB1B), a homolog of DREB1A, is a transcriptional activator that binds to the CRT/DRE element, in the promoter region of cold-regulated (*COR*) genes that respond to both low temperature and water deficit (Stockinger et al., 1997; Gilmour et al., 1998). Overexpression of the *CBF1* gene in Arabidopsis plants induces expression of *COR* genes and increases freezing tolerance in the absence of cold acclimation (Jaglo-Ottosen et al., 1998), suggestive of the role of a master switch of the CBF regulation (Thomashow, 2001). Besides its effect in Arabidopsis, overexpression of *CBF1* in canola oilseed rape (*Brassica napus*) also activates *COR* homologous genes and enhances freezing tolerance, indicating that the function of *CBF1* may be highly conserved in plants (Jaglo et al., 2001). Once ice crystals form in the extracellular

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spaces of plant cells, water moves out of the cell resulting in water deficit. Therefore, the mechanisms of freezing and water deficit tolerance may be similar to each other (Thomashow, 2001). The freezing, salt, and drought tolerance capabilities of transgenic Arabidopsis have also been achieved by the expression of *DREB1A* (*CBF3*) cDNA, driven by a 35S promoter or stress-inducible *RD29A* promoter (Liu et al., 1998; Kasuga et al., 1999). Therefore, overexpression of *CBF1* might also improve water deficit tolerance in Arabidopsis plants.

The existence of a *CBF1*-like expressed sequence tag (EST) in tomato (*Lycopersicon esculentum*; Jaglo et al., 2001) suggests that a pathways may exist in tomato that is similar to the Arabidopsis signal transduction. The objective of this experiment was to determine whether overexpression of *CBF1* in tomato enhanced water deficit tolerance, as is the case in Arabidopsis expressing *DREB1A* (*CBF3*). In this study, we present evidence that the transgenic tomato plants expressing *CBF1* are more resistant to water deficit than the wild-type plants.

RESULTS

Overexpression of Heterologous Arabidopsis CBF1 in Transgenic Tomato Plants

A DNA cassette consisting of an Arabidopsis *CBF1* cDNA driven by a cauliflower mosaic virus 35S promoter and a *nos* terminator was ligated into pCAMBIA2301, which contains β-glucuronidase (*GUS*) and *NPTII* reporter genes, to form pJLM1. This plasmid was transferred into the tomato genome by *Agrobacterium tumefaciens*-mediated transformation. After kanamycin selection, the putative transgenic tomato plants were assayed by GUS staining and Southern-blot analyses to identify the transgenic plants (data not shown). We obtained 22 unique transgenic tomato lines. Northern-blot analysis was performed to reveal the mRNA levels in transgenic T_1 plants, three independent T_1 lines from C5 (Fig. 1, lanes 2–4), three independent T_1 lines from C15 (Fig. 1, lanes 5–7), and two independent T_1 lines from C21 (Fig. 1, lanes 8 and 9). The heterologous *CBF1* and *GUS* transcripts accumulated only in transgenic T_1 plants. Interestingly, one transgenic T_1 plant did not show high expression of Arabidopsis *CBF1*, but *GUS* transcripts were detected (Fig. 1, lane 2). This phenomenon is probably due to segregation of T1 seeds. Transgenic T_1 plants expressing heterologous *CBF1* were evaluated for resistance to water deficit stress. As shown in Figure 1, levels of mRNA of *β-TUBULIN* and rRNA were used as internal control.

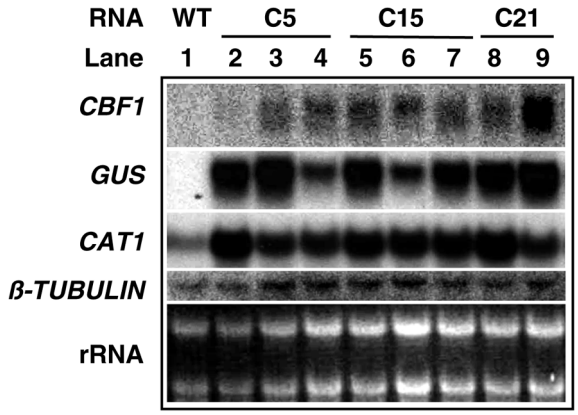

Figure 1. Northern-blot analysis of transgenic tomato plants. Total RNA (10 μg) was extracted from the wild-type (WT; lane 1) and transgenic T_1 plants overexpressing *CBF1* (lane 2–9). Probes used were ^{32}P-labeled Arabidopsis *CBF1* cDNA, the *GUS* reporter gene from pCAMBIA2301, tomato *CAT1*, and *β-TUBULIN*.

