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The Effect of Overexpression of Two *Brassica CBF/DREB1*-like Transcription Factors on Photosynthetic Capacity and Freezing Tolerance in *Brassica napus*

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The effects of overexpression of two *Brassica CBF/DREB1*-like transcription factors (*BNCBF5* and *17*) in *Brassica napus* cv. Westar were studied. In addition to developing constitutive freezing tolerance and constitutively accumulating *COR* gene mRNAs, *BNCBF5*- and *17*-overexpressing plants also accumulate moderate transcript levels of genes involved in photosynthesis and chloroplast development as identified by microarray and Northern analyses. These include *GLK1*- and *GLK2*-like transcription factors involved in chloroplast photosynthetic development, chloroplast stroma cyclophilin *ROC4* (AtCYP20–3), β-amylase and triose-P/Pi translocator. In parallel with these changes, increases in photosynthetic efficiency and capacity, pigment pool sizes, increased capacities of the Calvin cycle enzymes, and enzymes of starch and sucrose biosynthesis, as well as glycolysis and oxaloacetate/malate exchange are seen, suggesting that *BNCBF* overexpression has partially mimicked cold-induced photosynthetic acclimation constitutively. Taken together, these results suggest that *BNCBF/DREB1* overexpression in *Brassica* not only resulted in increased constitutive freezing tolerance but also partially regulated chloroplast development to increase photochemical efficiency and photosynthetic capacity.

Keywords: *Brassica* — CBF/DREB1 — Cold acclimation — Freezing tolerance — GLK —Photosynthesis.

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; COR, cold regulated; EPS, epoxidation state; EST, expressed sequence tag; ETR, electron transport; FBP, fructose 1,6-bisphosphatase; GLK, Golden 2-like transcription factor; LHC, light-harvesting complex; MDH, malate dehydrogenase; MV, methylviologen; PFD, photon flux density; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SLW, specific leaf weight; SPS, sucrose-phosphate synthase.

Introduction

Exposure of cold-hardy species to low, non-freezing temperatures induces genetic, morphological and physiological changes in plants, which result in the development of cold hardiness and the acquisition of freezing tolerance. The ability of plants to acquire freezing tolerance from cold acclimation has been shown to involve the reprogramming of gene expression networks (Seki et al. 2001, Fowler and Thomashow 2002, Kreps et al. 2002, Seki et al. 2002). Photosynthetic cold acclimation has been reported to be an essential component of the development of cold hardiness and freezing tolerance and requires the complex interaction of low temperature, light and chloroplast redox poise (Gray et al. 1997, Wanner and Junttila 1999). Furthermore, overlaps have been identified between genes induced by high light and cold or drought stress (Kimura et al. 2003). It is well established that both short- (hours to days) and long- (weeks to months) term exposure to low temperature results in similar trends for accumulation of soluble sugars, modifications of thylakoid membrane lipid composition, enhanced antioxidant and reactive oxygen species (ROS) scavenging capacities and increased non-photochemical quenching (Gray et al. 1996, Savitch et al. 1997, Uemura and Steponkus 1997, Demmig-Adams and Adams 1992, Savitch et al. 2000b, Savitch et al. 2001, Savitch et al. 2002). Indeed, the accumulation of transcripts implicated in ROS scavenging has been observed in low temperature-induced reprogramming of overlapping gene networks in *Arabidopsis* (Fowler and Thomashow 2002, Kreps et al. 2002, Seki et al. 2002). However, it has been observed that low temperature stress applied to plants previously developed at normal growth temperatures results in a restriction in phosphate recycling and photophosphorylation leading to a limitation in the supply of ATP in the chloroplast, with concomitant reductions in rates of photosynthetic electron transport and CO_2 assimilation (Labate and Leegood 1988, Sharkey 1990, Pammenter et al. 1993, Savitch et al. 1997, Savitch et al. 2000a). In contrast, development of the plants (leaves) under low temperature stress (weeks to months) results in photosynthetic adjustment that includes the recovery of high plant photosynthetic capacity (Gray et al. 1996, Gray et al. 1997) associated with increased pigment pool size, increased protein content as well as increased capacities of Calvin cycle enzymes and enzymes of sucrose biosynthesis (Gray et al. 1996, Strand et al. 1999, Savitch et al. 2000a, Stitt and Hurry 2002, Leonardos et al. 2003). This long-term cold acclimation has also been shown to be associated with morpho-

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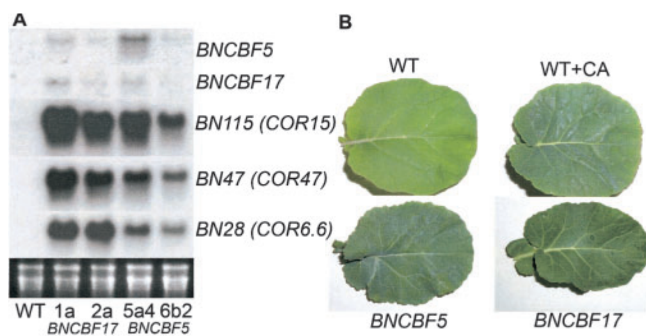

Fig. 1 mRNA accumulation of *BNCBF5*, *17* and BN *COR* genes in transgenic *Brassica napus* (A) and effect of cold acclimation (CA) and *BNCBF5* and *17* overexpression on leaf morphology (B). A 10 µg aliquot of total RNA extracted from leaves was loaded in each lane. The bottom panel is an ethidium bromide-stained RNA gel before transfer. Staining was also performed after transfer to ensure equal transfer in all lanes. WT, wild type; and independent transgenic lines overexpressing either *BNCBF17* or *5*. The complete *BNCBF5* and *17* random-primed probes, which cross-hybridize equally to both *BNCBF5* and *17*, were used to detect the level of *BNCBF* mRNA accumulation. *BNCOR47* (accession no. BQ704412, 660, 705018) is a *B. napus* ortholog of the *Arabidopsis COR47* and was obtained from the ECORC EST clonal depository.

logical changes, such as compact dwarf morphology, increased leaf thickness caused by increased mesophyll cell size, increased specific leaf weight, marked decrease of leaf water content and an increase in cytoplasmic volume relative to vacuole volume (Huner et al. 1981, Gray et al. 1997, Stefanowska et al. 1999, Strand et al. 1999, Stefanowska et al. 2002). In fact, it has been suggested that such structural changes might be necessary to account for the increase in stromal and cytosolic enzymes and metabolites in cold-acclimated leaves (Strand et al. 1999).

In *Arabidopsis thaliana*, as well as in *Brassica napus*, specific *COR* (cold-regulated) genes that are up-regulated in response to low temperature are characterized by common specific CRT/DRE *cis*-elements in their 5′-regulatory regions (Yamaguchi-Shinozaki and Shinozaki 1994, Baker et al. 1994, Jiang et al. 1996, Seki et al. 2002). A family of transcriptional activators (*CBF/DREB1*s) that interact specifically with these elements to direct the transcription of *COR* genes has been isolated and characterized in *Arabidopsis* (Stockinger et al. 1997, Gilmour et al. 1998, Liu et al. 1998, Kanaya et al. 1999, Medina et al. 1999, Sakuma et al. 2002). Overexpression of *Arabidopsis CBF/DREB1* can activate the simultaneous transcription of *COR* genes as well as confer constitutive freezing tolerance in transgenic *Arabidopsis* and oilseed *Brassica* (Jaglo-Ottosen et al. 1998, Kasuga et al. 1999, Jaglo et al. 2001, Fowler and Thomashow 2002, Gilmour et al. 2004).

Studies of the effects of *CBF/DREB1* overexpression on photosynthetic cold acclimation are limited. Previous reports have indicated that heterologous expression of *Arabidopsis CBF1* in tomato (Hsieh et al. 2002) imparts resistance to low temperature-induced photoinhibition, management of ROS and chlorophyll accumulation. Also, overexpression of *Arabidopsis CBF1*, *2* and *3* independently resulted in constitutive accumulation of proline and soluble sugars and in leaf anatomical and augmentative alterations partially associated with cold acclimation (Gilmour et al. 2000, Gilmour et al. 2004). Taken together, this suggests that *CBF/DREB* overexpression might affect photosynthetic development and mimic some photosynthetic responses to cold temperature. Nevertheless, factors that might directly contribute to *CBF/DREB* overexpression-induced changes in photosynthetic development and capacity have not been elucidated. Moreover, it is not clear if *CBF/DREB* overexpression-induced photosynthetic responses reflect adjustments associated with short-term (hours to days) low temperature stress or modulate photosynthetic cold acclimation responses associated with long-term (weeks to months) growth at low temperature.

