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RESEARCH PAPER

Overexpression of the *CBF2* transcriptional activator in Arabidopsis delays leaf senescence and extends plant longevity

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Abstract

Leaf senescence is a programmed developmental process governed by various endogenous and exogenous factors, such as the plant developmental stage, leaf age, phytohormone levels, darkness, and exposure to stresses. It was found that, in addition to its well-documented role in the enhancement of plant frost tolerance, overexpression of the C-repeat/dehydration responsive element binding factor 2 (*CBF2*) gene in *Arabidopsis* delayed the onset of leaf senescence and extended the life span of the plants by approximately 2 weeks. This phenomenon was exhibited both during developmental leaf senescence and during senescence of detached leaves artificially induced by either darkness or phytohormones. Transcriptome analysis using the Affymetrix ATH1 genome array revealed that overexpression of *CBF2* significantly influenced the expression of 286 genes in mature leaf tissue. In addition to 30 stress-related genes, overexpression of *CBF2* also affected the expression of 24 transcription factor (TF) genes, and 20 genes involved in protein metabolism, degradation, and post-translational modification. These results indicate that overexpression of *CBF2* not only increases frost tolerance, but also affects other developmental processes, most likely through interactions with additional TFs and protein modification genes. The present findings shed new light on the crucial relationship between plant stress tolerance and longevity, as reported for other eukaryotic organisms.

Key words: *Arabidopsis*, CBF, longevity, senescence, stress.

Introduction

Senescence is a complex and highly regulated process that occurs as part of plant development or can be prematurely induced by stresses (Buchanan-Wollaston *et al.*, 2003, 2005; Lim *et al.*, 2007). Although deteriorative in nature, leaf senescence is crucial for plant fitness, and is essential for the mobilization and recycling of nutrients from mature leaf tissues to the developing reproductive structures (seeds and fruit) (Buchanan-Wollaston *et al.*, 2003, 2005; Lim *et al.*, 2003, 2007; Lin and Wu, 2004). Leaf senescence occurs in an orderly manner, beginning with the degeneration of the chloroplast and hydrolysis and remobilization of macromolecules, followed by the degeneration of the mitochondrion and nucleus (Buchanan-Wollaston *et al.*, 2003, 2005; Lim *et al.*, 2003, 2007; Guo *et al.*, 2004; Lin and Wu, 2004; Van der Graaff *et al.*, 2006). Recent transcriptome studies using the *Arabidopsis* ATH1 genome array revealed thousands of genes that are up- or down-regulated during natural and dark-induced leaf senescence (Lin and Wu, 2004; Buchanan-Wollaston *et al.*, 2005; Van der Graaff *et al.*, 2006). Among these, it was reported that the transcript levels of 96 transcription factor (TF) genes were up-regulated at least 3-fold during developmental senescence, and that transcripts of 303 and 81 TFs were up- and down-regulated, respectively, during dark-induced leaf

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Table 1. Developmental stages and progression of senescence in wild-type (WS-2 ecotype) and *CBF2*-overexpressing *Arabidopsis* plants

Individual plants in 7×7×8 cm pots were grown at 22 °C under fluorescent lights (~100 μmol m^{-2} s^{-1}) and a 16 h photoperiod. Data are means ±SE of two separate experiments, each including measurements of 24–32 plants.

	Wild type (WS-2 ecotype)	*CBF2*-overexpression	Δ
No. of rosette leaves	9±1	11±1	
Time to bolting (d)	25±2	30±3	5
Time until 1st flower opened (d)	29±2	36±3	7
Begin yellowing of leaf no. 4 (d)	48±3	62±4	14
First silique shuttered (d)	48±2	55±3	7
Complete rosette yellowing (d)	72±3	88±4	16

senescence (Lin and Wu, 2004; Buchanan-Wollaston *et al.*, 2005). Thus, regulation and execution of leaf senescence form a complex process involving the activation of many biochemical and regulatory pathways. Further genetic and molecular studies have revealed the participation of the WRKY6 and WRKY53 TFs, and of AtNAP, an NAC family TF, in the signalling network involved in the regulation of senescence-specific gene expression (Robatzek and Somssich, 2001; Guo *et al.*, 2004; Miao *et al.*, 2004). In many monocarpic plants, such as soybean, the developing reproductive structures often govern the timing and onset of leaf senescence. In other monocarpic plants, however, such as *Arabidopsis*, the development of the reproductive structures indeed shortens the overall life span of the plant by preventing the regeneration of new leaves and bolts, but does not directly affect the longevity of individual leaves (Noodén and Penney, 2001).

Besides developmental control, leaf senescence is markedlyaffected by exposure to hormones: ethylene (Grbic and Bleecker, 1995), abscisic acid (ABA) (Zeevaart and Creelman, 1988), salicylic acid (SA) (Morris *et al.*, 2000), and jasmonic acid (JA) (He *et al.*, 2002) accelerate leaf senescence, whereas increased cytokinin levels delay senescence (Gan and Amasino, 1995). In addition, leaf senescence is affected by environmental factors, such as light intensity and nutrient supply (Buchanan-Wollaston *et al.*, 2003; Yoshida, 2003). Leaf senescence may also be induced by carbohydrate accumulation or by the availability of excess carbon relative to low levels of nitrogen (Wingler *et al.*, 2006).

In many aerobic organisms, including fungi, yeasts, nematodes, fruit flies, mice and humans, it was noted that increased resistance to stresses, especially oxidative stress, is correlated with extended longevity, and this has led to the 'stress resistance' theory of ageing, which hypothesizes that increased resistance to intrinsic and extrinsic stresses leads to a prolonged life span (Johnson *et al.*, 1996; Finkel and Holbrook, 2000; Murakami and Johnson, 2003; Pardon, 2007). In plants too, it was reported that there is a tight correlation between exposure to environmental stresses and determination of life span. In general, leaf senescence is accelerated by exposure to environmental stresses that have negative consequences for plant growth and development; these include extreme light or temperatures, radiation, drought, nutrient deficiency, pathogen infection, flooding, and the presence of toxic materials in the air, water or soil (Lers, 2007). In fact, exposure to stresses is estimated to be the primary cause of crop losses worldwide, because of premature senescence, which has the potential to reduce the average yield of main crops by more than 50% (Navabpour *et al.*, 2003). On the other hand, it was found that prolonged-life-span mutants exhibit enhanced tolerance to oxidative stresses, as exemplified by the *Arabidopsis* delayed-leaf-senescence mutants *ore1, ore3*, and *ore9*, and the long-lived mutant *gigantea* (Kurepa *et al.*, 1998; Woo *et al.*, 2004). Indeed, transcript profiling studies have revealed the occurrence of considerable cross-talk between stress responses and leaf senescence (Lim *et al.*, 2007). For example, among 43 TF genes that were found to be induced during senescence, 28 were also induced by exposure to various stresses (Chen *et al.*, 2002). Furthermore, the expression of many senescence-associated genes (SAGs), such as the *Arabidopsis SEN1* gene, is commonly regulated both by the initiation of leaf senescence and by exposure to stresses (Schenk *et al.*, 2005).

