Overexpression of the *CBF2* transcriptional Activator Enhances Oxidative Stress Tolerance in Arabidopsis Plants

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Abstract

The C-repeat/dehydration-responsive-element binding factor genes (*CBF1-3*) are transcriptional activators involved in governing plant responses to low temperatures; their overexpression enhances plant frost tolerance. We found that overexpression of *CBF2* in Arabidopsis enhanced oxidative stress tolerance as compared with wild-type plants, an effect that was manifested in: increased seed germination rates on Petri dishes containing H_2O_2; delayed leaf senescence following incubation with H_2O_2; and delayed wilting and senescence after spraying whole plants with paraquat, a generator of superoxide radicals. Transcript profiling analysis using the Affymetrix ATH1 genome array revealed that overexpression of *CBF2* did not affect expression of reactive oxygen-scavenging genes but rather, remarkably enhanced expression of oxidative-stress-responsive transcription factor genes. Overall, the present findings suggest that overexpression of *CBF2* in Arabidopsis enhances oxidative stress tolerance, most likely via activation of a network interaction among stress-related transcription-factor genes.

Keywords: Arabidopsis, CBF2, Oxidative stress, Stress tolerance, ROS

1. Introduction

Aerobic organisms utilize molecular oxygen as a terminal oxidant during respiration, and thus consistently generate reactive oxygen species (ROS) as a normal by-product of aerobic respiration (Finkel & Holbrook, 2000; Martin *et al.*, 1996). ROS include singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydroperoxyl radical (HO_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) – all of which are extremely reactive and capable of oxidizing biological molecules such as DNA, proteins, or lipids (Alscher *et al.*, 1997; Schopfer *et al.*, 2001). In addition to generating ROS in mitochondria during oxidative phosphorylation, plants also produce large amounts of ROS in the chloroplasts during photosynthesis and CO_2 fixation (Arora *et al.*, 2002; Mittler *et al.*, 2004; Munne-Bosch & Alegre, 2002). Furthermore, because plants are immobile and fixed in the soil they are constantly exposed to environmental stresses such as unfavorable temperatures (heating, chilling, and freezing), drought, salinity, flooding, pathogen attack, etc. Exposures to such stresses result in massive generation of ROS that interfere with the delicate balance of cellular redox homeostasis (Bolwell *et al.*, 2002; J. Dat *et al.*, 2000; Foyer & Noctor, 2005). Overall, their status as photosynthesizing organisms and their constant exposure to stresses make plants especially vulnerable to oxidative stresses. Because of the vital importance to control intercellular ROS levels, all aerobic organisms, including plants, developed enzymatic systems to detoxify

excessive accumulation of ROS, mainly ROS scavenging enzymes such as catalase and superoxide dismutase (Mittler *et al*., 2004). In addition, many plant transcription factor genes are also regulated by ROS accumulation (Gadjev *et al*., 2006).

Many plants, including Arabidopsis, increase their frost tolerance in response to low nonfreezing temperatures; a phenomenon known as "cold acclimation" (M. F. Thomashow, 1999). Transcript profiling experiments revealed that multiple regulatory pathways are activated during cold acclimation, and that one such important pathway involves the c-repeat binding factor (CBF) regulon (M. F. Thomashow, 1999; M.F. Thomashow, 2001). The *CBF* genes are members of a small family of three AP2 domain transcriptional activators, comprising *CBF1*, *CBF2* and *CBF*3 (Gilmour *et al.*, 2004; Shinwari *et al.*, 1998). Ectopic expression of *CBF1* in Arabidopsis induced expression of cold-regulated (COR) genes and significantly enhanced freezing tolerance even without cold acclimation(Gilmour *et al*., 2004; Jaglo-Ottosen *et al.*, 1998). In addition to frost tolerance, overexpression of *CBF* genes also induced plant tolerance towards other environmental stresses, such as drought and salinity (Kasuga *et al.*, 1999; Shinozaki & Yamaguchi-Shinozaki, 2000). Furthermore, it was reported that ectopic expression of *CBF1* also induced tolerance to water deficit, chilling, and salt stress in tomato plants (Hsieh *et al.*, 2002a; Lee *et al.*, 2003). Moreover, Hsieh *et al*. (2002b) suggested that overexpression of *CBF1* increased chilling tolerance in tomato by enhancing *CATALASE1* gene expression and enzyme activity, and oxidative stress tolerance (Hsieh *et al*., 2002b). In addition to its effects on induction of plant stress tolerance, we recently reported that overexpression of *CBF2* in Arabidopsis also considerably delayed leaf senescence and extended the life span of the plants by approximately 2 weeks as compared with wild-type plants (Sharabi-Schwager *et al*., 2010). In this study, we show that overexpression of *CBF2* enhanced oxidative stress tolerance in Arabidopsis, as manifested in: increased seed germination rates on Petri dishes containing H_2O_2; delayed leaf senescence following incubation with H_2O_2; and delayed wilting and senescence following spraying of whole plants with paraquat, a generator of superoxide radicals. Also, during growth and development the *CBF2*-overexpressing plants accumulated much lower levels of H_2O_2 and O_2^- radicals than wild-type plants. Moreover, transcriptome analysis with the Affymetrix ATH1 genome array revealed that overexpression of *CBF2* may have enhanced oxidative stress tolerance via activation of a network of oxidative-stress-responsive transcription factor genes.