Here we report the effect of homologous overexpression of two *B. napus BNCBF/DREB1*-like genes (Gao et al. 2002) on gene expression profiles, freezing tolerance and factors that affect photosynthetic capacity, chloroplast photosynthetic development as well as photosynthetic cold acclimation.

Results

Effect of BNCBF overexpression on COR gene expression

Homozygous *BNCBF5*- or *BNCBF17*-overexpressing plants with single insertion sites were used for analyses. Consistent with observations of constitutive *CBF/DREB1* overexpression in *Arabidopsis* (Liu et al. 1998), transgenic *B. napus* cv. Westar plants overexpressing *BNCBF*s exhibited various degrees of dwarf habit and longer time to flower. *BNCBF5*-overexpressing plants are marginally dwarfed compared with the wild type, whereas *BNCBF17*-overexpressing plants were more severely dwarfed and required a longer time to bolt and flower (data not shown). Under normal growth conditions, *BNCBF17*-overexpressing plants developed waxier dark green as well as thicker leaves comparable with wild-type plants that have undergone cold acclimation (Fig. 1B). This leaf phenotype is carried from seedlings to the mature flowering plant.

Northern blot analyses (Fig. 1A) of two independent transgenic lines overexpressing *BNCBF5* and *17* indicated that both transcription factors were efficient in constitutive accumulation of *BN115*, *BN28* and *BN47* (orthologs of the *Arabidopsis COR15*, *6.6* and *47*) mRNAs.

Effect of BNCBF overexpression on freezing tolerance

Three independent transgenic lines each of *BNCBF5*-overexpressing and *BNCBF17*-overexpressing plants were screened. All *BNCBF*-overexpressing lines showed increased constitutive freezing tolerance over the wild type, as indicated by leaf electrolyte leakage (LE_{50}). Average LE_{50}s of –5.3 and –10.2°C for *BNCBF5* and *BNCBF17* lines, respectively, compared with –3.7°C for the wild type were obtained (Supplementary material Table S1). *BNCBF17*-overexpressing plants, on

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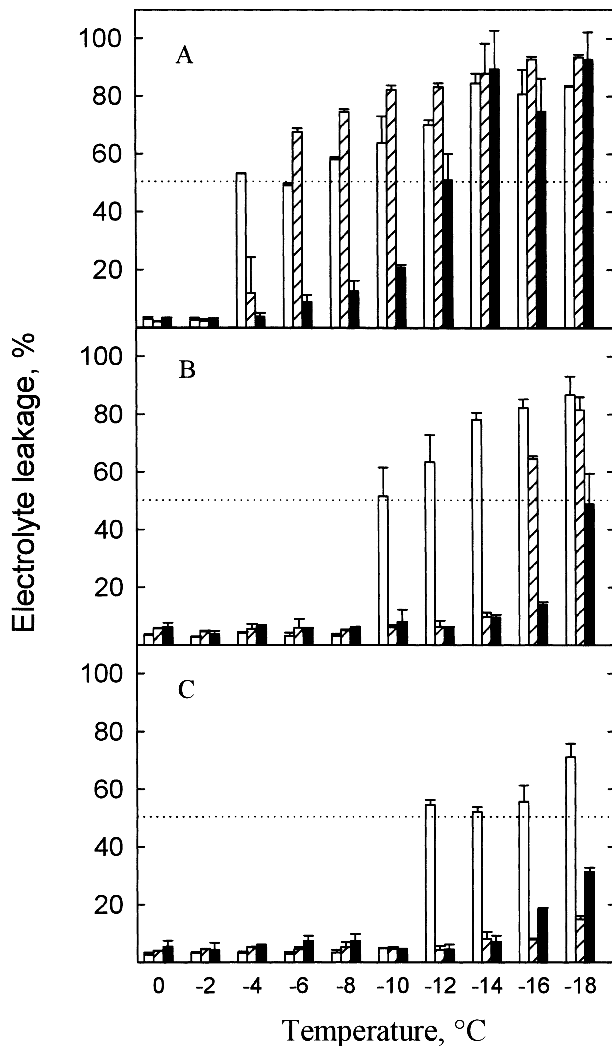

Fig. 2 Freezing survival of leaves of non-acclimated wild-type, *BNCBF17*- and *BNCBF5*-overexpressing transgenic plants as measured by electrolyte leakage. (A) No acclimation; (B and C) Cold acclimated at 4°C for 2 and 5 weeks, respectively. Empty bars, wild-type; dashed bars, *BNCBF5* transgenic line 5A4; solid bars, *BNCBF17* transgenic line 1A. Data obtained from each experiment were based on one leaf collected from each individual seedling and four leaves per freezing temperature point. Error bars for electrolyte leakage are standard deviations calculated from four leaves per freezing temperature point.

the other hand, were able to induce a higher level of constitutive freezing tolerance in the lines tested.

Fig. 2 shows comparisons of freeze-induced leaf electrolyte leakage between the hardiest *BNCBF5*- (5A4) and *17*-overexpressing (1A) lines and wild-type plants. Leaves of non-acclimated wild-type, *BNCBF5*- and *17*-overexpressing plants exhibited LE_{50} values of –4, –6 and –12°C, respectively (Fig. 2A). These differences were also evident in whole plant freeze tests where non-acclimated seedlings were frozen directly in pots and survival estimated 2 weeks after return to room temperature. One hundred and 75% of the *BNCBF17*-overexpressing plants survived freezing to –8 and –12°C, respectively, whereas only 37% of *BNCBF5*-overexpressing plants survived –8°C (Supplementary material Fig. S1). No wild-type plants survived freezing to –8°C. In addition, *BNCBF5*- and *17*-overexpressing plants cold acclimated more rapidly than the wild type and were able to maintain a superior level of freezing tolerance even under prolonged exposure (5 weeks) to the cold (Fig. 2B, C). These were also confirmed with whole plant freeze tests (data not shown). Overall, the results indicated that *BNCBF5* and *17* overexpression led to the development of constitutive freezing tolerance in non-acclimated plants and accelerated the acquisition of maximal freezing tolerance during cold acclimation.

Effects of BNCBF overexpression and cold stress on the gene expression profile in Brassica BNCBF17-overexpressing plants

The Arabidopsis RAFL microarray data (Supplementary material, Tables S2, S3) indicated that, for the most part, genes ‘highly’ up-regulated by *BNCBF17* overexpression paralleled those observed for cold stress and *CBF/DREB* overexpression in *Arabidopsis* (Kasuga et al. 1999, Seki et al. 2001, Fowler and Thomashow 2002, Kreps et al. 2002, Seki et al. 2002, Maruyama et al. 2004) and high light stress (Kimura et al. 2003), and confirm that the RAFL microarray could be used as a partial screening tool for *Brassica*. In contrast, down-regulation of some genes related to cell growth and cell wall biosynthesis was also observed (Supplementary material Table S3). Interestingly, the majority of genes observed to be ‘moderately’ up-regulated encode chloroplast targeted proteins (Table S3). Moreover, moderate constitutive up-regulation of chloroplast stroma cyclophilin *ROC4* (AtCYP20–3), stress-regulated chloroplast targeted elongation factor EF-Tu (Singh et al. 2004), cytosolic β-amylase, triose-P/Pi translocator and cpSRP54 controlling integration of light-harvesting chlorophyll proteins into the thylakoid membranes (Schuenemann et al. 1998) suggested that, at least in *Brassica*, photosynthetic development and carbon partitioning might be affected by *BNCBF17* overexpression (Fig. 3, Table S3)

Effects of BNCBF overexpression, cold stress and cold acclimation on gene expression profiles in Brassica plants

A 2000 element *Brassica* cDNA microarray constructed from leaves of *BNCBF17*-overexpressing plants was used for these studies (Supplementary material, Table S4). Microarray analyses identified that cold stress and/or cold acclimation resulted in a similar pattern of mRNA accumulation for *COR* genes, *GLK2* MYB transcription factor involved in chloroplast photosynthetic development (Cribb et al. 2001, Rossini et al. 2001, Fitter et al. 2002), chloroplast stroma cyclophilin *ROC4* (AtCYP20–3), cytosolic β-amylase and NADH dehydrogenase (Table S4). Conversely, low temperatures (cold stress and/or cold acclimation) decreased transcript levels of photosynthesis-related genes encoding light-harvesting complex I (LHCI),