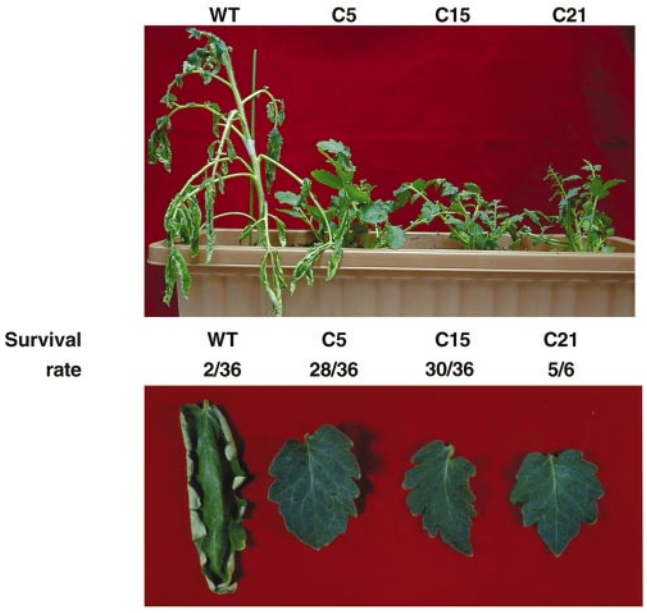

Figure 2. Transgenic tomato plants exhibited more resistance to water deficit stress than wild-type plants. Wild-type and three transgenic T_1 plants (WT, C5, C15, and C21) were grown at 24°C without watering for 21 d. Leaves of the wild-type plant significantly curled and wilted. For the survival rate test, wild-type (WT) and three T_1 transgenic plants (C5, C15, and C21) were grown at 24°C without watering for 28 d. Numbers of plants alive per total number of tested plants are indicated in the middle of the photograph.

Transgenic Tomato Plants Were More Resistant to Water Deficit Than the Wild-Type Plants

To evaluate the capacity for water deficit resistance, transgenic tomatoes and wild-type plants grown in the same pot with peat moss were not watered for 21 d. It was observed that the leaves of wild-type plant became wilted and curled, whereas the transgenics were not (Fig. 2). To examine the survival rates of the wild-type and transgenic plants under conditions of water deficit, the treatment (water deficit) was extended to 4 weeks. The wild-type plants were sick after 28 d without watering, and did

not recover during the 7-d period after rewatering. Compared with the survival rate of wild-type plants, transgenic tomatoes were apparently more resistant to water deficit after 4 weeks of water deprivation (Fig. 2). Less than 6% of the wild-type plants survived after 4 weeks of water deficit treatment, whereas 77.8%, 83.3%, and 83.3% of the transgenic tomato lines C5, C15, and C21 survived the treatment. These results suggest that overexpression of *CBF1* can significantly improve water deficit resistance in tomato, similar to the results obtained from transgenic Arabidopsis plants overexpressing *DREB1A* (*CBF3*; Kasuga et al., 1999).

The resistance to water deficit stress was revealed by chlorophyll fluorescence maximum photochemical efficiency of PSII in the dark-adapted state (F_v/F_m) values, measured at d 0, 7, 14, and 28 during water deficit treatment. PS II integrity was significantly more stable in the transgenic plants as compared with the wild-type plants during water deficit stress (Fig. 3A). F_v/F_m decreased in wild-type plants after 21 d without watering and did not recover after rewatering. The transgenic plants, however, maintained an F_v/F_m value of about 50% of their well-watered control even after 28 d without watering (Fig. 3A), and recovered almost completely after rewatering (data not shown).

To test the ability of maintaining water in the tissue, water contents of leaves (Fig. 3B) and roots (Fig. 3C) of water deficit-stressed transgenic tomato and wild-type plants were measured at various time points. Water content of transgenic plants remained high during water deficit treatments. In contrast, a marked reduction in water content was observed in the wild-type plants.

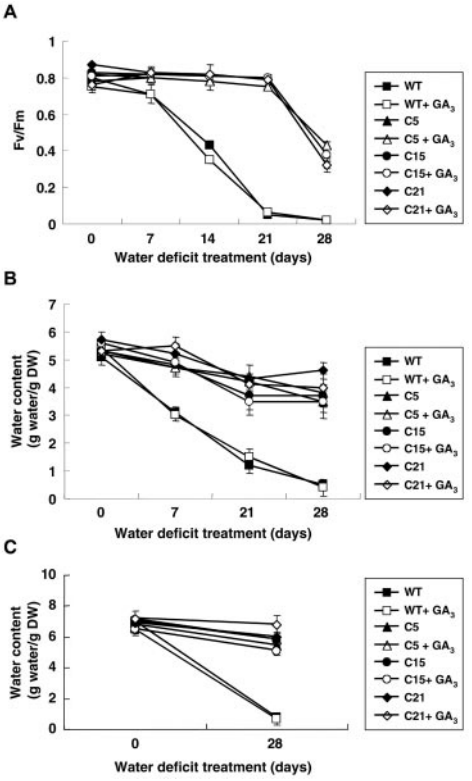

Figure 3. Improved resistance of *CBF1* transgenic tomato T_1 plants to water deficit stress not affected by gibberellic acid (GA_3) treatment. GA_3-treated and non-treated tomato plants were deprived of water for various times. F_v/F_m values (A) and water content of leaves (B) were measured on d 0, 7, 21, and 28. The water content of the roots (C) of water deficit-stressed transgenic tomato and wild-type plants was measured on d 0 and 28. Each value is the mean ± SD (n = 5 individual plants).