2. Materials and Methods

2.1 Plant Material and Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Wassilewskija (WS-2) and of transgenic plants overexpressing the *CBF2* gene in a WS-2 background were obtained from Prof. M. Thomashow of Michigan State University, MI, USA (Gilmour *et al*., 2004). Before sowing, seeds were sterilized in 5% bleach and immersed in water at 4°C for 48 h to ensure uniform germination. The plants were grown in 7 × 7 × 8 cm plastic pots filled with a commercial growing-soil mix, at a constant temperature of 22°C, and illuminated by cool-white fluorescent lamps at approximately 100 μmol m^{-2} s^{-1}, with a photoperiod of 16 h. Plants were grown at a density of four plants per pot. In some experiments, seeds were grown on Petri dishes containing 0.8% agar and 0.5 × Murashige and Skoog (MS) medium including Gamborg B5 vitamins (Duchefa Biochemie, Haarlem, the Netherlands) at pH 5.7, as described (Weigal & Glazebrook, 2002).

2.2 Chlorophyll Content

Chlorophyll content was measured in 5-mm-diameter leaf disc samples. Chlorophyll was extracted from two leaf discs placed in a microtube containing 1 mL of 80% acetone. The discs were homogenized with a fitted pestle and incubated overnight at 4°C. Chlorophyll content in the acetone extracts was measured spectrometrically according to Porra *et al*. (Porra *et al.*, 1989). Each measurement included four replications.

2.3 Electrolyte Leakage

Electrolyte leakage was measured by placing entire rosettes in scintillation vials containing 10 mL of double-distilled water. The first reading was taken after 2 h of incubation at room temperature with gentle agitation, and afterwards the rosettes were exposed to a high level of microwave radiation for 2 min, to destroy all living cells. The vials were then cooled to room temperature, and second readings were taken. Electrolyte leakage data are presented as percentages of the total amount of electrolytes present in the tissue.

2.4 Exposure to Oxidative Stress

Oxidative stress tolerance of wild-type and *CBF2*-overexpressing plants was evaluated by three different means. First, we examined seed germination rates following sowing on MS medium containing various concentrations (0-10 mM) of H_2O_2 in Petri dishes. The seed germination rate was determined as the percentage of seeds that survived with each concentration of H_2O_2 after 7 days at 22°C. Second, we evaluated the degree of yellowing of

detached leaves following incubation in 0-10 mM H_2O_2 solutions: leaves numbers 5 and 6 were detached from 36-day-old plants, and incubated for 72 h at 22°C, adaxial side up, in Petri dishes containing H_2O_2 at 0-10 mM. Third, 36-day-old wild-type and *CBF2*-overexpressing plants were sprayed with various concentrations (0-30 μM) of paraquat (Sigma, St Louis, MO, USA), a generator of superoxide radicals. In all these experiments, seeds, detached leaves, and whole plants were kept at 22°C under a 16-h photoperiod of illumination at ~100 μmol m^{-2} s^{-1}.

2.5 NBT and DAB Staining

To evaluate H_2O_2 and O_2^- levels in plant tissues we used 3,3'-diaminobenzidine (DAB) (J. F. Dat *et al.*, 2003), and nitroblue tetrazolium (NBT) (Le Deunff *et al*., 2004) staining procedures, respectively. Briefly, leaves numbers 5 and 6 were detached from wild-type and *CBF2*-overexpressing plants at various development stages and were vacuum infiltrated for 20 min in 2.5 mM DAB or 500 mM NBT solutions in citrate buffer (10 mM citrate, pH 6.0). Afterwards leaves were washed several times with 80% ethanol in order to remove the green chlorophyll, and the intensity of DAB or NBT staining was assessed visually by photography. In one experiment, entire rosettes of 36-day-old plants were stained in DAB and NBT solutions.

2.6 Transcript Profiling Analysis

Total RNA was isolated from leaves numbers 5 and 6 of 40-day-old wild-type and *CBF2*-overexpressing plants by phenol/chloroform extraction and precipitation with LiCl, according to standard procedures (Sambrook *et al*., 1992). For each treatment, we performed three separate RNA extractions, each involving leaves from 5 to 10 different plants. The RNA samples were prepared for hybridization according to the protocols outlined in the Affymetrix GeneChip Expression Analysis Technical Manual, and were hybridized to the Affymetrix Arabidopsis ATH1 Genome Array representing ~24,000 genes (Affymetrix, Santa Clara, CA, USA). Hybridizations were performed at the Department of Biological Services in the Weizmann Institute of Science, Rehovot, Israel. Data were analyzed with the Affymetrix Microarray Suite 5.0 (MAS5.0) statistical algorithms. Further advanced data analyses, including background subtraction, normalization and elimination of false positives, were performed with the Partek Genomics Suite (Partek GS) statistical and data visualization program. One-way analysis of variance was used to identify probe sets that exhibited significant changes in signal levels at $P \leq 0.05$.