Many plants, including *Arabidopsis*, increase their frost tolerance in response to low, non-freezing temperatures; a phenomenon known as 'cold acclimation' (Thomashow, 1998, 2001). Transcript profiling experiments have shown that multiple regulatory pathways are activated during cold acclimation, and one such important pathway involves the CBF/DREB1 regulon (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). The CBF/DREB1 proteins (CRT binding factor or DRE binding protein) are transcriptional regulators that bind specifically to the cold- and dehydration-responsive *cis*-element, designated the CRT (C-repeat)/DRE (dehydration response element), present in the promoter of *COR* (cold-regulated) genes and a multitude of other stress-responsive genes, collectively known as the CBF regulon (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Liu *et al.*, 1998). Ectopic expression of *CBF1* driven by the *CaMV-35S* constitutive promoter in *Arabidopsis* induced the expression of *COR* genes and significantly enhanced freezing tolerance (Jaglo-Ottosen *et al.*, 1998). Further studies confirmed that overexpression of *CBF1*, *CBF2*, and *CBF3* in *Arabidopsis* have matching functional activities that mimicked multiple biochemical changes associated with cold acclimation (Gilmour *et al.*, 2004). In addition to the effects on frost tolerance, overexpression of the *CBF1*, *CBF2*, and *CBF3* genes in *Arabidopsis* resulted in growth retardation and occurrence of a 'dwarf' phenotype (Liu *et al.*, 1998; Gilmour *et al.*, 2004). A recent study found that the constitutive expression of *CBF1* not only led to frost tolerance but also caused growth retardation by allowing the accumulation of DELLAs, a family of nuclear growth-repressing proteins, whose degradation is stimulated by gibberellins (GA) (Achard *et al.*, 2008).

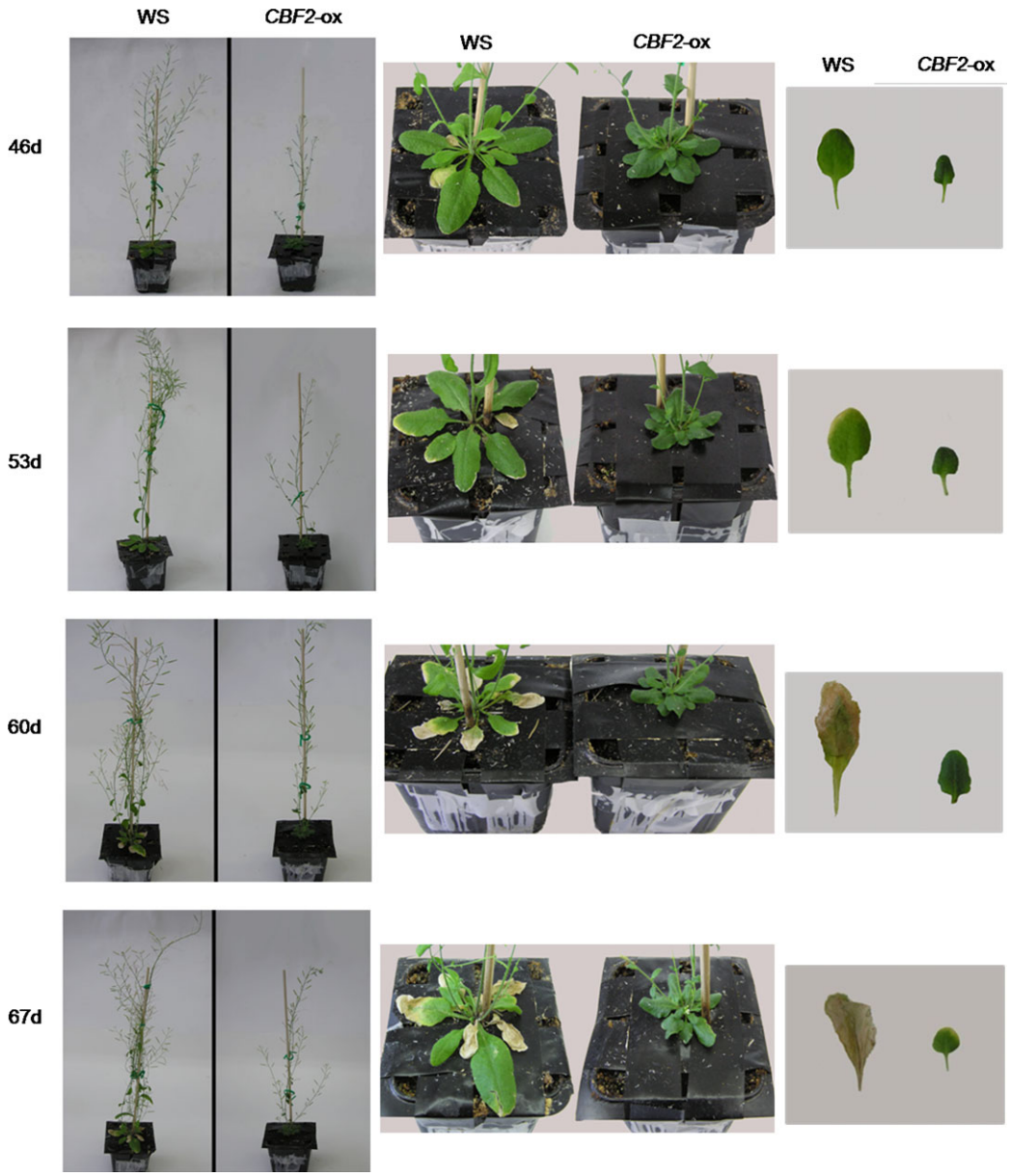

Fig. 1. Photographs of wild-type (WS-2 ecotype) and *CBF2*-overexpressing plants during developmental senescence. The left column shows the phenotypes of whole plants; the middle column enlargements of rosettes; and the right column the appearance of leaf number four in each rosette. The pictures were taken 46, 53, 60, and 67 d after sowing.

In the present study, it has been shown that, in addition to its well-defined role in the enhancement of plant frost tolerance, overexpression of the *CBF2* and *CBF3* genes in *Arabidopsis* also remarkably delayed the onset of developmental leaf senescence and extended the life-span of the plants by approximately 2 weeks compared with that of the wild-type plants (WS-2 ecotype). Furthermore, overexpression of these genes significantly delayed artificial leaf senescence induced by the phytohormones ethylene, ABA, SA, and JA, and by detachment from the plant. To explore the molecular mechanisms that might be involved in regulating the delay of leaf senescence and the extension of life span in *CBF2* overexpressing plants, the *Arabidopsis* ATH1 genome array was used to perform transcript profiling analysis of mature leaf tissues. Significant changes were observed in the abundance of various TFs and protein modification and post-transcriptional regulation genes, suggesting their possible roles in the regulation of senescence and longevity. Furthermore, among the 286 genes observed in the *CBF2*-regulon, more than 60% (175 genes) were specifically detected in mature leaves and not in seedling tissues (Vogel *et al.*, 2005), which suggests that overexpression of *CBF2* might have additional specific roles in mature tissues.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Wassilewskija (WS-2) and of transgenic plants overexpressing the *CBF2* (line E2),

and *CBF3* (line A28) genes in the WS-2 background were obtained from Professor 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. Illumination was provided from cool-white fluorescent lights at approximately 100 μmol m^{-2} s^{-1}, and a photoperiod of 16 h. In most experiments, plants were grown at a density of four plants per pot, except for the first experiment, in which developmental senescence was evaluated and a single plant grows in each pot. For physiological evaluations of leaf senescence, leaf numbers 5 and 6, harvested at 36–40 d after sowing were used.

Chlorophyll and protein contents

Chlorophyll and protein contents were measured in 5-mm-diameter leaf disc samples. For protein evaluation, two discs from different leaves were placed in a microtube in 150 μl of extraction buffer [50 mM TRIS-HCl, pH 7.5, 0.1% (w/v) SDS, and 10% (w/v) polyvinylpyrrolidone], and ground with a fitting pestle and a motorized drill. Samples were centrifuged at 10 000 *g* for 20 min at 4 °C, and the protein content in the supernatant were determined spectrometrically according to the Bradford assay, with a commercial protein assay kit (Bio-Rad, CA, USA). 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 was measured spectrometrically according to Porra *et al.* (1989). Each measurement included four replications, and data are presented based on leaf area or dry weight.