Water Deficit Resistance Was Not Affected by Applying GA_3 in Transgenic Tomato Plants

All the transgenic tomato plants were shorter than the wild-type plants due to their short internodes. Previously, internode length has been reported to have a positive correlation with GA content (Ross et al., 1989). Recently, we found that the internode length of transgenic plants could be recovered by applying GA_3 exogenously (Hsieh et al., 2002). It was of interest to know whether the resistance to water deficit stress of transgenic tomato plants would be affected after GA_3 treatment. GA_3-treated wild-type and transgenic tomato plants were subjected to the water deficit stress as previously described. F_v/F_m values and the water content of leaves and roots showed that transgenic tomato plants were still more resistant to water deficit stress than GA_3-treated wild-type plants (Fig. 3). It is also worth noting that GA_3 treatment of both wild-type and transgenic tomato plants seem to have little or no impact on water deficit resistance. There seems to be a correlation of GA content with internode length, but water deficit resistance seems to be independent of it. Hence, the ability to resist water deficit stress was not affected by GA_3 in transgenic tomato plants. These results suggest that the *CBF1*-mediated improvement of water deficit resistance in transgenic tomato plants was probably not due to a morphological change.

To identify the possible relationship between stomatal movement and water imbalance, changes in leaf conductance were determined and are shown in Figure 4. Leaf conductance in the wild-type plants followed a typical diurnal pattern. In all transgenic tomato and wild-type plants, the stomatal opening increased rapidly after the start of the light period, reached a maximal level at about 6 h, and then decreased (Fig. 4A). The stomata of the transgenic *CBF1* tomato plants closed rapidly after water deficit treatment with or without GA_3 pretreatment as compared with wild-type plants, which showed a similar pattern during regular watering (Fig. 4, A and B). The effects of *CBF1* expression apparently result in retained water, thus negating tissue damage; therefore,

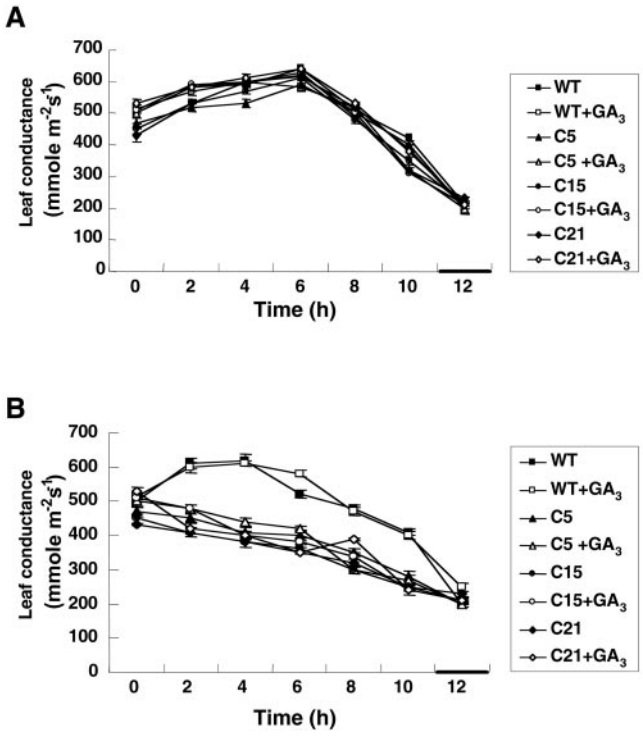

Figure 4. Transgenic tomato T_2 plants rapidly close stomata compared with wild-type plants under water deficit condition. Transgenic tomato and wild-type plants were grown at 24°C with regular watering (A) or no watering for 7 d (B). Horizontal bars in the axis of abscissas represent the dark period. The 2-h dark period extended from 10 to 12 h. Each value is the mean ± SD (n = 15 individual plants).

the phenotype of transgenic plants appeared normal under water deficit conditions.