3. Results

3.1 Effects of CBF2-Overexpression on Oxidative Stress Tolerance

We evaluated the effects of *CBF2*-overexpression on oxidative stress tolerance by three different means: a) evaluation of seed germination on MS medium containing various concentrations of 0-10 mM H_2O_2; b) evaluation of leaf yellowing following incubation in 0-10 mM H_2O_2 solutions; and c) evaluation of whole plant wilting following sprays with 0-30 μM paraquat. It can be seen that in all cases, *CBF2*-overexpressing plants were more tolerant to the imposed oxidative stresses than wild-type plants.

In the seed germination assay, we found that seeds of both wild-type and *CBF2*-overexpressing plants had high germination rates (~95%) on MS agar media containing up to 4 mM H_2O_2. However, at higher concentrations – of 6 and 8 mM H_2O_2 – germination rates were significantly higher in seeds of the transgenic line: 68 and 38%, respectively, in *CBF2*-overexpressing plants as compared with just 32 and 13%, respectively, in those of wild-type plants (Figure 1). At the high concentration of 10 mM H_2O_2 seed germination was very low (below 14%) in both wild-type and *CBF2*-overexpressing plants (Figure 1).

In the leaf-yellowing assay, we found that leaves of wild-type plants lost chlorophyll following incubation at the lowest concentration of 2.5 mM H_2O_2, whereas those of *CBF2*-overexpressing plants began to loose chlorophyll only after incubation at the higher concentration of 5 mM H_2O_2 (Figure 2). Overall, leaves of wild-type plants lost 50% of their chlorophyll content, as compared with leaves incubated in water alone, after incubation in 5 mM H_2O_2, whereas leaves of *CBF2*-overexpressing plants lost this proportion only after incubation in H_2O_2 at the highest concentration of 10 mM (Figure 2B). It can be seen (Figure 2A) that leaves of wild-type plants became transparent and lost almost all of their chlorophyll following incubation at 7.5 and 10 mM H_2O_2, whereas leaves of *CBF2*-overexpressing plants remained green and viable even after incubation at the highest H_2O_2 concentration of 10 mM.

In the paraquat spray assay, we found that *CBF2*-overexpressing plants were much more tolerant than wild-type plants to the generated superoxide. It can be seen (Figure 3) that wild-type rosettes suffered from slight necrosis and increased electrolyte leakage rates already after being sprayed with paraquat at the lowest concentration of 10 μM, whereas the *CBF2*-overexpressing plants still remained green and healthy. Furthermore, rosettes of

wild-type plants wilted completely after receiving 20-30 μM paraquat, whereas those of *CBF2*-overexpressing plants showed necrosis symptoms mainly at the high paraquat concentration of 30 μM (Figure 3A). Electrolyte leakage rates in rosettes of wild-type plants increased continuously from 33% without paraquat to 42, 56, and 76% following paraquat sprays at 10, 20, and 30 μM, respectively, whereas electrolyte leakage rates of *CBF2*-overexpressing plants increased above base level only after exposure to paraquat at the highest concentration of 30 μM (Figure 3B).

3.2 Effects of CBF2-Overexpression on H_2O_2 and O_2^- Contents in Leaves and Rosettes

We used DAB and NBT staining to evaluate the accumulation of H_2O_2 and O_2^- in leaves of wild-type and *CBF2*-overexpressing plants during plant development. Figures 4 and 5, respectively, show that in wild-type plants both H_2O_2 and O_2^- began to accumulate in leaf tissue during initiation of flowering, peaked at mid-flowering, and then declined. In contrast, we did not detect any accumulation of either H_2O_2 or O_2^- in leaves of *CBF2*-overexpressing plants at any stage of plant development (Figures 4 and 5). Figure 6 shows ROS accumulation in rosettes of wild-type and *CBF2*-overexpressing plants 36 days after sowing. Once again, it can be seen that rosettes of wild-type plants accumulated high levels of H_2O_2 and O_2^-, whereas those of *CBF2*-overexpressing plants contained only minor levels of these radicals (Figure 6).

3.3 Effects of CBF2 Overexpression on Transcript Levels of ROS-Scavenging and Oxidative-Stress-Responsive Transcription Factor Genes

In order to identify the molecular mechanisms that might be involved in governing the enhanced tolerance of *CBF2*-overexpressing plants to oxidative stress we analyzed transcript profiles with the Affymetrix ATH1 genome array, and examined the expression patterns of ROS-scavenging and oxidative-stress-responsive transcription factor genes. The list of Arabidopsis ROS-scavenging genes was taken from the review article by Mittler *et al*. (Mittler *et al*., 2004). Table 1 shows that none of the Arabidopsis ROS-scavenging genes, including *superoxide dismutase* (*SOD*)*, catalase* (*Cat*)*, ascorbate peroxidase* (*APX*)*, monodehydroascorbate reductase* (*MDAR*)*, dehydroascorbate reductase* (*DHAR*)*, glutathione reductase* (*GR*)*, glutathione peroxidase* (*GPX*)*, peroxiredoxin* (*PrxR*) and *ferritin* were induced or repressed by more than a factor of 2. Thus, overexpression of *CBF2* did not have any direct effect on ROS-scavenging transcript levels.