Electrolyte leakage

Electrolyte leakage was measured by placing entire rosettes in scintillation vials containing 10 ml of double-distilled water. The first reading was done 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 then were cooled to room temperature, and second readings were taken. Electrolyte leakage data are presented as leakage percentages of the total amount of electrolytes present in the tissue.

Senescence of detached leaves

Leaf numbers 5 and 6 were detached from rosettes 36 d after sowing. The leaves were enclosed in 2.0 l boxes fitted with inlet and outlet ports, and stored for up to 6 d in the dark at 20 °C, in order to promote senescence. The boxes were sealed and connected to a flow-through air supply bubbled through water to maintain a high humidity in the boxes as described by Canetti *et al.* (2002).

Hormones induce leaf senescence

To examine the stimulating effects of ethylene on leaf senescence, entire rosettes were placed in sealed boxes for 48 h. The boxes were fitted with inlet and outlet ports and connected to a flow-through system that passed air containing ethylene at 1 μl l^{-1}, at a rate of 100 ml min^{-1}. The ethylene mixture was bubbled through water to maintain a high humidity inside the box.

The effects of ABA, SA, and MeJA on leaf senescence were examined on leaf numbers 5 and 6, detached from plants 36 d after sowing. The detached leaves were rinsed briefly with sterile water and then placed adaxial side up in Petri dishes containing 50 μM ABA (Fan *et al.*, 1997), 50 μM MeJA (He *et al.*, 2002), 100 μM SA (Morris *et al.*, 2000), or water as a control. The leaves were incubated at 22 °C under a 16 h photoperiod of illumination at ~100 μmol m^{-2} s^{-1}. The leaves were incubated in ABA and SA for 48 h and in MeJA for 72 h. ABA and MeJA ware first dissolved in ethanol and then diluted in H_20 to a final concentration of 0.005% ethanol, therefore, this concentration of ethanol was included in all of the treatments, including the water controls.

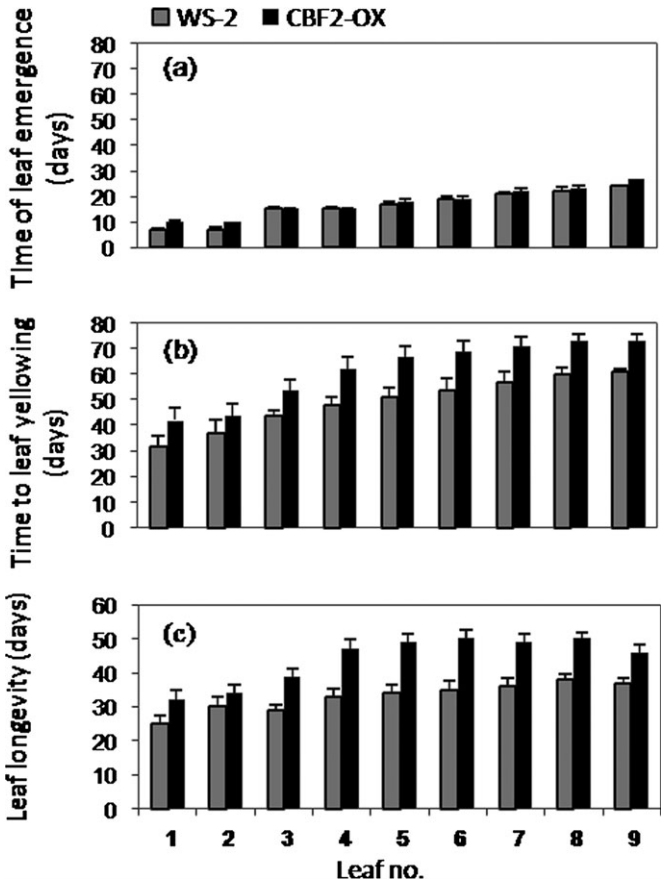

Fig. 2. Longevity of individual leaves of wild-type (WS-2 ecotype) and *CBF2*-overexpressing plants. (a) Time of leaf emergence. (b) Time until the beginning of leaf yellowing. (c) Leaf longevity (time from emergence until the beginning of yellowing). Data are means ±SE of 20 plants.

Transcript profiling analysis

Total RNA was isolated from leaf numbers 5 and 6 that had been collected 40 d after sowing from wild-type (Ws-2 ecotype) and *CBF2*-overexpressing plants, by phenol/chloroform extraction and precipitation with LiCl, according to standard procedures (Sambrook *et al.*, 1992). For the microarray experiment, three biological replicates were used per treatment, each including separate RNA extractions from leaves collected from 5–10 different plants. The RNA samples were further prepared for hybridization according to the protocols outlined in the GeneChip Expression Analysis Technical Manual, and 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 analysis was performed with the Affymetrix Microarray Suite 5.0 (MAS5.0) statistical algorithms (Affymetrix). Further advanced data analysis including background subtraction, normalization, and elimination of false positives was performed using the Partek Genomics Suite (Partek GS) statistical and data visualization program. One-way analysis of variance (ANOVA) was used to identify probe sets that exhibited significant changes in signal levels at $P \leq 0.05$. Among

these, genes differentially expressed by a factor of at least 2.5 were imported into the MapMan software (http://gabi.rzpd.de/projects/MapMan/) in order to perform functional categorization and to assign *CBF2*-regulon genes to corresponding metabolic pathways (Thimm *et al.*, 2004).

Results

Effects of CBF2 *overexpression on developmental leaf senescence*

To examine the effects of *CBF2* overexpression on plant development and natural leaf senescence, wild-type (WS-2 ecotype) and transgenic plants overexpressing the *CBF2* gene were grown at 22 °C under a 16 h photoperiod, and the progress of plant development and senescence was evaluated. In the first experiment a single plant was grown in each pot. It can be seen that *CBF2*-overexpressing plants had 11 leaves, as compared with just nine leaves in wild-type ones (Table 1). Overexpression of *CBF2* also delayed bolting by 5 d, and the opening of the first flower by 7 d (Table 1). However, overexpression of *CBF2* extended the time to yellowing of leaf number 4 by 2 weeks, and delayed complete yellowing of entire rosettes by 16 d (Table 1). Thus, overexpression of *CBF2* delayed leaf senescence and extended plant longevity much more than it delayed flowering and seed set (Table 1). The progress of leaf senescence in the wild type is compared with that of *CBF2*-overexpressing plants in Fig. 1. It can be seen the overexpression of *CBF2* considerably extended the time until the onset of leaf senescence from about 53 d to 67 d after sowing (Fig. 1).

Detailed measurements of the time of emergence and the beginning of yellowing of each rosette leaf revealed that, apart from the first two leaves, all other leaves in *CBF2*-overexpressing plants emerged at similar times to those of wild-type plants (Fig. 2A). However, the leaves of *CBF2*-overexpressing plants began to senesce 10–15 d later than the wild-type ones (Fig. 2B). Accordingly, the average longevity of rosette leaves of *CBF2*-overexpressing plants was ~47 d compared with ~33 d of wild-type leaves, leading to an overall ~40% increase in leaf longevity (Fig. 2C). Overall, in wild-type plants, leaf senescence was

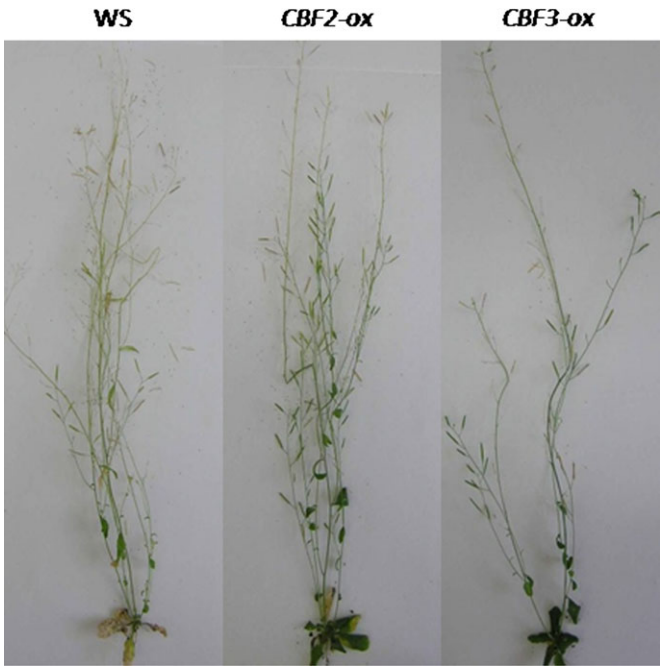

Fig. 3. Phenotypes of wild-type (WS-2 ecotype), *CBF2*- and *CBF3*-overexpressing plants with shuttered siliques. The pictures were taken 80 d after sowing.