More Pro Was Detected in Transgenic Tomato Plants

Many plants respond to water deficit by accumulating high concentrations of compatible solutes or osmolytes, such as Pro, mannitol, Fru, Gly betaine, and trehalose (Bajaj et al., 1999; Hoekstra et al., 2001). Because elevated Pro levels occur in transgenic Arabidopsis that overexpressed *DREB1A* (*CBF3*; Gilmour et al., 2000), we also measured Pro content in transgenic tomato plants under normal and water deficit conditions. The Pro content in transgenic tomato was higher than in the wild-type plants under both normal and water deficit conditions (Fig. 5). However, there is no further elevation in response to the water stress conditions in transgenic tomato plants, indicating that overexpression of *CBF1* under non-stress conditions protects the plants from subsequent stress. GA_3 treatment did not affect the Pro content in wild-type and transgenic tomato plants (Fig. 5). These results may suggest that transgenic tomato plants possess an inherent resistance to water deficit conditions, which is much higher than wild-type plants, consistent with results of F_v/F_m value and water content (Fig. 3).

Exogenous GA_3 Treatment Reversed Growth Retardation of Transgenic Tomato Plants without Affecting Water Deficit Resistance

The dwarf phenotype was only observed in transgenic tomato plants that overexpressed heterologous *CBF1*, not in transgenic tomato plants that only overexpressed the *GUS* reporter gene (data not shown). Hence, the dwarfism was a result of overexpression *CBF1*, not the transformation procedure itself. The transgenic tomato plants were not only shorter than wild-type plants, fruit and seed numbers and fresh weights were also less than those of wild-type plants under normal growth conditions (Table I). After exogenous GA_3 treatment, fruit, seed number, and fresh weight increased, suggesting GA_3 improved the growth of the transgenic tomato plants apparently. However, the seed number of transgenic plants treated with GA_3 did not reach the same level as that of the wild-type plants. After water deficit treatment, the fresh weights of transgenic tomato plants were higher than the wild-type plants that had wilted after treatment. No difference in fruit and seed number of transgenic tomato plants without GA_3 treatment under normal or water deficit treatment was observed (Table I). Moreover, there was no significant reduction in fruit, seed number, and fresh weights of transgenic plants treated with GA_3 under water deficit conditions. However, the production and fresh weight of wild-type plants was severely impaired after water deficit treatment, indicating that wild-type plants were not resistant to water deficit as compared with transgenic tomato plants. Exogenous GA_3 treatment showed the same results with non-GA_3 treatment, suggesting that the water deficit resistance was not affected in transgenic tomato plants, which can be observed from the fact that there were no change in fruit, seed number, and fresh weight

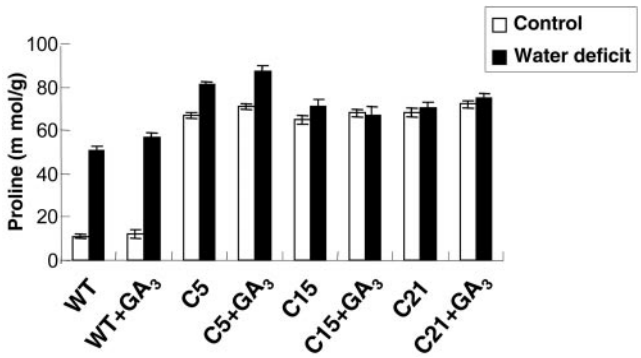

Figure 5. Transgenic tomato plants contain more Pro than wild-type plants. Wild-type and transgenic T_1 plants with or without GA_3 treatment were grown at 24°C with daily watering (control) or without watering for 28 d (water deficit). Pro content was measured. Each value is the mean ± SD (n = 5 individual plants).

Table I. *The effects of various treatments on the growth characteristics of transgenic tomato and wild-type plants*

The order of data shown in each treatment is: fruit no. per plant, seed no. per fruit, and fresh wt (g) per plant. Each value is the mean ± SD (n = 5 individual plants). Chilling treatment is incubated at 0°C for 7 d, then returned to 24°C. Water deficit treatment is without watering for 4 weeks. The measured plants are 3 months old. The stress treatment time is also included in the growth period.