In addition to the ROS-scavenging system, (Gadjev *et al*., 2006), on the basis of various ROS-related microarray experiments, defined a group of 32 regulatory Arabidopsis transcription-factor genes whose expression levels were elevated at least fivefold following exposure to various ROS, and Table 2 shows that the transcript levels of 18 out of these 32 oxidative-stress-responsive transcription factor genes were up-regulated at least threefold in *CBF2*-overexpressing plants. Furthermore, the transcript levels of 10 of these ROS-responsive transcription factor genes were remarkably up-regulated, by more than fivefold, in *CBF2*-overexpressing plants (Table 2). The ROS-responsive transcription-factor genes induced by *CBF2* overexpression belong to various families, including WRKY, NAM, C_2H_2 zinc finger, CCCH-type zinc finger AP2, HSF, MYB, ZAT (Table 2).

4. Discussion

In the present study, we showed that overexpression of *CBF2* further increased oxidative-stress tolerance in Arabidopsis plants, and we demonstrated this by various assays, including evaluation of seed germination, leaf yellowing and whole-plant wilting, in response to various ROS treatments (Figures 1-3). Although not reported before, the finding that overexpression of *CBF2* enhanced oxidative-stress tolerance in Arabidopsis is not very surprising; it is known that overexpression of *CBF* genes enhanced plant tolerance towards various environmental stresses, such as freezing, drought, and salinity (Kasuga *et al*., 1999; Shinozaki & Yamaguchi-Shinozaki, 2000). Furthermore, it was previously reported that ectopic expression of *CBF1* increased tolerance of chilling and oxidative stress also in tomato plants (Hsieh *et al*., 2002b).

Since generation of ROS and activation of oxidative processes are known to provide integral components of the senescence syndrome in all aerobic organisms, including plants (Finkel & Holbrook, 2000; Martin *et al*., 1996; Prochazkova *et al.*, 2001; Zimmermann & Zentgraf, 2005), we hypothesize that overexpression of *CBF2* might have delayed leaf and whole-plant senescence, at least in part, by increasing oxidative-stress tolerance and/or by reducing ROS accumulation during plant development. Indeed, our present findings clearly demonstrate that overexpression of *CBF2* increased oxidative stress tolerance and remarkably reduced accumulation of H_2O_2 and O_2^- radicals in leaf tissue during development, as compared with that in wild-type plants (Figures 4-6).

Therefore, by analogy with previous studies, which reported that the Arabidopsis delayed-leaf-senescence mutants *ore1, ore3*, and *ore9,* and the long-living mutant *gigantea* exhibited enhanced tolerance to oxidative stresses (Kurepa *et al.*, 1998; Woo *et al*., 2004), we hereby suggest that ectopic expression of *CBF2* also might

have delayed senescence and extended plant longevity via enhancement of oxidative stress tolerance. Similar correlations between oxidative stress resistance and extension of life span were reported in various aerobic organisms, ranging from yeasts to mammals (Johnson *et al.*, 1996; Kapahi *et al.*, 1999; Orr & Sohal, 1994).

Finally, we do not yet know for certain the exact mechanism by which overexpression of *CBF2* might have increased oxidative stress tolerance. In tomato, it was reported that overexpression of *CBF1* increased chilling tolerance by enhancing *CATALASE1* gene expression and enzyme activity (Hsieh *et al*., 2002b). However, in the current study, we could not detect any induction of genes involved in ROS-scavenging, but rather detected a remarkable up-regulation of transcript levels of 18 out of 32 Arabidopsis ROS-responsive transcription-factor genes (Tables 1-2) (Gadjev *et al*., 2006). Therefore, we suggest that overexpression of *CBF2* might have enhanced oxidative stress tolerance in Arabidopsis via activation of a network of oxidative-stress-responsive transcription-factor genes.

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Table 1. Expression levels of reactive oxygen species (ROS) scavenging network genes in mature leaves of wild-type (WS ecotype) and *CBF2*-overexpressing plants.