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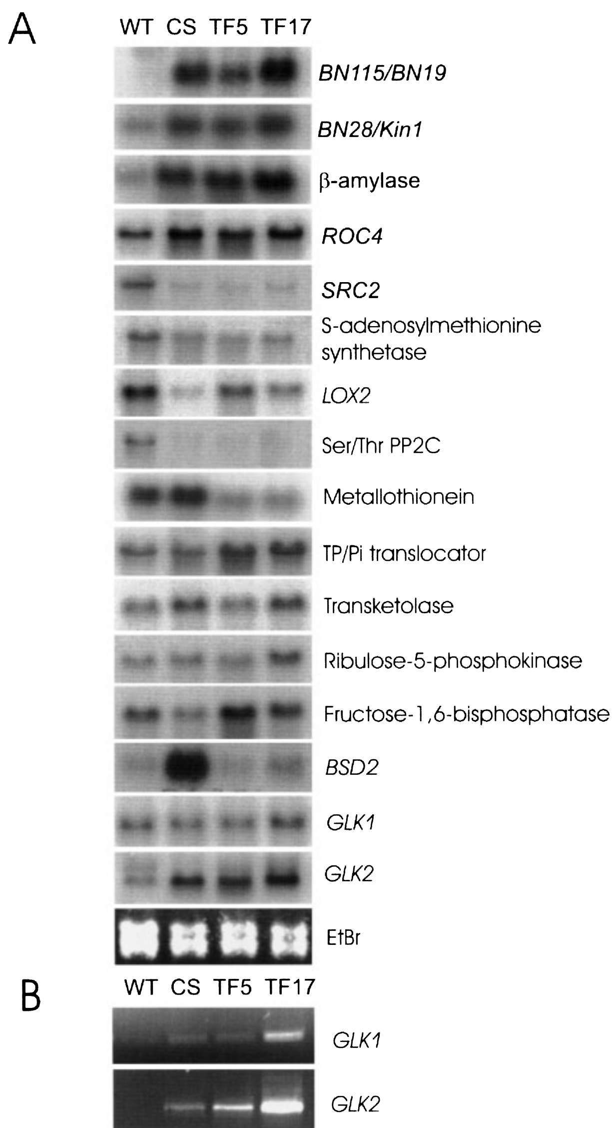

Fig. 3 Comparative analyses of transcript accumulations of selective genes identified by microarray analysis. (A) Northern blot analysis. A 10 μg aliquot of total RNA isolated from leaves of wild type (WT), cold-stressed wild type (CS), *BNCBF5* (TF5) and *BNCBF17* (TF17) overexpressing plants were loaded in each line. (B) RT–PCR analysis of *GLK1* and *GLK2* transcript accumulation.

LHCII, OEE1, OEE3 and subunits of PSII and PSI reaction centres. This is consistent with the notion that post-transcriptional mechanisms also play a crucial role in photosynthetic acclimation (Walters 2005). Both cold stress and cold acclimation resulted in accumulation of *BSD2* (Fig. 3, Table S4), a protein involved in post-transcriptional control of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (*rbcL*) assembly (Roth et al. 1996, Brutnell et al. 1999). In addition, cold stress and cold acclimation resulted in expected differential expression of early light-inducible proteins (ELIPs) and differentially affected transcripts encoding 40S and 60S ribosomal proteins (Table S4).

Moderate (compared with *COR* genes) increases in the levels of transcript accumulations for *GLK2* MYB transcription factor, chloroplast stroma cyclophilin *ROC4* and cytosolic β-amylase were observed for cold stress, cold acclimation and *BNCBF5/17* overexpression (Table S4, Fig. 3). In contrast, SRC2, *S*-adenosylmethionine synthetase, Ser/Thr protein phosphatase 2C and chloroplast targeted lipoxygenase (*LOX2*) were down-regulated under cold stress, cold acclimation as well as *BNCBF* overexpression. On the other hand, the expression of chloroplast inner envelope triose-P/Pi translocator and metallothionein-like proteins was differentially regulated by cold treatment and *BNCBF5/17* overexpression (Fig. 3). In this context, it is interesting to note that a mutant of *Arabidopsis* lacking triose-P/Pi translocator is defective in photosynthetic acclimation to light (Walters et al. 2003).

Microarray analyses identified that mRNA accumulation for the *GLK2* MYB transcription factor was similarly regulated by cold stress, cold acclimation and *BNCBF* overexpression in *Brassica.* However, because it has been observed that chloroplast development in diverse plant species is regulated by *GLK* gene pairs, *GLK1* and *GLK2* (Fitter et al. 2002, Yasumura et al. 2005), we analyzed transcript accumulation of *GLK1* even though it was not identified in our microarrays. Northern blot and reverse transcription–PCR (RT–PCR) analyses indicated that GLK1 mRNA also increased accumulation in response to cold and by *BNCBF* overexpression although not to the same extent as *GLK2* (Fig. 3).

Effects of BNCBF overexpression on photosynthetic capacity

To evaluate if *BNCBF* overexpression resulted in any alterations in photochemical efficiency and photosynthetic capacity, we measured light- as well as light–CO_2-dependent responses of CO_2 assimilation rates and fluorescence parameters in the non-acclimated wild-type, and *BNCBF5*- and *17*-overexpressing plants (Fig. 4). The data suggest that *BNCBF5/17* overexpression resulted in increases in light-saturated rates of CO_2 assimilation (32–38%) associated with increased photochemical efficiency of photosynthesis (Fv′/Fm′), higher rates (42–69%) of PSII-driven linear electron transport (ETR), lower (12–19%) redox state of the electron transport chain (1 – qP) and, as a result, lower light energy dissipation through non-photochemical events (NPQ, qN) when compared with the respective wild-type plants (Fig. 4A, B, E–G). It is important to note that despite similar light-saturated rates of CO_2 assimilation for both *BNCBF*-overexpressing plants, *BNCBF17*-overexpressing plants had 18% higher ETR and dissipated less energy through the mechanism of antenna quenching (NPQ) than *BNCBF5*-overexpressing plants (Fig. 4D, G). To determine the maximum photochemical efficiencies and photosynthetic capacities, we measured CO_2 responses of CO_2 assimilation rates and fluorescence parameters at light-saturated conditions. CO_2 enrichment resulted in both *BNCBF*-

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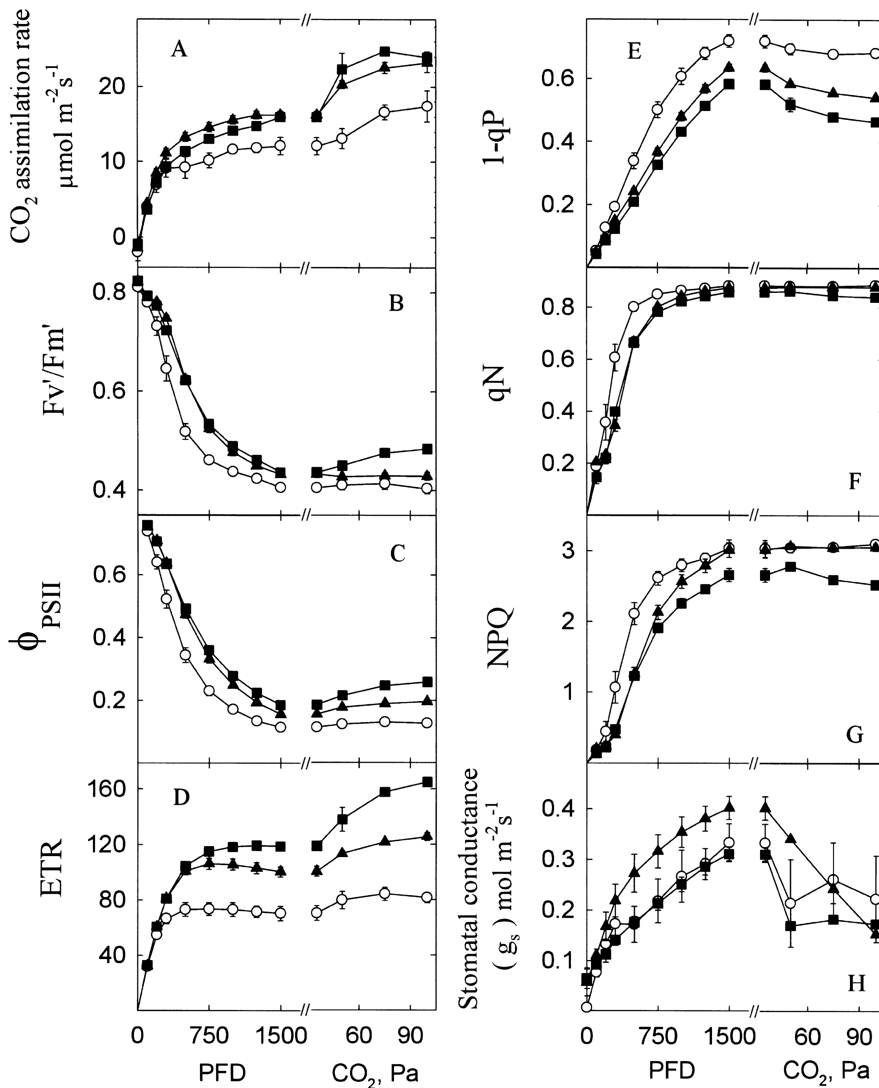