	WT	C5	C15	C21	WT+GA_3	C5+GA_3	C15+GA_3	C21+GA_3
Control	22.6 ± 4.3	6.0 ± 1.6	7.2 ± 1.6	1.6 ± 1.1	26.6 ± 4.1	24.8 ± 3.6	22.4 ± 3.2	17.4 ± 5.8
	49.7 ± 9.5	8.4 ± 2.7	6.8 ± 1.3	2.4 ± 0.9	43.7 ± 9.2	25.4 ± 3.0	22.6 ± 2.6	29.6 ± 14.8
	131.4 ± 5.1	80.6 ± 5.1	106.8 ± 9.2	85.0 ± 3.9	147.4 ± 7.1	133.4 ± 13.8	138.8 ± 13.6	127.6 ± 8.7
Chilling	1.1 ± 0.9	6.6 ± 0.9	8.2 ± 0.8	2.4 ± 0.5	2.3 ± 0.6	21.8 ± 2.4	24.4 ± 4.5	15.2 ± 2.2
	1.5 ± 1.1	9.0 ± 1.5	9.0 ± 1.5	2.0 ± 0.8	1.1 ± 0.8	27.0 ± 8.6	24.8 ± 7.8	10.6 ± 2.8
	32.5 ± 6.7	84.2 ± 5.4	120.0 ± 17.3	84.4 ± 7.1	31.5 ± 5.8	123.0 ± 21.4	130.2 ± 8.5	131.2 ± 6.9
Water deficit	6.2 ± 2.2	8.8 ± 1.2	8.2 ± 0.8	2.6 ± 1.8	2.2 ± 0.5	23.6 ± 2.4	21.4 ± 2.1	15.6 ± 3.9
	3.5 ± 1.1	7.6 ± 2.4	9.0 ± 1.5	1.8 ± 0.9	4.1 ± 1.7	37.0 ± 8.2	37.7 ± 6.5	27.7 ± 13.3
	39.8 ± 27.1	101.2 ± 11.6	120.0 ± 17.3	88.4 ± 6.1	41.2 ± 26.7	123.4 ± 15.9	129.8 ± 14.4	128.8 ± 14.7

(Table I). Because the phenomenon of chilling treatment was similar to water deficit treatment, we also calculated the fruit, seed number, and fresh weight after chilling treatment. Results of chilling treatment were similar to water deficit treatment, indicating that the transgenic tomato plants were more resistant to chilling and water deficit stress than the wild-type plants.

Enhancement of Catalase Activity, and Reduction of Hydrogen Peroxide (H_2O_2) Concentration in Transgenic Tomato Plants

Transgenic tomato plants were analyzed at the molecular level using subtractive hybridization techniques against wild-type plants. Subtractive hybridization experiments were performed to identify any up-regulated genes belonging to the transgenic tomato plant (data not shown). No known *COR* homologous genes were isolated from the subtractive hybridization experiment, and Arabidopsis *COR* genes, such as *COR47*, *KIN1*, and *COR15a*, did not cross hybridize with any tomato RNA even in low-stringency hybridization condition (data not shown). Using tomato dehydrin *TAS14*, which is also responsive to stresses (Godoy et al., 1990, 1994; Parra et al., 1996), as a probe, we failed to detect any mRNA transcripts expressed in any transgenic tomato plants (data not shown). The *CAT1* gene, however, was one of the numbers of up-regulated genes we isolated. Northern-blot analysis indicated that transcriptional levels of *CAT1* were higher in transgenic tomatoes than in wild-type plants under normal (Figs. 1 and 6A) or water deficit (Fig. 6A) conditions. The catalase and H_2O_2 concentrations of plants grown under normal conditions with or without watering for 28 d were measured. Catalase activity of transgenic tomato plants was higher than that of wild-type plants under normal or water deficit conditions (Fig. 6B). H_2O_2 concentrations were lower in transgenic tomato than in wild-type plants under normal or stressed conditions (Fig. 6C). Our results indicate that *CAT1* expression and catalase activity increased and H_2O_2 concentration reduced in transgenic tomato plants.

DISCUSSION

CBF genes are considered as "master switches" that activate expression of *COR* genes, increasing freezing tolerance in transgenic Arabidopsis plants in the absence of cold stimulation (Thomashow et al., 2001). Overexpression of *DREB1A* (*CBF3*) not only increases freezing tolerance, but also salt loading and drought tolerance in transgenic Arabidopsis (Kasuga et al., 1999). In this study, we found that overexpression of Arabidopsis *CBF1* increases water deficit resistance in transgenic tomato plants. Results of survival rate, F_v/F_m, and water content show that transgenic tomato plants are more resistant to water deficit stress than wild-type plants (Figs. 2 and 3). Results of stomatal movement and Pro content imply that transgenic plants have the ability to cope with water deficit conditions better than wild-type plants. These results suggest that heterologous *CBF1* could improve environmental stress resistance in agriculturally important crop plants.

CBF1, however, also severely reduced growth in tomato (Table I). Similar effects were found in Arabidopsis plants overexpressing *DREB1A* (*CBF3*; Kasuga et al., 1999), suggesting heterologous *CBF1* also affects developmental processes in transgenic tomato plants. The transgenic tomato plants showed a decrease in fruit, seed number, and fresh weight as compared with wild-type plants under normal conditions. This phenomenon could be reversed by GA_3 treatment (Table I). However, seed number of transgenic plants treated with GA_3 could not be improved as compared with wild-type plants (Table I). Furthermore, there is no obvious difference of fruit, seed production, and fresh weights of transgenic tomato plants between normal or water deficit conditions regardless of GA_3 treatment (Table I). These results suggested that overexpression of the CBF1 protein interferes with production in transgenic plants. The increased resistance could have been due to less water evaporation from the dwarf phenotype of the transgenic tomato plants. However, when the growth retardation was reversed by exogenous application of GA_3, the water deficit resistance of the transgenic

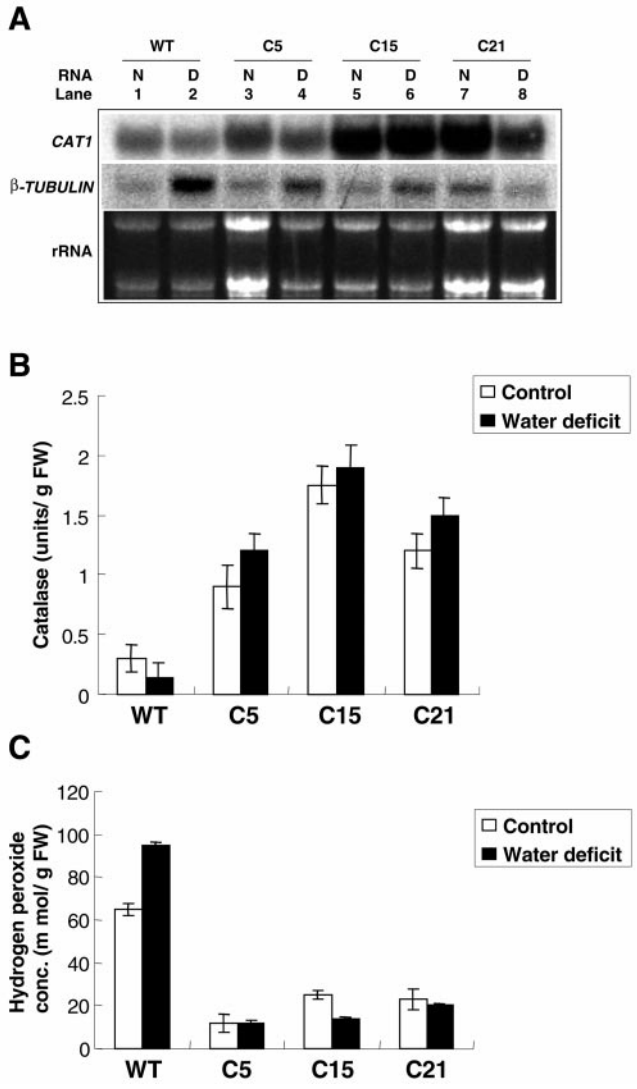

Figure 6. Transgenic tomatoes exhibit increased catalase activity but a reduction in H_2O_2 concentrations under normal and water deficit conditions. Ten micrograms of RNA was extracted from wild-type (WT) and three transgenic plants (C5, C15, and C21) grown under control conditions (N) or without watering for 12 d (D), were used to for northern-blot analysis. Probes used were the ^{32}P-labeled tomato *CAT1* gene and *β-TUBULIN* (A). Plants were grown at 24°C with regular watering (control) or without watering for 28 d (water deficit), catalase activity (B), and H_2O_2 concentration (C) were measured.

tomato plants was not lost (Fig. 3). These results indicate that the dwarf phenotype may not be a major factor determining resistance to water deficit. As shown in Figure 4, the stomata of the transgenic *CBF1* tomato plants closed rapidly after water deficit treatment as compared with wild-type plants. These results indicate that *CBF1* might have an effect on the apparent retention of water to avoid damage resultant from water deficit.

Previously, inhibition of growth under nonstressed conditions could be prevented by replacing the constitutive 35S promoter with the *RD29A* stress-inducible promoter in transgenic Arabidopsis plants expressing *DREB1A* (*CBF3*; Kasuga et al., 1999; Smirnoff and Bryant, 1999). However, the stress tolerance of transgenic Arabidopsis plants was not affected by replacing this *RD29A* stress-inducible promoter. We also conducted experiments replacing the cauliflower mosaic virus 35S promoter, used to drive the *CBF1* gene in transgenic tomatoes, with the barley *ABRC1* and Arabidopsis *COR15A* stress-inducible promoter (J. Lee, P.-T. Yang, J.-F. Wu, Y. Charng, T.H.D Ho, and M.-T. Chan, unpublished data). It was found that no plant growth retardation was derived from swapping the promoter. Moreover, water deficit resistance was also not affected in transgenic *ARBC1-CBF1* tomato plants.