Enzyme and Reaction	Locus	Name	Fold change (*CBF2*-ox / WS)
Superoxide Dismutase (SOD)	At4g25100.3	FeSOD (FSD1)	-1.05
$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	At5g51100.1	FeSOD (FSD2)	-1.04
	At5g23310.1	FeSOD (FSD3)	-1.03
	At1g08830.1	Cu/ZnSOD (CSD1)	1.24
	At2g28190.1	Cu/ZnSOD (CSD2)	1.33
	At5g18100.1	Cu/ZnSOD (CSD3)	1.34
	At3g10920.1	MnSOD (MSD1)	1.08
	At3g56350.1	MnSOD-like	nd
Ascorbate Peroxidase (APX)	At1g07890.1	APX1	-1.18
$2\ Asc + H_2O_2 \rightarrow 2\ MDA + 2H_2O$	At3g09640.1	APX2	nd
	At4g35000.1	APX3	-1.00
	At4g09010.1	APX4	1.29
	At4g35970.1	APX5	nd
	At4g32320.1	APX6	-1.01
	At1g33660.1	APX7	nd
	At4g08390.2	stromal-APX	1.21
	At1g77490.1	thylakoid-APX	1.07
Monodehydroascorbate Reductase (MDAR)	At1g63940.4	MDAR1	1.38
$MDA + NAD(P)H + H^+ \rightarrow Asc + NAD(P)^-$	At3g09940.1	MDAR2	nd
	At3g27820.1	MDAR3	1.07
	At3g52880.1	MDAR4	1.21
	At5g03630.1	MDAR5	-1.83
Dehydroascorbate Reductase (DHAR)	At5g16710.1	DHAR1	1.01
$DHA + 2\ GSH \rightarrow Asc + GSSG$	At5g36270.1	DHAR2	nd
	At1g75270.1	DHAR3	-1.12
	At1g19550.1	DHAR4	nd
	At1g19570.1	DHAR5	1.77
Glutathione Reductase (GR)	At3g24170.1	GR1	1.09
$GSSG + NAD(P)H \rightarrow 2\ GSH + NAD(P)^-$	At3g54660.1	GR2	1.05
Catalase (Cat)	At1g20630.1	Cat1	1.29
$2H_2O_2 \rightarrow 2H_2O + O_2$	At4g35090.1	Cat2	-1.10
	At1g20620.1	Cat3	-1.86
Glutathione Peroxidase (GPX)	At2g25080.1	GPX1	-1.10
$H_2O_2 + 2\ GSH \rightarrow 2H_2O + GSSG$	At2g31570.1	GPX2	-1.15
	At2g43350.1	GPX3	-1.07
	At2g48150.1	GPX4	nd
	At3g63080.1	GPX5	-1.08
	At4g31870.1	GPX7	1.11
	At1g63460.1	GPX8	-1.17
	At4g11600.1	Phospholipid GPX6	1.33
Ferritin	At5g01600.1	Ferritin 1	1.02
$Fe + P \rightarrow P\text{-}Fe$	At3g56090.1	Ferritin 2	-1.08
	At2g40300.1	Ferritin 3	1.22
	At3g11050.1	Ferritin 4	nd
Peroxiredoxin (PrxR)	At1g48130.1	1- cys PrxR	nd
$2P\text{-}SH + H_2O_2 \rightarrow P\text{-}S\text{-}S\text{-}P + 2H_2O$	At3g11630.1	2-cys PrxR A	1.07
	At5g06290.1	2-cys PrxR B	1.13069
	At3g06050.1	2-cys PrxR F	1.15
	At3g26060.1	PrxR Q	1.08
	At1g65990.1	Type 2 PrxR A	nd
	At1g65980.1	Type 2 PrxR B	-1.11
	At1g65970.1	Type 2 PrxR C	nd
	At1g60740.1	Type 2 PrxR D	nd
	At3g52960.1	Type 2 PrxR E	1.09
	At3g03405.1	Type 2 PrxR-related	nd

The list of Arabidopsis ROS-scavenging genes was taken from Mittler *et al*. (2004). For gene expression analysis, RNA was isolated from leaves numbers. 5 and 6 of wild-type and CBF2-overexpressing plants 40 days after sowing. Expression values are from gene profiling experiment using the Affymetrix ATH1 genome array. nd, not detected (expression level below background).

Table 2. Expression levels of oxidative stress-responsive transcription factor genes in mature leaves of wild-type (WS ecotype) and *CBF2*-overexpressing plants.

Locus	**Name**	**Fold change (*CBF2*-ox / WS)**
At5g13080	AtWRKY75	nd
At1g62300	AtWRKY6	4.01§
At1g10585	bHLH transcription factor	2.77
At3g04070	NAM transcription factor	3.23¥
At2g38340	DREB transcription factor	nd
At2g38250	GT-1-like transcription factor	nd
At2g30250	AtWRKY25	4.56§
At1g52890	NAM transcription factor	4.37§
At2g43000	NAM transcription factor	nd
At3g15500	AtNAC3	nd
At5g63790	NAM transcription factor	6.27£
At2g26150	AtHSFA2	nd
At5g05410	DREB2A	2.86
At4g23810	AtWRKY53	4.82§
At1g43160	ERF/AP2 transcription factor	2.28
At4g17230	scarecrow-like transcription factor	4.48§
At4g17490	AtERF6	nd
At4g17500	AtERF1	nd
At5g04340	C_2H_2 zinc finger	5.18£
At5g59820	ZAT12	6.09£
At5g47220	AtERF2	nd
At3g50260	DREB transcription factor	3.27¥
At4g27410	NAM transcription factor	7.66£
At4g18880	AtHsfA4A	6.03£
At1g25560	AP2 transcription factor	5.54£
At1g18570	AtMYB51	nd
At3g23250	AtMYB15	nd
At1g77450	NAM transcription factor	5.88£
At1g27730	ZAT10	6.43£
At1g80840	AtWRKY40	4.17§
At2g40140	CCCH-type zinc finger	5.10£
At2g38470	AtWRKY33	6.13£

The list of transcription factors commonly up-regulated (at least fivefold) in Arabidopsis by oxidative stress is according to Gadjev *et al*. (2006). Changes by factors of 3-4, 4-5, and >5 are marked by **¥**, **§** and **£**, respectively. For gene expression analysis, RNA was isolated from leaves numbers 5 and 6 of wild-type and CBF2-overexpressing plants 40 days after sowing. Expression values are from gene profiling experiment with the Affymetrix ATH1 genome array. nd, not detected (expression level below background).