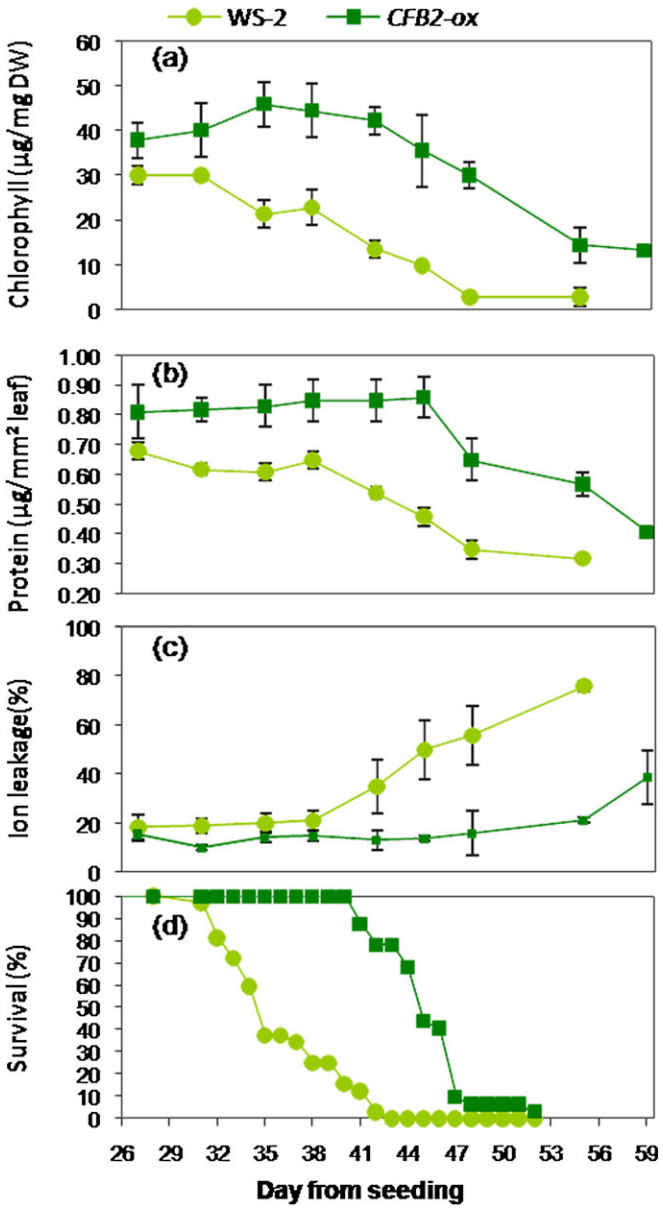

Fig. 4. Biochemical and physiological changes occurring during developmental senescence of wild-type (WS-2 ecotype) and *CBF2*-overexpressing plants. (a) Chlorophyll content. (b) Protein content. (c) Ion leakage. (d) Survival curve: the percentage of plants in which leaf number 4 was still green. Measurements were taken every 4 d from 27 until 59 d after sowing. (A–C) Data are means ±SE of four replications; those of (D) represent means of 32 plants.

initiated in parallel with silique formation and seed set, whereas, in *CBF2*- and *CBF3*-overexpressing plants, leaf senescence was decoupled from reproductive development, and after 80 d mature plants were observed with shuttered siliques that still had green rosettes (Fig. 3).

In further experiments, four plants were grown per pot, which resulted in plants with fewer leaves and shorter life spans than single plants, but the differences in leaf senescence delay and in plant longevity between *CBF2*-overexpressing and wild-type plants were unchanged. Biochemical and physiological analysis of leaf number 4 of these plants revealed that *CBF2* overexpression significantly delayed the degradation of chlorophyll and soluble leaf protein content and the increase in electrolyte leakage during developmental senescence by approximately 2 weeks (Fig. 4A–C). Chlorophyll levels on the basis of leaf DW began to decline in wild-type plants after 35 d, but decreased below its initial levels in *CBF2*-overexpressing plants only after 49 d (Fig. 4A). Overall, chlorophyll content declined by 50% (to 15 μg mg^{-1} leaf DW) after 42 d in wild-type plants but only after 53 d (to 19 μg mg^{-1} leaf DW) in *CBF2*-overexpressing plants (Fig. 4A). *CBF2*-overexpressing leaves also had somewhat higher protein contents than the wild type (0.81 versus 0.68 μg mm^{-2}). Like chlorophyll, leaf soluble protein decreased by 50% (to 0.34 μg mm^{-2}) after 48 d in wild-type plants but only after 59 d (to 0.41 μg mm^{-2}) in *CBF2*-overexpressing plants (Fig. 4B). Similarly, electrolyte leakage began to increase in wild-type plants after 42 d, but only after 57 d in *CBF2*-overexpressing plants (Fig. 4C). A survival curve representing the percentage of plants in which leaf number 4 began to turn yellow shows that 50% of wild-type and *CBF2*-overexpressing plants turned yellow after 34 d and 45 d, respectively (Fig. 4D).

Effects of CBF2 *and* CBF3 *overexpression on dark-induced senescence of detached leaves*

The progress of leaf senescence (yellowing) of detached leaves of wild-type and *CBF2*- and *CBF3*-overexpressing plants in the dark is shown in Fig. 5A. It can be seen that leaves of wild-type plants began to turn yellow after 4 d whereas those of the transgenic plants did so only after 6 d (Fig. 5A). Indeed, chlorophyll content in wild-type leaves decreased by 50% in 4 d and by 88% in 6 d (Fig. 5B, C), whereas the chlorophyll content in leaves of *CBF2*- and *CBF3*-overexpressing plants declined by just 0–19% at 4 d, and by 32–52% at 6 d (Fig. 5B, C). Overall, *CBF3*-overexpressing plants were somewhat greener than *CBF2*-overexpressing plants, and showed an even greater delay in leaf senescence in the dark (Fig. 5A–C). A similar delay in the progression of senescence of detached leaves of *CBF2*- and *CBF3*-overexpressing plants also occurred when the plants were grown under short-day conditions (8 h photoperiod) (data not shown). Under these photoperiodic conditions, all plants remained vegetative and had not begun flowering, thus, proving that the delay in leaf senescence in the transgenic plants was not related to possible differences in their developmental stage as compared with wild-type plants.