Fig. 4 Effects of *BNCBF5* and *17* overexpression on the photosynthetic capacity and photochemical efficiency of non-acclimated *Brassica* plants. Measurements of light response curves of CO_2 assimilation rates and fluorescence parameters were performed on control grown plants at 20°C and a $p(CO_2)$ of 35 Pa. To assess the maximum photosynthetic efficiency of photosynthesis, measurements continued at 20°C/1,500 PFD and a $p(CO_2)$ of 35, 50, 75 and 100 Pa. Variants represent leaves of wild-type (open circles), *BNCBF5*-overexpressing (filled triangles) and *BNCBF17*-overexpressing (filled squares) plants. Data are the average of three independent experiments with $n = 3$ for each experiment, ±SE.

overexpressing plants having 38% higher CO_2-saturated rates of CO_2 assimilation, higher ETR (54–102%) and lower 1 – qP values (21–32%) than the respective wild-type plants (Fig. 4A, D, E). Overall, our data indicate that *BNCBF* overexpression led to increased photochemical efficiency of photosynthesis (Fig. 4B) and increased capacities for linear electron transport and CO_2 assimilation (Fig. 4A, D). In addition, *BNCBF17*-overexpressing plants have shown a greater capacity for linear electron transport, a greater stimulatory effect of CO_2 enrichment on Fv′/Fm′, ϕ_{PSII} and ETR, lower redox state of the electron transport chain and decreased levels of light energy dissipation than *BNCBF5*-overexpressing plants (Fig. 4D, E, G). Measurements of stomatal conductance (Fig. 4H) indicated that under ambient $p(CO_2)$ conditions, a 20–30% increase in g_s values were observed in *BNCBF5*-overexpressing but not in the wild-type and *BNCBF17*-overexpressing plants. CO_2 enrichment, however, resulted in a reduction of stomatal conductance to the level that was similar for all the plants. This suggests that differential capacities for linear electron transport and CO_2 assimilation (Fig. 4A, D) observed between wild-type and *BNCBF*-overexpressing plants under CO_2-enriched conditions were not the result of differential stomatal conductance.

Effects of BNCBF overexpression, cold stress and cold acclimation on specific leaf weight, content and composition of photosynthetic pigments

Improved photochemical efficiency may result from alterations in chlorophyll content and composition as well as changes in the activities of the Calvin cycle enzymes. To ascertain if this is the case, measurements were made on three types of leaves from the hardiest *BNCBF5* and *17* lines and wild-type plants: (i) leaves fully developed at 20°C, i.e. non-acclimating control leaves (N leaves); (ii) leaves fully developed at 20°C and then ‘temperature-shifted’ to 4°C (NS leaves), i.e. cold-stressed leaves; and (iii) leaves fully developed at 4°C (C leaves), i.e. cold acclimated. This allowed us (i) to evaluate the

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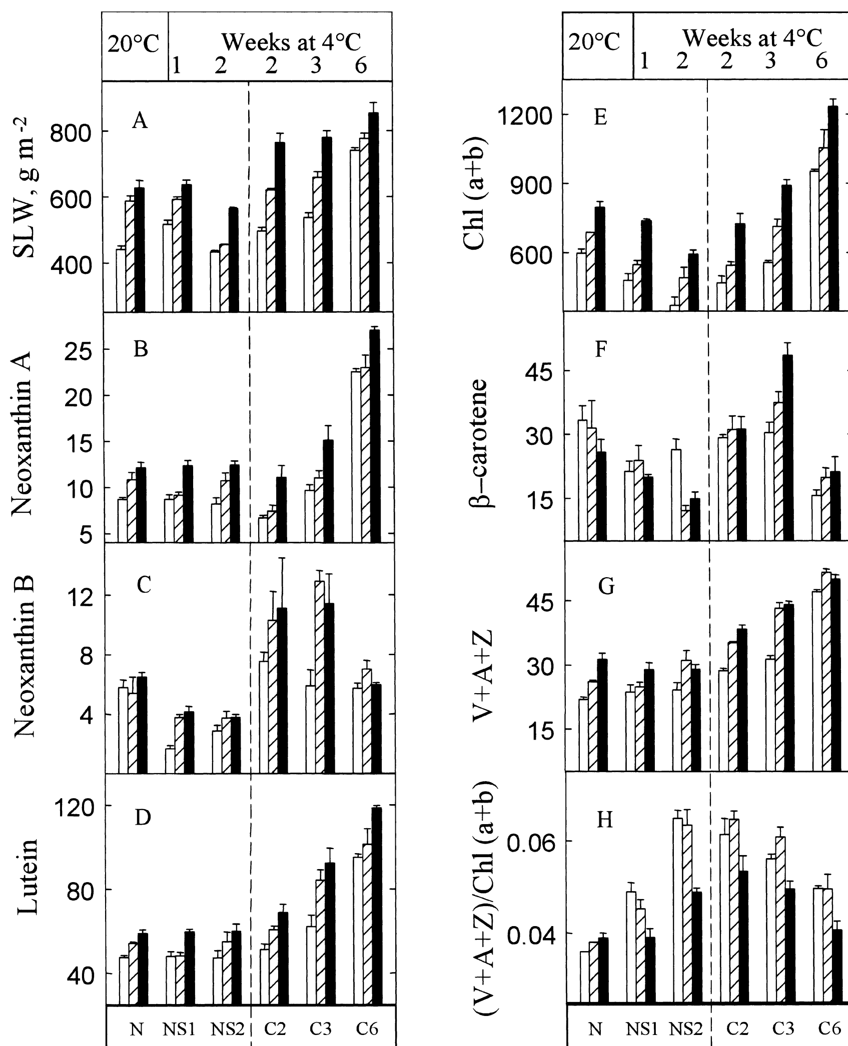

Fig. 5 Effects of cold stress and cold acclimation on specific leaf weight (SLW), and pigment content and composition in leaves of wild-type, *BNCBF5* and *17* transgenic plants. Plants were maintained at either control growth conditions (20/16°C and 250 PPFD) or cold stress/cold acclimation conditions (4/4°C and 250 PPFD). N, leaves developed at 20°C; NS1 and NS2, leaves developed at 20°C and cold stressed at 4°C for a period of 1 and 2 weeks, respectively; C1, C3 and C6, leaves developed totally at 4°C for periods of 2, 3 and 6 weeks, respectively, as indicated. Leaf samples were collected 8 h after the onset of the photoperiod. Bars represent leaves of wild-type (open bars), *BNCBF5*-overexpressing (dashed bars) and *BNCBF17*-overexpressing (filled bars) plants. The level of individual pigments was expressed in mg m^{-2}. Data are the average of three independent experiments with $n = 2$ for each experiment, ±SE.

effect of *BNCBF* overexpression on non-acclimated controls and on cold stress as well as cold acclimation; and (ii) to identify if *BNCBF* overexpression mimics photosynthetic cold acclimation responses.