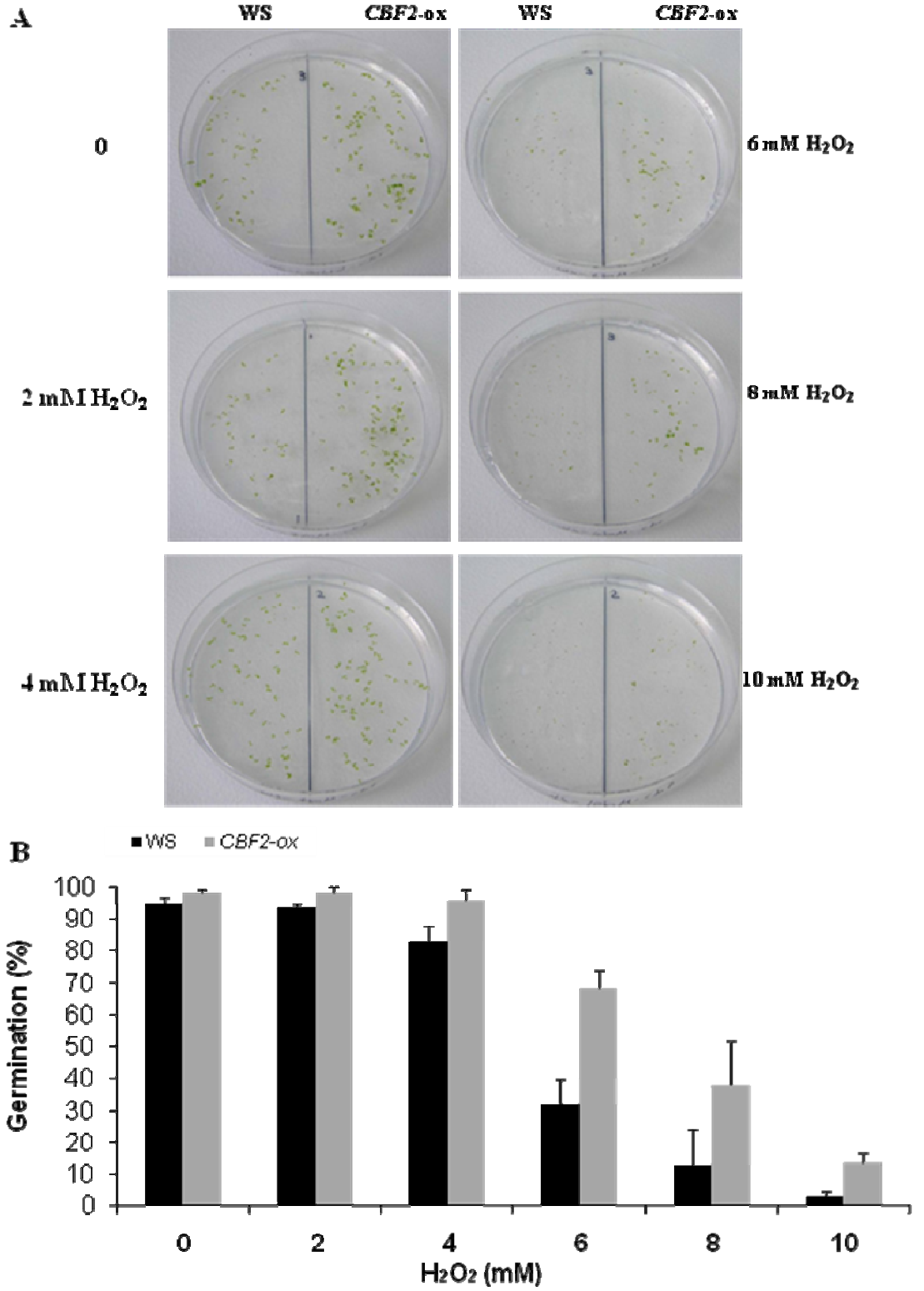

Figure 1. Effects of hydrogen peroxide (H_2O_2) on seed germination of wild-type and *CBF2*-overexpressing plants. Seeds were grown in Petri dishes in MS-agar in the presence of several different concentration (0-10 mM) of H_2O_2 A, Photographs taken after 7 days of growth at 22˚C. B, Seed germination rates following growth in the presence of various concentrations of H_2O_2. Data are means ± S.E. of two different experiments, each including three replications.