Leaf senescence artificially induced by plant hormones

To examine the effects of overexpression of *CBF* genes on leaf senescence further, the responses of detached leaves or

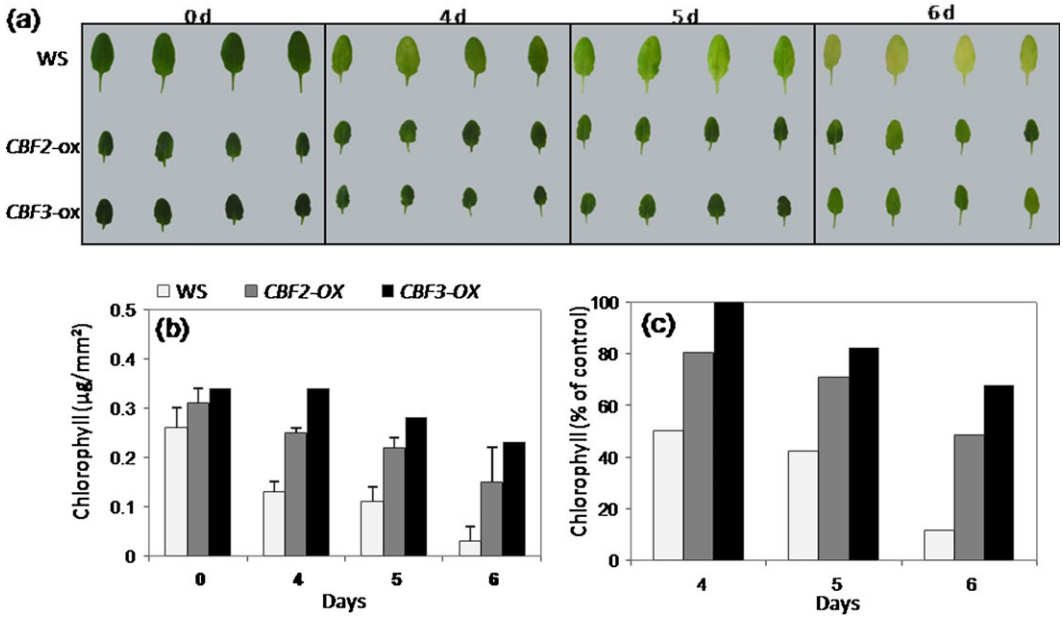

Fig. 5. Dark-induced senescence of detached leaves of wild-type (WS-2 ecotype), *CBF2*-and *CBF3*-overexpressing plants. (a) Visual appearance. (b) Chlorophyll content. (c) Percentage of chlorophyll content after 4, 5, and 6 d as compared with initial levels at time zero. In all cases, leaf numbers 5 and 6 were detached from the rosettes 36 d after sowing. Data in (B) are means ±SE of three different experiments, each including four replications.

rosettes to the senescence-promoting hormones ethylene, ABA, SA, and methyl jasmonate (MeJA) were evaluated. It can be seen that exposure to ethylene at a concentration of 1 μl l^{-1} for 48 h caused marked yellowing of wild-type rosettes but barely affected the rosettes of *CBF2*-overexpressing plants (Fig. 6A). In wild-type leaves, the chlorophyll content decreased from 0.18 to 0.14 μg mm^{-2} after 48 h in air. However, exposure to ethylene reduced the chlorophyll content to 0.05 μg mm^{-2} (Fig. 6B). By contrast, the chlorophyll content in leaves of *CBF2*-overexpressing plants decreased from 0.22 μg mm^{-2} to 0.15 μg mm^{-2} after 48 h in air, while exposure to ethylene only slightly reduced chlorophyll content to 13.05 μg mm^{-2} (Fig. 6B). Thus, following 48 h of exposure to ethylene, leaves of the wild-type plants lost over 60% more of their chlorophyll content than those exposed to air alone, whereas leaves of

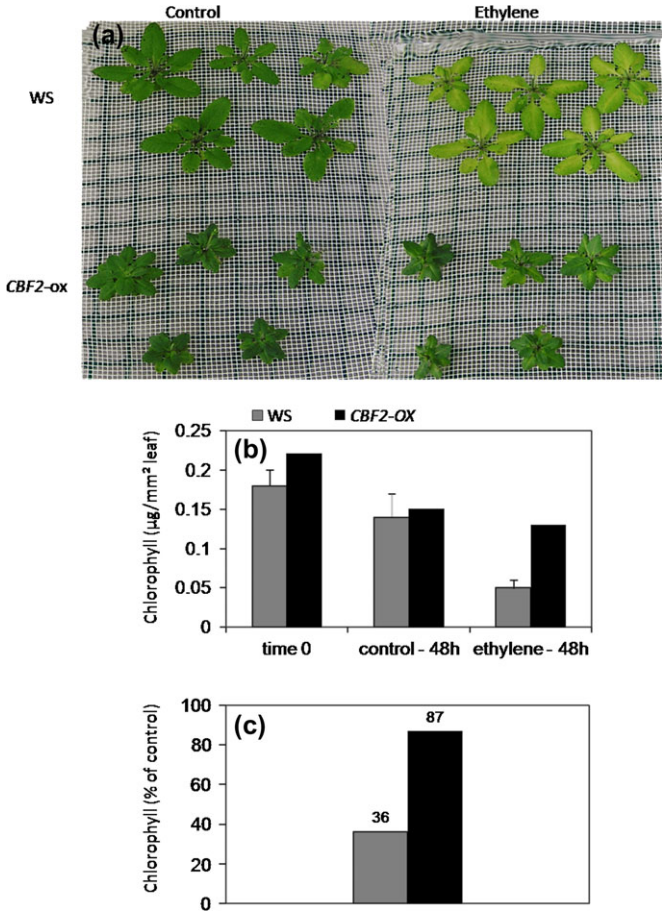

Fig. 6. Ethylene-induced senescence of detached rosettes of wild-type (WS-2 ecotype) and *CBF2*-overexpressing plants. Rosettes were harvested 36 d after sowing and exposed to air (control) or ethylene at 1 μl l^{-1} for 48 h. (a) Photographs of rosettes after 48 h exposure to air (left) or ethylene (right). (b) Chlorophyll content. (c) Percentage of chlorophyll content after 48 h of exposure of rosettes to ethylene as compared with that of those exposed to air. Chlorophyll measurements were performed in leaves 5 and 6 detached from the rosettes harvested 36 d after sowing. Data in (B) are means ±SE of three different experiments, each including four replications.

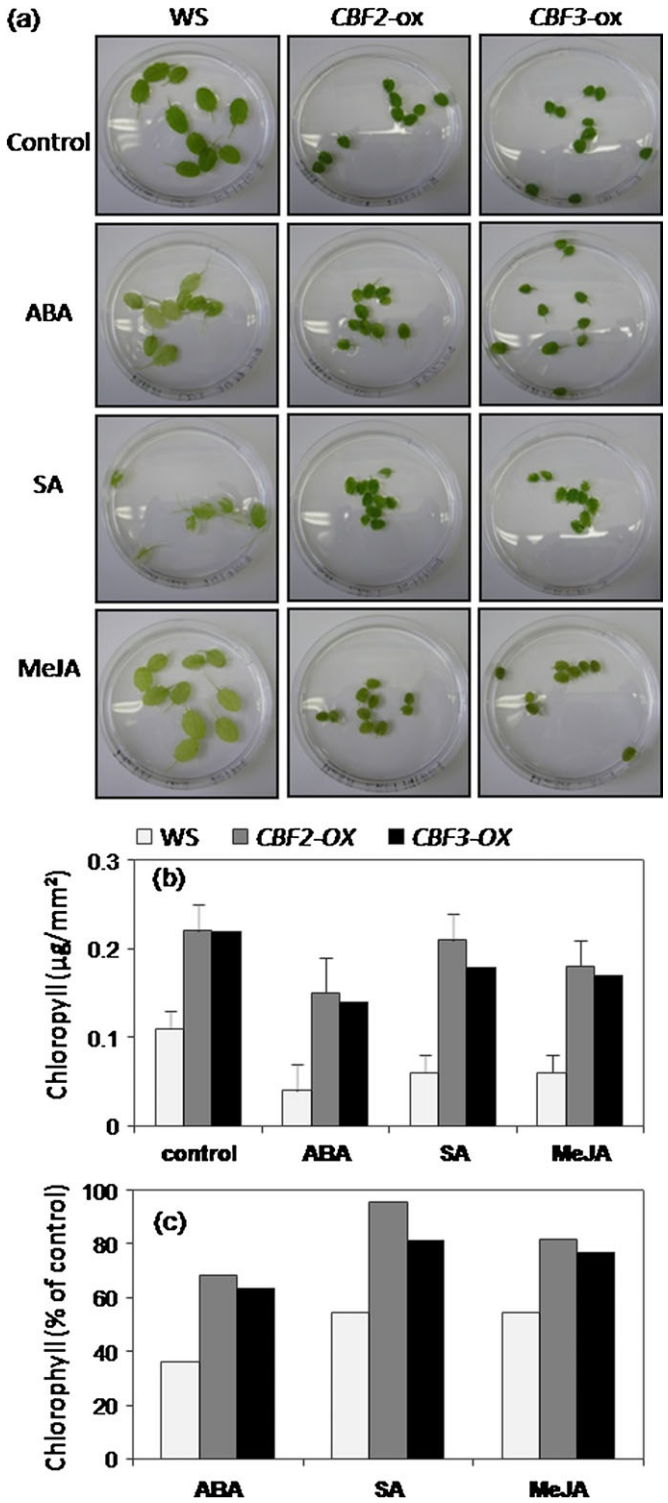

Fig. 7. Phytohormone-induced senescence of detached leaves of wild-type (WS-2 ecotype), and *CBF2*- and *CBF3*-overexpressing plants. (a) Photographs of detached leaves after incubation in water (control), ABA (50 μM), SA (100 μM) and MeJA (50 μM). (b) Chlorophyll content. (c) Percentage of chlorophyll content remaining after exposure to the various hormones, as compared with that in leaves incubated in water. Chlorophyll measurements were performed in leaves 5 and 6 detached from the rosettes harvested 36 d after sowing. Data in (B) are means ±SE of three different experiments, each including Four replications.

CBF2-overexpressing plants lost only 13% more of their chlorophyll in ethylene than in air (Fig. 6C).

The effects of ABA, SA, and MeJA on leaf senescence were evaluated by incubating expanded leaf numbers 5 and 6 in hormone solutions under light. It can be seen that leaves of wild-type plants turned yellow after exposure to all the various hormones, whereas those of the *CBF2*- and *CBF3*-overexpressing plants stayed green (Fig. 7A). Chlorophyll measurements revealed that exposure to ABA, SA, and MeJA resulted in major decreases in chlorophyll content to just 36, 55, and 55%, respectively, of their contents after incubation in water alone (Fig. 7B, C). By contrast, exposure to ABA, SA, and MeJA decreased chlorophyll content to between 68% and 95% of its levels in water alone in *CBF2*-overexpressing plants and to between 64% and 82% in *CBF3*-overexpressing plants (Fig. 7B, C).

Effects of CBF2 *overexpression on the transcriptome of mature leaves*

In order to evaluate the molecular mechanisms involved in the response to overexpression of the *CBF2* transcriptional activator, which might be responsible for the inhibition of leaf senescence, transcript profiling analysis was performed by using the Affymetrix ATH1 genome array. To do so, RNA was isolated from leaf numbers 5 and 6, that had been harvested from mature rosettes 40 d after sowing (Fig. 8). At this stage, leaves 5 and 6 were still green and had not yet undergone senescence in either the wild-type or the transgenic plants (Fig. 8). Overall, 286 probe sets were detected whose expression patterns were significantly different at $P \leqslant 0.05$ and that were up- or down-regulated by factors of at least 2.5 (see Supplementary Table S1 at *JXB* online). Overall, the assigned *CBF2*-regulon of mature leaves included 210 up-regulated and 76 down-regulated genes (see Supplementary Table S1 at *JXB* online).

The currently identified *CBF2*-regulon of mature leaves (a total of 286 probe sets) were then compared with that previously described from microarray analysis studies of 10–12-d-old seedlings (a total of 197 probe sets) (Vogel *et al.*, 2005). It was found that a core set of 111 *CBF2*-regulated genes were common to the two microarray experiments. However, 84 probe sets were specifically expressed only in young seedlings, whereas a much larger number of 175 probes sets were detected specifically in mature leaf tissue. Thus, many of the genes in the *CBF2*-overexpressing plants were expressed specifically in mature leaves, and might perform additional functions besides the enhancement of frost tolerance.

Functional categorization of the *CBF2* differentially expressed genes with the MapMan software (Thimm *et al.*, 2004) revealed that the three main functional categories affected were 'stress' (30 genes), 'RNA regulation of transcription' (24 genes), and 'protein' (20 genes) (Table 2). In addition, there were two more relatively large groups categorized as 'misc' (32 genes) and 'not assigned' (81 genes)

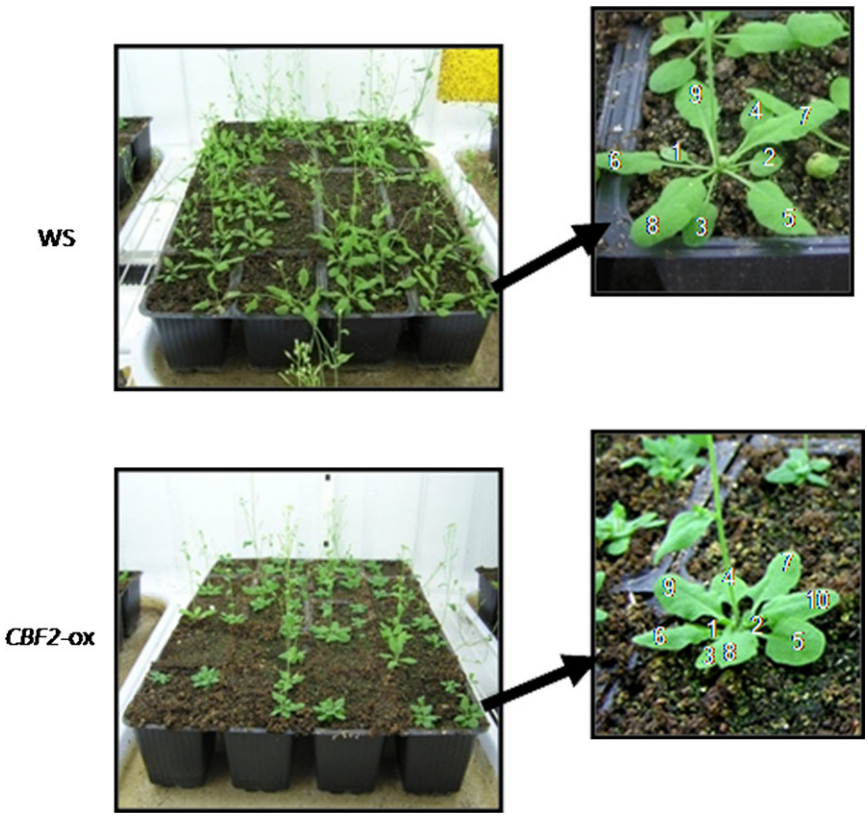

Fig. 8. Phenotypes of wild-type (WS-2 ecotype) and *CBF2*-overexpressing plants taken for transcriptome analysis studies. Photographs were taken 40 d after sowing. The pictures on the right are enlargements of the rosettes, including leaf numbers.

(Table 2). Among the 30 'stress' genes, 25 were up-regulated and only five down-regulated. Furthermore, most of the up-regulated 'stress' genes (18 out of 25) were known *COR*-genes, manifesting the pivotal role of the *CBF* transcriptional activators in conferring frost tolerance (Gilmour *et al.*, 1998; Thomashow, 1998) (Table 2; see Supplementary Table S2 at *JXB* online). Moreover, seven out of the top 10 most highly up-regulated genes in the *CBF2*-regulon (showing at least a 35-fold induction) were *COR* genes (see Supplementary Table S1 at *JXB* online).

The second largest group of genes in the *CBF2*-regulon of mature leaves was classified as 'RNA regulation of transcription', and included 24 TFs—15 up-regulated and nine down-regulated (Table 2). Among the 15 up-regulated TFs, a family of four *AP2/EREBP* genes was identified (including *CBF2*), whereas the nine down-regulated TFs included a family of four *bHLH* genes (Table 3). By comparing the list of TFs in the *CBF2*-regulon with *Arabidopsis* TFs known to be up- or down-regulated during senescence (Lin and Wu, 2004), it was found that two TFs normally up-regulated during senescence, *ANAC013* and *ATMYC2*, were down-regulated in the *CBF2*-regulon, whereas two other TFs whose expression is down-regulated during senescence, *RAP2* and *RAV1*, were instead up-regulated in the *CBF2*-regulon (Table 3). Furthermore, some of the TFs in the *CBF2*-regulon of mature leaves appear to be involved in the regulation of plant growth and

Table 2. Functional categorization of *CBF2*-regulon genes in mature leaves

Functional categorization was performed according to MapMan (http://gabi.rzpd.de/projects/MapMan/). The functional groups of 'Stress', 'RNA regulation of transcription', and 'Protein' are shaded.

Functional categorization	Up-regulated	Down-regulated	Total
PS	1	–	1
Major CHO metabolism	4	–	4
Minor CHO metabolism	5	1	6
Glycolysis	–	–	–
Fermentation	1	–	1
Gluconeogenese/glyoxylate cycle	–	–	–
OPP	–	1	1
TCA/org. transformation	–	1	1
Mitochondrial electron transport/ ATP synthesis	–	–	–
Cell wall	6	3	9
Lipid metabolism	8	1	9
N–metabolism	1	–	1
Amino acid metabolism	3	–	3
S–assimilation	1	–	1
Metal handling	2		2
Secondary metabolism	7	2	9
Hormone metabolism	8	3	11
Tetrapyrrole synthesis	1	–	1
Stress	25	5	30
Redox regulation	–	3	3
Polyamine metabolism	–	–	–
Nucleotide metabolism	1	–	1
Biodegradation of xenobiotics	–	–	–
C1–metabolism	–	–	–
Misc	17	15	32
RNA regulation of transcription	15	9	24
DNA	1	1	2
Protein	19	1	20
Signalling	5	–	5
Cell	5	1	6
Micro RNA, natural antisense etc	–	–	–
Development	3	2	5
Transport	10	6	16
Not assigned	61	21	81

Table 3. List of transcription factors genes up- or down-regulated in the *CBF2*-regulon

Family	Locus[a]	Fold	Description
Up-regulated			
Ap2/EREBP	At2g23340**	32	AP2 domain transcription factor (RAP family)
	At4g25470	52.8	DRE binding protein (DREB1C,CBF2)
	At1g46768	7.5	AP2 domain transcription factor RAP2.1
	At5g25810	5.0	Transcription factor TINY
C2C2-CO-like	At2g47890	2.7	COL13/unknown protein
C2C2-YABBY	At2g26580	3.2	Identical to Axial regulator YABBY 5 (YAB5)
C2H2(Zn)	At1g13260**	4.5	RAV1 (Related to ABI3/VP1 1)
	At5g04340	3.9	Putative c2h2 zinc finger transcription factor
SBP (squamosa)	At1g76580	2.5	SPL16/unknown protein
Aux/IAA	At1g04240	3.3	SHY2/IAA3 (SHORT HYPOCOTYL 2)
AB13/VP1	At3g45260	2.5	DNA-binding protein (RAV1)
GRAS	At5g17490	2.9	RGL 3/RGA-like protein
Trihelix	At5g28300	2.6	GTL1 - like protein
MYB	At4g34990	4.1	AtMYB32/myb family transcription factor
bZIP	At1g59530	7.9	AtbZIP4 /bZIP protein
Down-regulated			
bHLH	At5g04150	–2.5	AtbHLH101/myc - like protein
	At3g56980	–3.0	AtbHLH039/putative protein
	At2g43060	–2.5	Similar to cDNA bHLH (bHLH zeta gene)
	At1g32640*	–2.8	ATMYC2 (JASMONATE INSENSITIVE 1)
NAC	At1g32870*	–2.4	ANAC013 (Arabidopsis NAC domain containing protein 13)
HB	At5g47370	–3.0	Homeobox-leucine zipper protein-like
SBP (squamosa)	At1g53160	–4.4	spl4/transcription factor, putative
WRKY	At2g24570	–2.4	AtWRKY17/WRKY family
MYB	At1g56650	–4.5	AtMYB75/myb-related protein anthocyanin2, putative

[a] *Genes up-regulated during senescence but down-regulated in the *CBF2-regulon*; **Genes down-regulated during senescence but up-regulated in the CBF2-*regulon.*

development. For example, *SHY2*/*IAA3* (a negative regulator of auxin signal transduction) (Tian *et al.*, 2002); *RAV1* (known to be down-regulated by brassinsteroids) (Hu *et al.*, 2004); and *RGL3* (encoding a DELLA protein which is a nuclear growth-repressing protein) (Tyler *et al.*, 2004; Achard *et al.*, 2008), were up-regulated in the *CBF2*-regulon and are known as inhibitors of growth and flowering. By contrast, the *ATMYC2* (JASMONATE INSENSITIVE 1) (Dombrecht *et al.*, 2007) gene involved in JA signalling was down-regulated in the *CBF2*-regulon (Table 3). Overall, overexpression of *CBF2* in leaf tissue resulted in the activation of a network of TFs that may simultaneously affect senescence and growth and developmental processes.

The third main functional category identified in the *CBF2*-regulon was 'protein'; it included 20 genes, of which 19 were up-regulated (Table 2). In-depth analysis of this category revealed that most of these genes belonged to two main sub-groups: 'protein degradation' (11 genes), and 'protein post-translational modification' (five genes) (Table 4). Two other genes in the 'protein' category were involved in 'protein glycosylation' and one protein involved in each of 'amino acid activation' and 'protein targeting' (Table 4). The 'protein degradation' group included induction of four ubiquitin.E3.RING genes; three serine protease genes, and two cysteine protease genes (Table 4). Thus, *CBF2* overexpression may result in targeting specific proteins towards degradation, via several different proteolysis pathways.

Finally, a remarkable up-regulation of *AtOEP16* (32.3-fold) was noted. *AtOEP16* encodes a transporter specifically involved in the import of protochlorophyllide oxidoreductase A (PORA), the key enzyme of the chlorophyll A biosynthesis pathway, into the chloroplast (Reinbothe *et al.*, 2004*a*, *b*; Drea *et al.*, 2006) (Table 4). Thus, the remarkable induction of *AtOEP16* (the transporter of PORA) together with an observed 4-fold increase in protochlorophyllide oxidoreductase B (PORB), the key enzyme involved in chlorophyll B biosynthesis, may explain why *CBF2*-overexpressing plants were greener and had higher leaf chlorophyll contents than the wild-type ones (see Supplementary Tables S1 and S2 at *JXB* online) (Armstrong *et al.*, 1995; Buhr *et al.*, 2008).

Table 4. List of genes in the 'Protein' functional categorization group within the *CBF2*-regulon

Functional category	Locus[a]	Fold	Description
Degradation	At1g20160	–2.6	Subtilase family protein
	At5g04250	2.5	Cysteine protease family protein
	At4g16500	4.1	Cysteine protease inhibitor family protein
	At5g47040	2.7	Serine protease (ATP-dependent proteolysis)
	At1g47710	4.5	Serine-type endopeptidase inhibitor
	At2g22980	2.5	Serine protease (serine carboxypeptidase)
	At3g54400	10.0	Aspartyl protease family protein
	At1g27910	2.9	Ubiquitin.E3.RING (protein ubiquitination)
	At4g23450	3.3	Ubiquitin.E3.RING(C3HC4-type RING finger)
	At5g01520	2.7	Ubiquitin.E3.RING(C3HC4-type RING finger)
	At1g02860	4.0	Ubiquitin.E3.RING(NLA protein binding)
Glycosylation	At4g18270	4.1	Glycosylation (protein amino acid), lipid metabolic process
	At1g05170	2.9	Glycosylation (protein amino acid).
Post-translational modification	At2g28930	2.7	Protein kinase APK1B, chloroplast precursor (APK1B)
	At5g25110	14.3	CBL-interacting protein kinase 25 (CIPK25)
	At3g04910	5.6	Serine/threonine protein kinase, whose transcription is regulated by circadian rhythm
	At3g23340	2.7	CKL10 (Casein Kinase I-like 10)
	At3g54030	2.7	Protein kinase family protein
aa activation	At3g50960	3.4	PLP3A (PHOSDUCIN-LIKE PROTEIN 3 HOMOLOG);
Targeting	At2g28900	32.3	AtOEP16, plastid import of protochlorophyllide oxidoreductase A

Discussion

The CBF cold-response pathway plays a central role in cold acclimation and protects plants from freezing temperatures. However, constitutive expression of *CBF* genes also causes various pleiotropic effects on plant growth and development, especially growth retardation, dwarfism, and the delay of flowering (Jaglo-Ottosen *et al.*, 1998; Gilmour *et al.*, 2004; Achard *et al.*, 2008). Indeed, in a recent study, it was demonstrated that *CBF1* overexpression restrained growth by reducing endogenous gibberellin (GA) content, thus allowing the accumulation of DELLAs, a family of nuclear growth-repressing proteins, whose degradation is stimulated by GA (Achard *et al.*, 2008). In the present study, it was found that overexpression of the *CBF2* and *CBF3* transcriptional activators in *Arabidopsis* also elicited another phenomenon—a delay in leaf senescence and an extension of life span. This phenomenon was exhibited both during developmental leaf senescence (Figs 1–4) and during senescence of detached leaves artificially induced by either darkness (Fig. 5) or phytohormones (Figs 6, 7). Moreover, detailed characterization of growth and development characteristics of *CBF2*-overexpressing plants revealed that there was a 5–7 d delay in development (time to bolting, flowering, and silique shuttering) but a rather longer delay of 14–16 d in the onset of leaf senescence, and a similar extension of life span (Table 1). Currently, it is not yet certain whether the observed delay in leaf senescence in *CBF2*-overexpressing plants is related to the accumulation of DELLA proteins, as reported for the delay in flowering and dwarfism phenotypes (Achard *et al.*, 2008), or rather is a DELLA-independent effect, as noticed for the increase in sugar levels in *CBF1*-overexpressing plants (Wingler and Roitsch, 2008).

Transcriptome analysis of mature leaves (leaves 5 and 6, harvested 40 d after sowing) with the Affymetrix ATH1 genome array revealed that constitutive expression of *CBF2* affected the expression of genes within three main functional categories: 'stress', 'RNA regulation of transcription', and 'protein' (Table 2). Thus, besides the activation of stress and defence responses, constitutive expression of *CBF2* also governs cellular regulatory networks at both the transcriptional and the protein levels. Our findings show that *CBF2* overexpression significantly affected the expression levels of 23 other, varied TFs, some of which are also involved in the regulation of development and senescence (Table 3). For example, in the *CBF2*-regulon, significant down-regulation of *WRKY17* and *ANAC013* genes was observed. These genes belong to classes of TFs that have some members known to be involved in the regulation of leaf senescence (Eulgem *et al.*, 2000; Guo *et al.*, 2004; Lin and Wu, 2004). Furthermore, among the up-regulated TFs in the *CBF2*-regulon, several genes were identified that are involved in the regulation of growth and development. For example, constitutive expression of *CBF2* induced the expression of *SHY2/IAA3*, a negative regulator of auxin-induced gene expression (Tian *et al.*, 2002); *RAV1*, a negative regulator of growth and development (Hu *et al.*, 2004); and *RGL3,* a DELLA protein which, as mentioned above, acts as a nuclear growth-repressing protein (Tyler *et al.*, 2004; Achard *et al.*, 2008). In addition, a 2.2-fold induction of FLC, a MADS-box TF that serves as a repressor of flowering (data not shown) (Sheldon *et al.*, 1999; Kim *et al.*, 2006) was observed. Overall, these observed changes in the expression patterns of various TFs within the *CBF2*-regulon may explain, at least in part, the observed phenotypes of growth retardation, and delayed flowering and senescence.

Our microarray analysis data suggest that *CBF2* overexpression may also affect development and senescence processes by modifications of cellular regulation at the protein level (Table 4). In this respect, the effects of *CBF2* on the expression of genes associated with protein degradation and post-translational modification are worth noting. For example, in the *CBF2*-regulon a 4-fold induction of *NLA*, a RING-type ubiquitin ligase (Table 4) was observed. It was reported that a mutation in this locus disrupts the adaptability of *Arabidopsis* to nitrogen limitation, and thereby leads to premature senescence (Peng *et al.*, 2007). *CBF2* may also regulate protein activity via post-translational modifications, especially via activation of various protein kinases (Table 4).

Overall, in light of the present findings, and taking an evolutionary perspective, it is suggested that, upon exposure to low temperatures, natural induction of the *CBF1-3* transcription activators act to enhance plant frost tolerance, but also act to slow growth, delay flowering and leaf senescence, and extend plant longevity in order to enable survival of the plants through winter until temperatures rise in spring (Fig. 9). This CBF-governed growth retardation and delay of flowering and senescence enables plants to extend their life span in order to pass the winter period; hence successfully completing their life cycle.

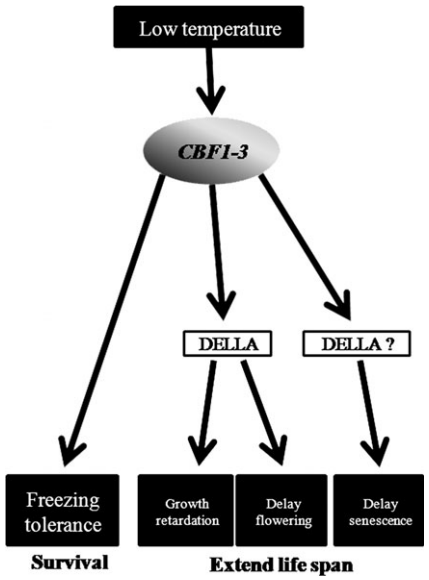

Fig. 9. Schematic diagram illustrating the effects of CBF transcriptional activators on frost tolerance enhancement, on the one hand, and on growth retardation and delay of flowering and senescence, on the other hand. Growth retardation and delay of flowering in *CBF*-overexpressing plants is governed by the accumulation of DELLAs, whereas their possible role in regulation of leaf senescence is still uncertain.

Finally, our findings that *CBF2*-overexpression simultaneously enhances plant frost tolerance, and delays leaf senescence and extends life span support the 'stress resistance' theory of ageing in plants. Thus, as found in various animal models, enhanced resistance to internal or external stress also prolongs life span and longevity (Johnson *et al.*, 1996; Murakami and Johnson, 2003).

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