Under non-acclimating conditions (N), leaves of *BNCBF*-overexpressing plants showed significantly higher specific leaf weight (SLW) and Chl ($a + b$) contents than wild-type plants (Fig. 5A, E). Similar trends were observed for accumulations of neoxanthin A, lutein and xanthophyll cycle pigments (V + A + Z) (Fig. 5B, D, G). As a result, no changes in (V + A + Z)/Chl ($a + b$) ratios were observed in response to *BNCBF* overexpression (Fig. 5H). Cold stress (NS), however, led to the differential increases in carotenoid/chlorophyll ratios as a result of progressive differential degradation of chlorophylls and no alterations in the content of photoprotective pigments (lutein and V + A + Z) in all plants tested (Fig. 5D, E, G, H). The effect of cold stress-induced chlorophyll degradation was less pronounced in *BNCBF5*- and *17*-overexpressing plants than in wild-type plants (Fig. 5E). Cold acclimation, however, was associated with an increase in SLW, levels of Chl ($a + b$), photoprotective carotenoids and carotenoid/chlorophyll ratios (Fig. 5A, D, E, F, G, H). Overall, cold acclimation-induced accumulation of chlorophylls, photoprotective pigments and increase in SLW was more pronounced in *BNCBF5*- and *17*-overexpressing plants than in wild-type plants (Fig. 5A, D, E–G).

Effects of BNCBF overexpression, cold stress and cold acclimation on resistance to low temperature-induced photoinhibition and photoprotection

The effects of cold stress as well as cold acclimation on photoinhibition of photosynthesis (Fv/Fm), epoxidation state (EPS) of xanthophyll cycle pigments and anthocyanin accumulation in leaves were studied (Fig. 6A–C). During photoinhibition of photosynthesis at growth conditions, a 15% decrease in Fv/Fm was observed only in the wild-type leaves developed at 20°C and cold stressed for a period of 1 and 2 weeks (Fig. 6A). This photoinhibitory effect on wild-type leaves was associated with a transient decrease in the epoxidation state of xantho-

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phyll cycle pigments (Fig. 6B) during the first week of stress and anthocyanin accumulation during the second week of stress (Fig. 6C).

To exacerbate the photoinhibitory damage, additional treatment was carried out on all plants at an irradiance of 1,000 photon flux density (PFD) for 6 h at 4°C (Fig. 6D). One week cold-stressed *BNCBF5*- and *17*-overexpressing plants showed a smaller decrease (20 and 10%) in Fv/Fm compared with the wild type (35%). After 2 weeks, cold-stressed wild-type plants developed some resistance to photoinhibition probably as a result of screening (Havaux and Kloppstech 2001) by stress-induced anthocyanin accumulation. Cold-stressed *BNCBF*-overexpressing plants on the other hand continued to show small decreases in Fv/Fm. The photoinhibition results correlated with the previously observed differential degree of Chl ($a + b$) degradation under the stress conditions, i.e. leaves of wild-type, *BNCBF5*- and *17*-overexpressing plants decreased the Chl ($a + b$) level by 30, 23 and 9%, respectively, after 1 week of stress and by 38, 28 and 18%, respectively, after 2 weeks of stress (Fig. 5E, 6D–F). As expected, during cold acclimation (C leaves), all genotypes increased tolerance to low temperature-induced photoinhibition (Fig. 6D) as well as levels of Chl ($a + b$) and photoprotective carotenoids (Fig. 5D, E, G).

Increased production of antioxidants and oxygen-scavenging enzymes is important with respect to the sensitivity to low temperature-induced photoinhibition. Monitoring Fv/Fm changes under methylviologen (MV)-induced oxidative damage was used to assess the plant's capacity to tolerate ROS-induced photooxidative damage through the capacity of ROS-scavenging enzymes (Fig. 6E). Control (N) leaves showed drastic decreases in the maximum photochemical efficiencies (decreases in Fv/Fm) in all genotypes tested. Cold stress (NS) and initial stages of cold acclimation (C), however, resulted in a progressive increase in ROS scavenging capacity that is similar for all genotypes. Cold acclimation in the long term, however, differentially decreased the resistance to MV treatment in wild-type, *BNCBF5*- and *17*-overexpressing plants (Fig. 6E).

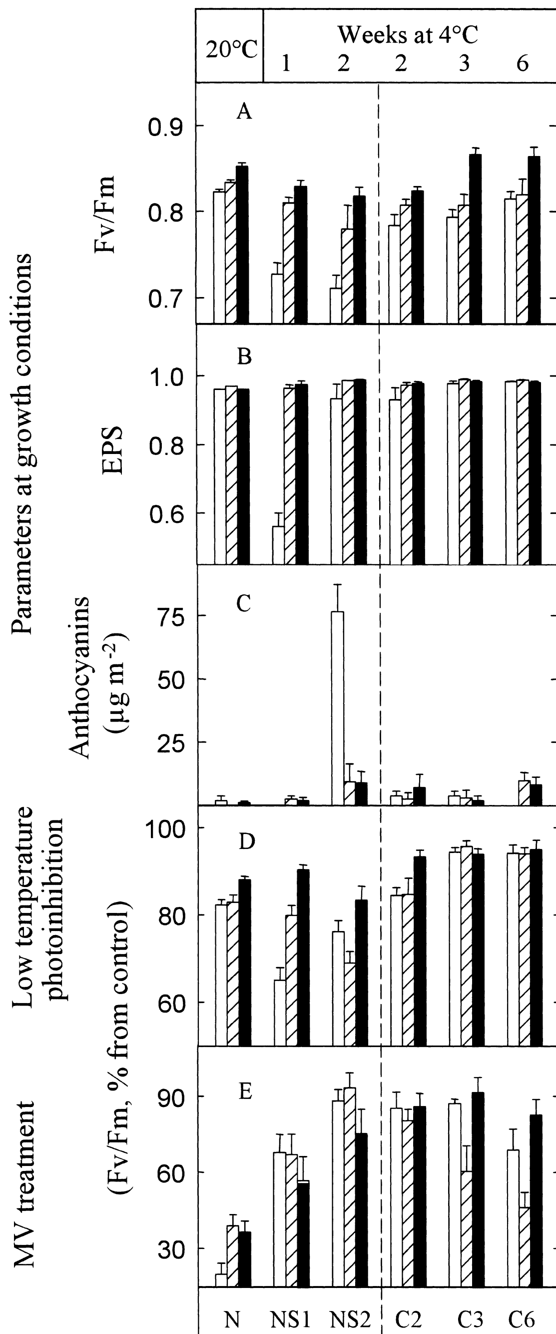

Fig. 6 Effects of cold stress and cold acclimation on resistance to low temperature-induced photoinhibition, methylviologen (MV)-induced oxidative stress, epoxidation state of the xanthophyll cycle pigments (EPS) and anthocyanin accumulation in leaves of wild-type, *BNCBF5*- and *17*-overexpressing plants. N, leaves developed at 20°C; NS1 and NS2, leaves developed at 20°C and cold stressed at 4°C for a period of 1 and 2 weeks, respectively; C1, C3 and C6, leaves developed totally at 4°C for periods of 2, 3 and 6 weeks, respectively, as indicated. Bars represent leaves of wild-type (open bars), *BNCBF5*-overexpressing (dashed bars) and *BNCBF17*-overexpressing (filled bars) plants. (B) and (C) reflect changes in EPS and anthocyanin content in leaves collected 8 h after the onset of the photoperiod from plants maintained at either control growth conditions (20/16°C and 250 PPFD) or cold stress/cold acclimation conditions (4/4°C and 250 PPFD). (A), (D) and (E) reflect changes in Fv/Fm, maximum photochemical efficiency of PSII in response to (A) exposure of leaves to growth conditions of either 20/16°C and 250 PPFD or 4/4°C and 250 PPFD; (D) exposure of all leaves to additional photoinhibitory treatment at 4°C/1,000 PFD for 6 h and (E) exposure of all leaves to 10 µM MV solution for 6 h at 20°C/500 PFD. Fv/Fm values of non-acclimated plants were used as controls for calculations of the data presented in (D) and Fv/Fm values of leaves exposed to 0 µM MV (H_2O) for 6 h at 20°C/500 PFD were used as controls for calculations of the data presented in (E). Data are the average of three independent experiments with $n = 2$ for each experiment, ±SE.