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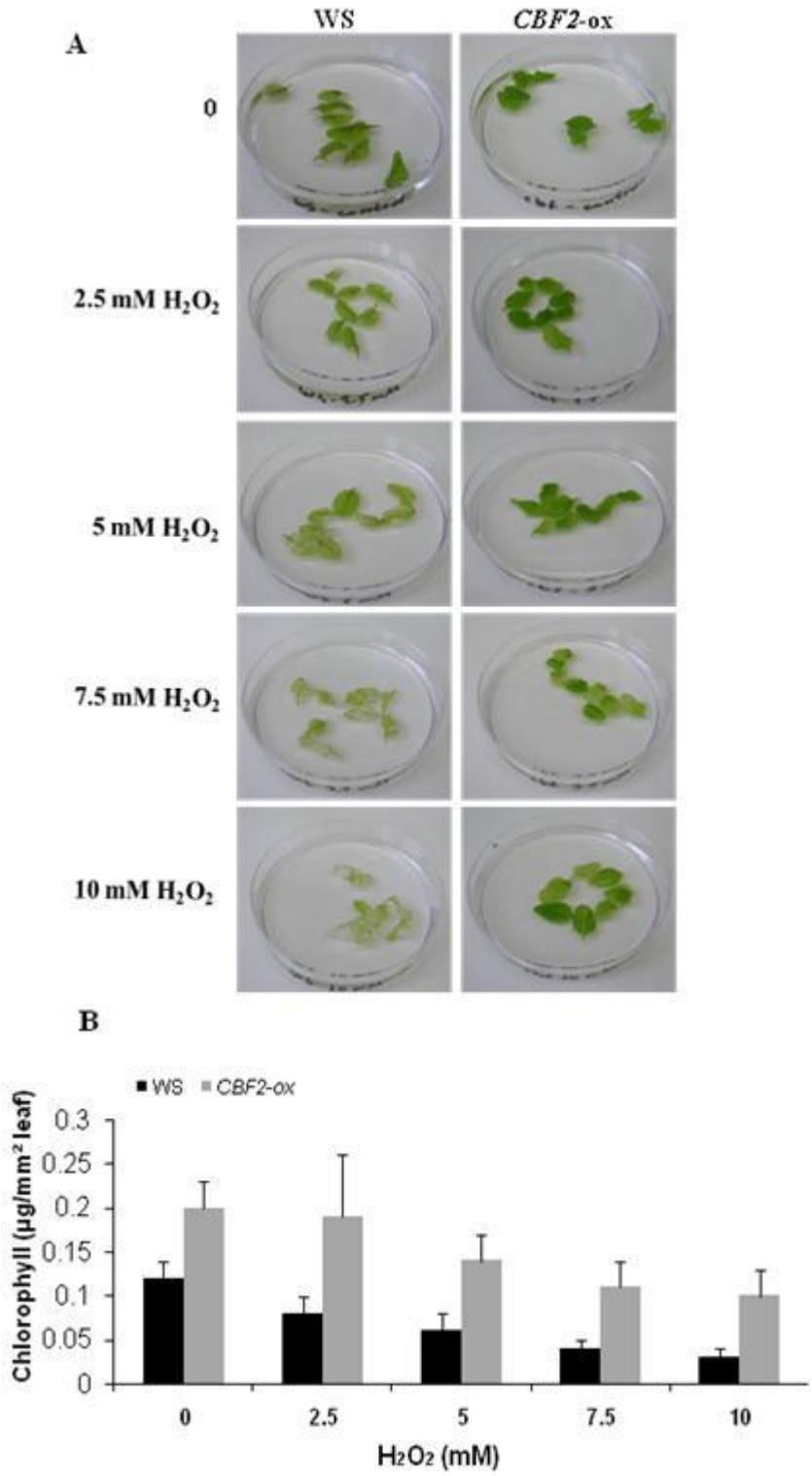

Figure 2. Effects of hydrogen peroxide (H_2O_2) on yellowing of detached leaves of wild-type and *CBF2*-overexpressing plants. A, Photographs of detached leaves after 72 h of incubation in 0-10 mM H_2O_2. B, Chlorophyll contents of detached leaves after incubation in 0-10 mM H_2O_2. Data are means of three different experiments, each including four replications.

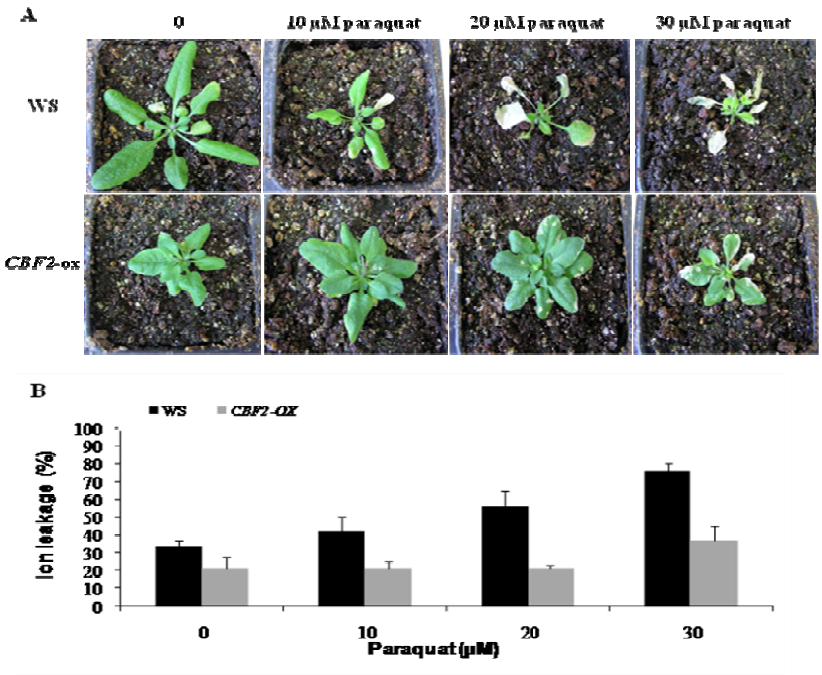

Figure 3. Effects of paraquat sprays on wilting and necrosis and of wild-type and *CBF2*-overexpressing plants. A, Photographs taken 7 days after spraying the plants with 10-30 μM paraquat. B, Ion leakage rates after spraying the plants with 10-30 μM paraquat. Data are means of three different experiments, each including four replications.