Overexpression of *CBF1* can activate expression of *COR* genes in Arabidopsis plants (Jaglo-Ottosen et al., 1998) and these induced *COR* genes may play an important role in freezing tolerance. It was expected that tomato endogenous *COR* homologs may also exist and be induced by the overexpression of a CBF1 transcriptional factor. Surprisingly, the use of known Arabidopsis *COR* genes as probes, such as *KIN1*, *COR15a*, *COR47*, and *RD29A*, did not lead to cross hybridization of any RNA transcripts from transgenic tomato plants, even under low-stringency hybridization condition (data not shown). Moreover, we did not detect any mRNA transcript in transgenic tomato plants using tomato dehydrin *TAS14*, one of the *LEA* genes, as a probe (data not shown). Although we have obtained many tomato EST clones and unknown cDNA fragments from subtractive hybridization experiments, we have not obtained any known Arabidopsis *COR*- or *RD*-like genes from our subtractive library (T.-H. Hsieh and M.-T. Chan, unpublished data). These results may be due to the low homology between Arabidopsis *COR* or *RD* gene probes and tomato endogenous homologs, or unknown tomato *COR*-like, *RD*-like, or *LEA* genes induced by heterologous *CBF1*. More evidence is needed to confirm or reject this hypothesis.

Antioxidant enzymes, such as glutathione reductase and superoxide dismutase activity, increase in response to water deficit stress (Ingram and Bartels, 1996), and overexpression of antioxidant genes improves tolerance to pathogens, paraquat, and osmotic stresses (e.g. chilling, salinity, and drought; Bray et al., 2000). *CAT1* is one of the responsive genes we isolated from subtractive hybridization. Both mRNA level and catalase activity increased in transgenic tomato as compared with wild-type plants (Figs. 1 and 6, A and B). The H_2O_2 concentration was reduced in transgenic tomatoes as compared with wild-type plants (Fig. 6C). Our results support the hypothesis that activation of antioxidant genes converge the resistance of transgenic tomato plants to water deficit. The up-regulation of *CAT1* might be a

consequence of the overexpression of *CBF1*. More evidence is needed to determine if heterologous *CBF1* activates these genes directly or indirectly.

Overexpression of Δ^1-pyrroline-5-carboxylate synthase (P5CS) in transgenic tobacco plants increases Pro content by 10- to 18-fold as compared with wild-type plants, resulting in better growth under water deficit conditions (Kavi et al., 1995). The mRNA transcripts of *P5CS2* and Pro content were highly increased in transgenic Arabidopsis plants expressing *CBF3* (Gilmour et al., 2000). Pro concentrations were higher in transgenic tomato plants than in wild-type plants (Fig. 5), similar to the overexpression results of *DREB1A* (*CBF3*) in Arabidopsis (Gilmour et al., 2000). This study implies that the tomato *P5CS* gene(s) may also be induced in transgenic tomato plants. Therefore, we did detect a low-stringency hybridized band in the northern-blot analysis using the tomato EST440219 clone (accession no. BF112629), which is similar to the *P5CS* gene, as a probe. However, there was no significant difference in expression between wild-type and transgenic tomato plants (data not shown). These results suggest that other *P5CS* genes may be up-regulated in transgenic *CBF1* tomato plants. We had many unknown cDNA fragments and EST clones from subtractive hybridization (data not shown). In future experiments, we will isolate the tomato *P5CS* genes and other genes responsive to heterologous *CBF1* that are important in transgenic tomato plants with water deficit resistance. Characterization of these responsive genes will contribute to an understanding of stress resistance, and help to decipher the stress signal transduction pathways in tomato plants.

It is interesting that the transcriptional activator similar to *CBF1* does exist in different plant species (Jaglo et al., 2001), indicating that stress signal transduction pathways may be conserved in various plant species. Overexpression of *DREB1A* (*CBF3*) increases tolerance to freezing and water deficit stresses (Kasuga et al., 1999), suggesting that different stress signal transduction pathways might cross talk between each other. Recent findings indicate that there is cross talk between two stress-signaling pathways in Arabidopsis (Shinozaki and Yamaguchi-Shinozaki, 2000). Recently, we also observed that transgenic *CBF1* tomato plants have enhanced chilling tolerance as compared with wild-type plants (Hsieh et al., 2002). These results in combination with the results in this study confirm that the enhancement of stress tolerance phenomenon in transgenic tomato plants might be conferred by multiple defense systems. These activated defense systems may also protect transgenic plants from other stress conditions. We believe that a similar approach might be applicable to other important crops, such as rice, maize (*Zea mays*), wheat (*Triticum aestivum*), and barley, to improve tolerance against stress conditions. This may be accomplished by transferring several (e.g. three–four) key regulatory genes, rather than a large number of stress-related genes under inducible promoters. Overall, the engineering of stress-tolerant crops by incorporating (a) master switch gene(s) like *CBF1* may be an efficient approach to minimize stress damage.

MATERIALS AND METHODS

Plant Materials

Seeds of tomato (*Lycopersicon esculentum* L. Miller cv CL5915-93D_4-1-0-3) were provided by the Asian Vegetable Research and Development Center (Tainan, Taiwan, Republic of China). Seeds were soaked in water at 32°C for 1 h, surface sterilized for 10 min with 1% (v/v) NaOCl, washed twice with sterile water for 5 min, and subsequently germinated on Murashige and Skoog medium under a 16-h photoperiod at 26°C.

DNA Construction and *Agrobacterium tumefaciens*-Mediated Tomato Transformation

A *CBF1* gene was isolated by reverse transcriptase-PCR from 3-week-old Arabidopsis leaves as described previously (Chan and Yu, 1998). The transformation procedure followed was as described previously (Hsieh et al., 2002).

Identification of Transgenic Tomato Plants

Total RNA was isolated using a Triazole (Life Technologies/Gibco-BRL, Cleveland) solution, the DNA- and RNA-blot analyses were performed according to Chan et al. (1994). The *GUS* DNA, excised from the *Bam*HI-*Sac*I restriction fragment of plasmid pBI221 (CLONTECH Laboratories, Palo Alto, CA), and the *CBF1* gene, isolated from pT7Blue-CBF1 (Hsieh et al., 2002), were used as probes. Tomato *β-TUBULIN* cDNA fragment was isolated by reverse transcriptase-PCR from 3-month-old tomato plant leaves. The 5′ primer (5′-CCCGGGCACACTTGATCCCATTCGT-3′, *Sma*I site underlined) and the 3′ primer (5′-CCCGGGCATTCTGTCTGGGTACTCT-3′, *Sma*I site underlined) were chosen to amplify the 539-bp *β-TUBULIN* partial cDNA fragment. The PCR fragments were cloned into pT7Blue(R) and the DNA sequences were determined by a PRISM 373 automatic DNA sequencing system (ABI, Sunnyvale, CA). *CAT1* (accession no. M93719) isolated from subtractive hybridization was also used as a probe. All fragments were labeled with [α-^{32}P]dCTP using the random primer method (Feinberg and Vogelstein, 1983). Tomato seeds produced from transgenic tomato plants were collected and selection procedures were performed as described previously (Hsieh et al., 2002).

Analysis of Transgenic Plants under Water Deficit Conditions

Wild-type and transgenic tomato plants were grown under similar conditions, in pots with peat moss and watered every alternate day. Day temperature was maintained at 26°C ± 2°C and night temperature at 22°C ± 2°C. Relative humidity was maintained at 50% ± 10%. Plants were grown under 16/8 h light (about 120 μmol m^{-2} s^{-1}). Survival rate of water deficit treatment was defined as healthy plant number divided by total plant number. Pictures were taken to record the phenotypes. The water deficit treatment time was included in the growth period. After 3 months, these plants were harvested, weighed for fresh weight, and the fruit and seed numbers calculated.

For water deficit treatment, wild-type and transgenic T_1 plants were grown at 24°C and without water supply for various time periods (0, 7, 14, 21, and 28 d). For GA_3 treatment, 3-week-old wild-type and transgenic T_1 plants were sprayed with 5 μL L^{-1} GA_3 three times in a week. Three leaves or five roots were detached from each plant and weighed for fresh weight, with sampling and measurements repeated five times. Detached leaves or

rootswere then dried at 65°C for 2 d to determine dry weight. The water content was calculated based on the following equation:

$$\text{Water content} = (\text{fresh weight} - \text{dry weight})/(\text{dry weight})$$

$$= g \cdot H_2O/g \cdot \text{dry weight}$$

Chlorophyll fluorescence values were measured using a pulse-activated modulation fluorimeter (Walz, Effeltrich, Germany) according to the method described by Oberschall et al. (2000).

Leaf conductance measurements were taken from the third and fourth leaves of intact transgenic *CBF1* T_2 and wild-type plants which were growing under normal (control) and water deficit (7 d) conditions. The results were measured at an interval of a 10 h of light and 2 h of dark. Leaf conductance was measured with an LI-1600 steady-state porometer (LI-COR, Lincoln, NE).

Pro Content, Catalase Activity, and H_2O_2 Concentration Analyses

Leaves detached from plants were extracted using 3-sulfosalicylic acid, and the supernatant collected after centrifugation. Ninhydrin and acetic acid was added to the supernatant and incubated at 100°C for 60 min. It was snap chilled on ice to terminate the reaction, and then toluene was added and the absorbance at A_{520} measured.

Wild-type and transgenic T_1 plants were grown at 24°C without water for 28 d as described previously (water deficit treatment). The leaf catalase activity was measured according to Pinhero et al. (1997). The H_2O_2 concentration was analyzed as described by O'Kane et al. (1996).

Subtractive Hybridization

Poly(A^+) RNA (0.7 μg) was extracted from leaves of wild-type and transgenic tomato plants that were grown in normal conditions, and used to perform subtractive hybridization according to the CLONTECH PCR select cDNA subtraction kit manual. After PCR amplification, the PCR products were cloned into the pT7Blue(R) vector (Novagen, Madison, WI). DNA sequences were determined by an ABI PRISM 373 automatic DNA sequencing system.

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