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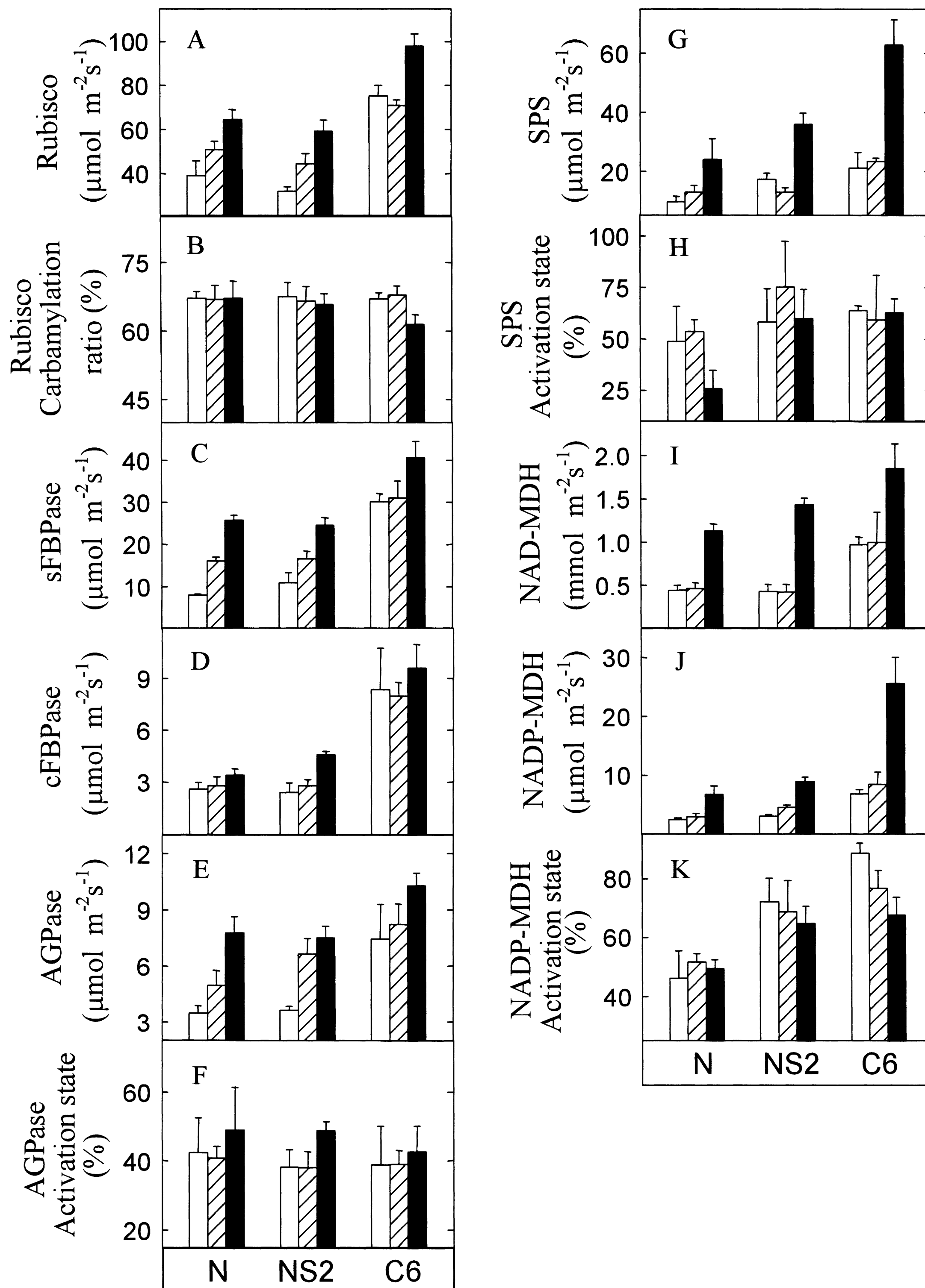

Fig. 7 Effects of cold stress and cold acclimation on enzymes of the photosynthetic carbon metabolism in leaves of wild-type, *BNCBF5*- and *17*-overexpressing plants. N, leaves developed at 20°C; NS2, leaves developed at 20°C and cold stressed at 4°C for a period of 2 weeks; C1, C3 and C6, leaves developed totally at 4°C for a period of 2, 3 and 6 weeks, as indicated. Bars represent leaves of wild-type (open bars); *BNCBF5*-overexpressing (dashed bars) and *BNCBF17*-overexpressing (filled bars) plants. Leaf samples were collected 8 h after the onset of the photoperiod at prevailing growth conditions. Data are the average of three independent experiments with $n = 2$ for each experiment, ±SE.

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Table 1 Effects of *BNCBF5* and *17* overexpression and cold acclimation on enzyme activities of photosynthetic carbon metabolism in leaves of wild-type, *BNCBF5*- and *17*- overexpressing plants

Enzyme	N			C6		
	Wild-type	*BNCBF5*	*BNCBF17*	Wild-type	*BNCBF5*	*BNCBF17*
Rubisco	235 ± 40	266 ± 20	293 ± 20	285 ± 19	242 ± 9	285 ± 17
sFBPase	48 ± 2	84 ± 5	116 ± 6	114 ± 7	106 ± 13	119 ± 11
AGPase	21 ± 2	26 ± 4	35 ± 4	28 ± 7	28 ± 4	30 ± 2
cFBPase	16 ± 2	15 ± 3	15 ± 2	32 ± 9	27 ± 3	28 ± 4
SPS	58 ± 12	68 ± 12	109 ± 15	80 ± 20	80 ± 4	183 ± 25
NADP-MDH	15 ± 2	16 ± 3	30 ± 7	26 ± 3	29 ± 7	75 ± 13
NAD-MDH	2.6 ± 0.4	2.4 ± 0.4	5.1 ± 0.4	3.7 ± 0.3	3.4 ± 0.2	5.4 ± 0.8

Variants represent leaves developed at 20°C (N) and developed at 4°C for a period of 6 weeks (C6). Maximum enzyme activities are expressed as μmol mg^{-1} Chl h^{-1} and mmol mg^{-1}Chl h^{-1} (NAD-MDH). Data are the average of three independent experiments with $n = 2$ for each experiment, ±SE.

Effects of BNCBF overexpression, cold stress and cold acclimation on photosynthetic carbon metabolism

Even in the absence of cold acclimation (N), a 30 and 65% increase in Rubisco, a 101 and 223% increase in stromal fructose 1,6-bisphosphatase (sFBPase) and a 43 and 123% increase in ADP-glucose pyrophosphorylase (AGPase) activities were observed for *BNCBF5*- and *17*-overexpressing plants, respectively (Fig. 7A, C, E). While increases in Rubisco, sFBPase and AGPase were associated with cold acclimation in all plants tested (Fig. 7A, C, E), *BNCBF17*-overexpressing plants maintained higher enzymes activities than the wild-type and *BNCBF5*-overexpressing plants even after 5 weeks of cold acclimation. However, neither *BNCBF* overexpression nor cold stress/cold acclimation affected Rubisco carbamylation ratios and AGPase activation states (Fig. 7B, F).

Changes in activities of the cytosolic FBPase (cFBPase) and sucrose-phosphate synthase (SPS) might be used as indicators of the potential capacity for sucrose synthesis, and increases in their activities were reported to be associated with cold acclimation (Savitch et al. 1997, Strand et al. 1999, Savitch et al. 2000a). Our results indicate that cFBPase and SPS activities increased substantially as a result of cold acclimation in all plants tested (Fig. 7D, G). However, while activities of cFBPase and SPS were similar for both *BNCBF5*-overexpressing and wild-type plants, *BNCBF17*-overexpressing plants had higher SPS activity in all conditions tested (Fig. 7G).

Further, we examined the effect of *BNCBF* overexpression on NAD-malate dehydrogenase (MDH) and NADP-MDH activities as increases in the activities of these enzymes have been implicated in stimulation of the glycolytic pathway and chloroplast oxaloacetate/malate exchange associated with cold acclimation (Savitch et al. 2000a, Savitch et al. 2000b, Savitch et al. 2001). Our results indicate that an increase in NAD-MDH and NADP-MDH activities (Fig. 7I, J) was a common response to cold acclimation in all plants tested. However, *BNCBF17*-overexpressing plants had much higher NAD-MDH and NADP-MDH activities in all conditions tested. The NADP-MDH activation state can be used as an estimate of the reduction state of the chloroplast stroma (Savitch et al. 2000b, Savitch et al. 2001). As expected, cold stress and cold acclimation resulted in increases in the NADP-MDH activation state in all genotypes tested (Fig. 7K). However, cold-acclimated *BNCBF5*-and *17*-overexpressing plants had 14–24% lower NADP-MDH activation states than the respective wild-type plants. This suggests that cold-acclimated *BNCBF*-overexpressing plants are less limited at the level of photosynthetic carbon assimilation than cold-acclimated wild-type plants.