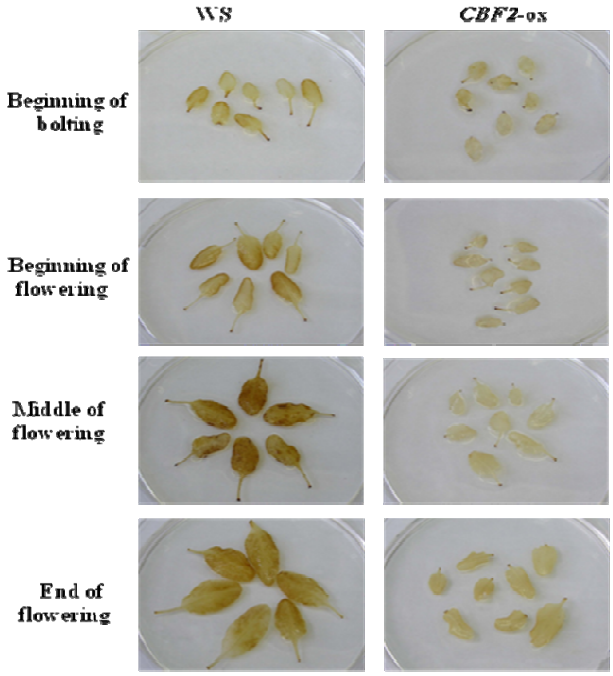

Figure 4. Evaluation of hydrogen peroxide (H_2O_2) contents and accumulation in detached leaves of wild-type and *CBF2*-overexpressing plants following 3,3'-diaminobenzidine (DAB) staining. Leaves in positions 5 and 6 were harvested from rosettes at various stages of plant development, including beginning of bolting, beginning of flowering, middle of flowering, and end of flowering.

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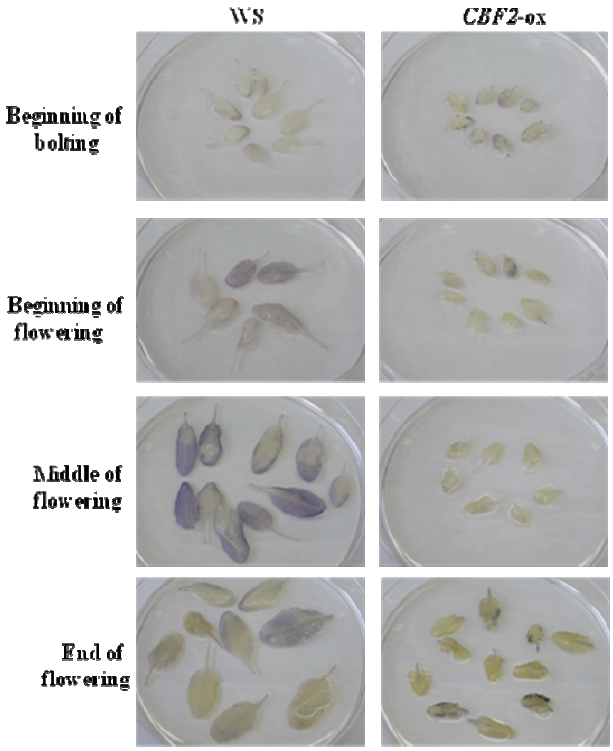

Figure 5. Evaluation of superoxide radical (O_2^-) content and accumulation in detached leaves of wild-type and *CBF2*-overexpressing plants following nitroblue tetrazolium (NBT) staining. Leaves in positions 5 and 6 were harvested from rosettes at various stages of plant development, including beginning of bolting, beginning of flowering, middle of flowering, and end of flowering.

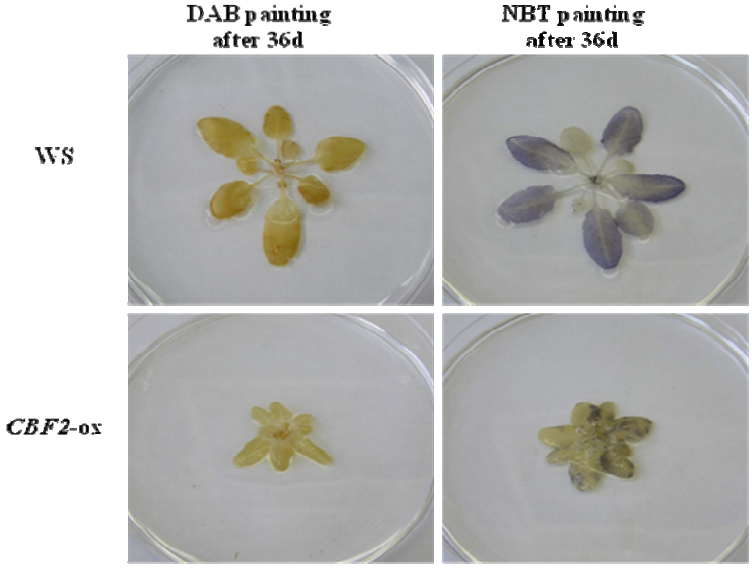

Figure 6. Evaluation of hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) contents in 36-day-old rosettes of wild-type and *CBF2*-overexpressing plants following 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining.