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

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Received 13 July 2009; Revised 10 September 2009; Accepted 11 September 2009

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 senescence. This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details).
Table 1. Developmental stages and progression of senescence in wild-type (WS-2 ecotype) and CBF2-overexpressing Arabidopsis plants

|                      | Wild type           | 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    |

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.

Leaf 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 markedly affected by exposure to hormones: ethylene (Grebic 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 DELLLAs, a family of nuclear growth-repressing proteins, whose degradation is stimulated by gibberellins (GA) (Achard et al., 2008).
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 \textit{CBF2} and \textit{CBF3} genes in \textit{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 \textit{CBF2} overexpressing plants, the \textit{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 \textit{CBF2}-regulon, more than 60\% (175 genes) were specifically detected in mature leaves and not in seedling tissues (Vogel \textit{et al.}, 2005), which suggests that overexpression of \textit{CBF2} might have additional specific roles in mature tissues.

\textbf{Materials and methods}

\textit{Plant material and growth conditions}

Seeds of \textit{Arabidopsis thaliana} (L.) Heynh. ecotype Wassilewskija (WS-2) and of transgenic plants overexpressing the \textit{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 7x7x8 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

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 ≤0.05. Among

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.

for 48 h and in MeJA for 72 h. ABA and MeJA were first dissolved in ethanol and then diluted in H₂O to a final concentration of 0.005% ethanol, therefore, this concentration of ethanol was included in all of the treatments, including the water controls.
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.

| WS | CBF2-ox | CBF3-ox |
|----|---------|---------|

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 photo-period) (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.](https://academic.oup.com/jxb/article-abstract/61/1/261/570198)
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.

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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 $P \leq 0.05$ 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)
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 development.

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

| 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     |
| Gluconeogene/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 | Locusa | 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 TIYY |
|            | At4g237890 | 2.7  | COL13/unknown protein |
|            | At2g26580 | 3.2  | Identical to Axial regulator YABBY 5 (YAB5) |
|            | Atig04340 | 3.9  | Putative c2h2 zinc finger |
| SBP (squamosa) | At1g76580 | 2.5  | SPL16/unknown protein |
| Aux/IAA    | At1g04240 | 3.3  | SHY2/IAA3 (SHORT HYPOCOTYL 2) |
| AB13/VP1   | At3g15260 | 2.5  | DNA-binding protein (RAV1) |
| GRAS       | At5g17490 | 2.9  | RGL 3/RGA-like protein |
| Trichloro | At5g28300 | 2.6  | GTL1 - like protein |
| MYB        | At4g34990 | 4.1  | ATMYB32/myb family transcription factor |
| bZIP       | Atig59530 | 7.9  | AtbZIP4 / bZIP protein |
| Down-regulated |      |      |             |
| bHLH       | At5g04150 | –2.5 | AtbHLH101/myc - like protein |
|            | At3g56980 | –3.0 | AtbHLH399/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  | AMYB75/myb-related protein antimycin2, putative |

* 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 (POR). The key enzyme of the chlorophyll A biosynthesis pathway, into the chloroplast (Reinbothe et al., 2004a, b; Drea et al., 2006) (Table 4). Thus, the remarkable induction of *AtOEP16* (the transporter of POR) 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).

**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 siliquester 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 over-expression 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.

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).

Acknowledgements

Our thanks are due to Professor Michael F Thomashow and Dr Sarah J Gilmour, of Michigan State University, MI, USA, for providing the seeds of CBF2- and CBF3-overexpressing plants. This manuscript is contribution no. 539/09 from the Agricultural Research Organization, the Volcani Center, Bet Dagan 50250, Israel.

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