Overall these results indicate that overexpression of either *BNCBF5* or *17* results in increased activities of Calvin cycle enzymes and increased capacity for carbon utilization in starch biosynthesis. In addition, overexpression of *BNCBF17* resulted in an increased flux of metabolites through the glycolytic and sucrose biosynthetic pathways. These results are in agreement with the increased photosynthetic capacity of *BNCBF5*- and *17*-overexpressing plants observed earlier (Fig. 4A). Moreover, constitutive increases in activities of NAD-MDH and NADP-MDH observed only in *BNCBF17*-overexpressing plants suggest that stimulation of the respiratory pathway and the oxaloacetate/malate 'valve' in the chloroplast might account for some discrepancies between CO_2 response of CO_2 assimilation and electron transport rates observed in *BNCBF17*-overexpressing plants (Fig. 4A, D).

Enzyme activities presented in Fig. 7 were recalculated and expressed on a Chl basis (Table 1). As expected in common responses to cold acclimation, wild-type plants increased maximum activities of enzymes by 21–140%. It is evident, that even in control growth conditions (N), *BNCBF5*-overexpressing plants modulated wild-type cold acclimation responses at the level of sFBPase activity and *BNCBF17*-overexpressing plants modulated wild-type cold acclimation responses at the level of Rubisco, sFBPase, AGPase, SPS, NAD-MDH and NADP-MDH activities. It is important to note that cold acclimation adjusted enzyme capacities to similar lev-

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els in wild-type and *BNCBF5*-overexpressing plants. In contrast, cold acclimation (C6) of *BNCBF17*-overexpressing plants resulted in 1.6- to 2.3-fold higher SPS, NAD-MDH and NADP-MDH activities than cold acclimation of wild-type and *BNCBF5*-overexpressing plants (Table 1).

Discussion

Overexpression of BNCBF5 and BNCBF17 is capable of conferring constitutive freezing tolerance as well as more rapid cold acclimation

Homologous overexpression of two *Brassica CBF/DREB*-like genes resulted in the development of constitutive freezing tolerance in transgenic *Brassica.* In addition, *BNCBF*-overexpressing plants acclimate more rapidly and achieve a higher degree of freezing tolerance than the wild type.

As in *Arabidopsis*, *BNCBF*-overexpressing *Brassica* constitutively accumulates orthologs of *COR15A*, *COR6.6*, *COR47* and *COR78* transcripts (Fig. 1). Compared with *BNCBF5*, however, *BNCBF17*-overexpressing plants exhibit a higher level of *cor* gene mRNA accumulation. This difference was not correlated with the respective levels of *BNCBF* transcripts in the transgenic plants and may reflect the differences between the *BNCBF17* and other *Brassica CBF/DREB1*-like transcription factors (Gao et al. 2002). This prompted us to (i) carry out transcript analyses on *BNCBF17*-overexpressing leaves using the RAFL 8K microarray as a screening tool as well as a smaller targeted *B. napus* cDNA array spotted from a cDNA library of leaves of *BNCBF17*-overexpressing plants grown at room temperature; and (ii) examine the effects of *BNCBF* overexpression on photosynthetic development and function. While *BNCBF* overexpression induced the accumulation of transcripts of some of the more highly up-regulated genes already shown in *CBF/DREB1* overexpression in *Arabidopsis* (Seki et al. 2001, Fowler and Thomashow 2002), a large number of the moderately up-regulated genes were identified to be either targeted to the plastid or associated with photosynthetic cold acclimation or chloroplast development (Fig. 3, Table S3). We show for the first time that overexpression of a *BNCBF/DREB1* transcription factor results in constitutive accumulation of mRNA for *GLK1* and *GLK2* MYB transcription factors involved in chloroplast photosynthetic development (Cribb et al. 2001, Rossini et al. 2001, Fitter et al. 2002, Yasumura et al. 2005), chloroplast stroma cyclophilin *ROC4* (AtCYP20–3), β-amylase and triose-P/Pi translocator (Fig. 3), suggesting that, at least in *Brassica*, chloroplast photosynthetic development and carbon partitioning might be affected by *CBF/DREB1* overexpression.

Northern blot analyses showed that on average, constitutive accumulations of putative *cor* and *GLK1/GLK2* transcripts were higher in the *BNCBF17*- than the *BNCBF5*-overexpressing plants (Fig. 1, 3), even when constitutive levels of *BNCBF5* transcripts were similar. Alignment of the deduced peptide sequences of the *BNCBF*s indicated that *BNCBF17* is different from the other known *Brassica* and *Arabidopsis CBF/DREB*s (Jaglo et al. 2001, Gao et al. 2002). This may translate into different transcriptional efficiencies between the two factors. Even in *Arabidopsis*, global analyses of gene expression have indicated that within the same set of genes, the levels of expression could differ between *CBF1*, *2* and *3* (Fowler and Thomashow 2002, Gilmour et al. 2004). DNA gel shift assays of mutagenesis of the CCGAC core of the CRT/DRE *cis*-domain in the promoter of the *Brassica cor* genes (*BN115* and *28*) suggested that there is a less stringent specificity requirement for binding by *BNCBF17* than *BNCBF5* (Gao et al. 2002). The regulons for *BNCBF5* and *17* have to be identified to resolve unequivocally the differential phenotypes observed.

In addition, we show that cold stress and cold acclimation of wild-type plants was also associated with moderate accumulations (although not to the same extent as *BNCBF17* overexpression) of mRNA for *GLK1*, *GLK2*, *ROC4* and β-amylase, suggesting the involvement of these genes in a process of cold acclimation. Increased accumulation of transcripts for β-amylase was reported previously for cold-stressed *Arabidopsis* plants (Fowler and Thomashow 2002) and suggested the possible role of the resultant maltose accumulation as compatible-solute stabilizing factor in the chloroplast stroma (Kaplan and Guy 2004).

Leaf thickness, mesophyll structure and specific leaf weight are the important determinants of the rate of leaf photosynthesis (Terashima et al. 2005). Involvements of *Arabidopsis CBF1*, *2* and *3* in constitutive accumulation of soluble sugars and in leaf anatomical alterations such as increased leaf thickness and cell density were reported (Gilmour et al. 2004). In this study, we present data to support the physiological results of multifunctional effects of the role of *CBF* by showing that *BNCBF5*- and *17*-overexpressing plants exhibit altered chloroplast photosynthetic development, higher photosynthetic capacity and photochemical efficiency, an increased level of photosynthetic pigments, and elevated activities of the Calvin cycle enzymes and key regulatory enzymes of starch and sucrose biosynthesis.

Roles of BNCBF5 and BNCBF17 overexpression in modulation of plant photosynthetic responses to cold stress and cold acclimation

This study also showed that *BNCBF5* and *17* overexpression, even in non-acclimating conditions, increases resistance to low temperature-induced photoinhibition, pigment accumulation, SLW, photochemical efficiency and capacity for linear electron transport and CO_2 assimilation rates associated with increased activities of Calvin cycle enzymes and enzymes of starch biosynthesis. Only *BNCBF17* overexpression affected glycolytic and sucrose biosynthetic pathways and chloroplast oxaloacetate/malate exchange as indicated by increased NAD-MDH, SPS and NADP-MDH activities, respectively. Increased carbon flux through SPS relative to that through cFBPase was reported previously for cold-

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acclimated plants and was suggested to be a result of re-utilization of hexose by phosphorylation and re-synthesis into sucrose (Collis and Pollock 1992, Savitch et al. 2000a) and/or resulted from inhibition of cFBPase by fructose 2,6-bisphosphate associated with an increased flux of metabolites through the glycolytic and pentose phosphate pathways (Savitch et al. 2000a). Results of cold acclimation of wild-type *Brassica* presented in this study, as well as previous reports of plant cold acclimation responses (Gray et al. 1996, Savitch et al. 1997, Strand et al. 1997, Strand et al. 1999, Savitch et al. 2000a, Savitch et al. 2000b, Savitch et al. 2001, Leonardos et al. 2003), suggest that overexpression of *BNCBF5* and *17*, even in non-acclimating conditions, leads to partial modulation of plant photosynthetic responses typically associated with cold acclimation. It is important to note that while both *BNCBF5* and *17* overexpression affected chloroplast photosynthetic development, only *BNCBF17* overexpression affected cytosolic carbon metabolism.

Increased (V + A + Z)/Chl ($a + b$) ratios and low EPS ratios of the xanthophyll cycle pigments have been suggested to reflect an increased capacity for non-photochemical dissipation of light absorbed by the photosynthetic antennae (Demmig-Adams and Adams 1992, Müller et al. 2001). To this effect, cold acclimation has been shown to increase photoprotective carotenoids and carotenoid/chlorophyll ratios (Gray et al. 1996, Strand et al. 1999, Savitch et al. 2002). While similar changes were observed during cold acclimation for all genotypes in this study, no alterations in chlorophyll/carotenoid ratios were observed in non-acclimated *BNCBF5*- and *17*-overexpressing plants, indicating that *BNCBF* overexpression affected only pigment content but not pigment composition. Changes in pigment composition typically associated with cold acclimation were reported to represent not so much a response to low temperature, but rather a response to the changes in chloroplast redox poise (Gray et al. 1997, Wilson and Huner 2000). In that respect, *BNCBF* overexpression constitutively affected neither the redox state of the electron transport chain (1 – qP) nor the redox state of the chloroplast stroma (NADP-MDH activation state).

Cold acclimation has been shown to result in increased production of antioxidants and oxygen-scavenging enzymes (Savitch et al. 2000b). Again, while this was observed during cold acclimation of all genotypes in this study, *BNCBF* overexpression did not affect ROS management mechanisms. Thus, the results obtained here only partially agree with reports that management of ROS-scavenging enzymes was the factor in increased resistance to photoinhibition resulting from heterologous expression of *Arabidopsis CBF1* in tomato (Hsieh et al. 2002). Our results suggest that *BNCBF5*- and *17*-overexpressing plants increase tolerance to photoinhibition through increased photosynthetic capacity. In addition, an increased NADP-MDH capacity observed in *BNCBF17*-overexpressing plants might also be a factor contributing to the differential susceptibility to photoinhibition. An increased capacity of oxaloacetate/malate exchange in the chloroplast has been reported to be a factor in mitigation of the sensitivity to photoinhibition at low temperatures (Savitch et al. 2000b).

It has been suggested that *CBF* signaling pathways might be involved in photosynthetic cold acclimation (Stitt and Hurry 2002). The combination of light and temperature (Crosatti et al. 1999, Fowler et al. 2001, Takumi et al. 2003), light stress (Kimura et al. 2003) and/or redox states of the chloroplast (Gray et al. 1997, Ndong et al. 2001, Dal Bosco et al. 2003) as well as mitochondria-mediated whole cell redox status can affect cold-regulated gene expression (Dutilleul et al. 2003, Lee et al. 2002). In addition, interactions between *CBF* pathways and light signaling, mediated by phytochrome (Kim et al. 2002) and the ABA-dependent pathway (Shinozaki and Yamaguchi-Shinozaki 2000), and identification of genes controlled by *DREB1A* (*CBF3*) but lacking CRT/DRE elements in their promoters (Seki et al. 2001) have also been reported and are consistent with the observation here of increased transcript accumulation for *ROC4*, *GLK1*, *GLK2* and triose-P/Pi translocator. Moreover, the regulation of *CBF* signaling downstream of *CBF* expression by *sfr6* mutation leading to a decrease in freezing tolerance associated with a decreased cold-inducible expression of *COR* genes as well as genes with and without CRT/DRE elements in their promoters, including a number of photosynthetic genes (Knight et al. 1999, Boyce et al. 2003), also suggests a multifunctional outcome as a result of *CBF* expression during cold acclimation.

In summary, we have shown that homologous overexpression of two *B. napus BNCBF/DREB1*-like genes, in addition to the expected effects on *COR* gene expression and freezing tolerance, regulated photochemical efficiency and photosynthetic capacity through its effects on pigment accumulation, capacities of the Calvin cycle enzymes and key regulatory enzymes of starch and sucrose biosynthesis, glycolysis and oxaloacetate/malate exchange. In addition, the up-regulation of *GLK1* and *GLK2* may indicate a potential for affecting chloroplast development.

Materials and Methods

Plant material and growth conditions

Seeds of wild-type *B. napus* cv. Jet neuf, cv. Westar and transgenic *B. napus* cv. Westar were germinated in soil flats, and seedlings were grown for 3 weeks in a Conviron E-15 cabinet under controlled environmental conditions with a temperature regime of 20°C/16°C (day/night), a 16 h photoperiod and a PFD of 250 μmol m^{-2} s^{-1} for control (non-acclimating) conditions (N leaves). To study the effect of low temperature stress and cold acclimation, 3-week-old, four-leaf stage plants (leaf size of 2–4 cm across) were transferred to a temperature regime of 4°C/4°C (day/night) with light conditions identical to the control grown plants. During growth at low temperature, the leaves previously developed at 20°C (N) were considered cold-stressed (NS) while the new leaves developed at 4°C (C) were considered cold-acclimated. The effect of cold stress on N leaves was monitored after 1 and 2 weeks of plant growth at 4°C while the effect of cold accli-

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mation on C leaves was monitored after 2, 3 and 6 weeks of plant growth at 4°C.

cDNA library construction and isolation of BNCBFs

A cDNA library was constructed in Uni-Zap (Stratagene, La Jolla, CA, USA) using mRNA isolated from leaves of *B. napus* cv. Jet neuf cold-acclimated for 4 d under the conditions described above. Total RNA was isolated using the Trizol kit as per the manufacturer's instructions (Invitrogen, Burlington, Ontario, Canada). mRNA was isolated using the MessageMaker kit (Gibco-BRL, USA). A 333 bp amplicon was generated by RT–PCR performed on the same mRNA employing semi-nested primers [first PCR, 5′Tfprimer 1, ATT TAC AGA GGA GTT CGT CAA, 3′poly$(T)_{19;}$ second PCR, 5′Tfprimer 1, 3′Tfprimer 2, ATA AAT AGC TTC CAC AT CGT] designed from the sequence of the *Arabidopsis CBF1* cDNA (Stockinger et al. 1997). This amplicon was used to screen the cDNA library using the PCR/ DIG Probe labeling kit (Roche Molecular Biochemicals). Thirty-five positive clones were isolated and sequenced using a LiCOR automated sequencer. Based on the sequence alignments, four different *BNCBF*s were identified (Gao et al. 2002).

cDNA library construction for EST sequencing

A second cDNA library using mRNA isolated from leaves of homozygous enhanced 35S-*BNCBF17* plants grown under non-acclimating conditions as described above was constructed in Bluescript SK+/*Xho*I–*Eco*RI (Stratagene, La Jolla, CA, USA) for expressed sequence tag (EST) sequencing using an automated LiCOR sequencer. The ESTs generated were blasted and deposited in the NCBI EST database (DbEST identifier 11009).

Agrobacterium-mediated transformation of Brassica napus cv. Westar

Cassettes containing chimeric fusions of the coding regions of *BNCBF17* (1,218 bp *Sma*I–*Kpn*I fragment) and *BNCBF5* (1,047 bp *Xba*I–*Kpn*I fragment) placed under the control of the cauliflower mosaic virus (CaMV)-enhanced 35S promoter (Timmermans et al. 1990) were constructed. These cassettes were ligated into the binary vector pHS723 (Nair et al. 2000), a gift from R. Datla, PBI/NRC, Saskatoon, Saskatchewan, Canada, to produce the final transformation vectors *pBNCBF5* and *17*. The resultant constructs were introduced into competent *Agrobacterium tumefaciens* strain GV3109pmp90. Cotyledons from *B. napus* cv. Westar were transformed using the cotyledon/petiole co-cultivation method (Moloney et al. 1989) with modifications (White et al. 1994). Transformed plants were selected on kanamycin and further confirmed by histochemical screening for expression of the GUS gene which is fused to NPTII in the pHS723 binary vector. Transformants with a single or a low number of transgene insertions were identified by genomic Southern analyses. Homozygous lines were then obtained by screening seedlings germinated from seeds of T1 plants with β-glucuronidase (GUS).

Northern blot analyses

Transgenic and wild-type plants were grown normally or cold-acclimated for the required periods as described above. Total RNA were extracted from 100 mg of leaf tissue using the FastRNA kit–Green (Qbiogene, Carlsbad, CA, USA) and the FP120 FastPrep cell disruptor (Savant Instruments, Holbrook, NY, USA). A 10 µg aliquot of total RNA per lane was run on a MOPS denaturing gel, transferred on a nylon membrane, UV cross-linked and then hybridized in Church buffer (Church and Gilbert 1984) with the appropriate probes. The probes were ^{32}P-labeled by random priming using the HexaLabel DNALabeling Kit (MBI Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. For northern blot analyses of *BNCBF17* accumulation during cold acclimation, a *BNCBF17*-specific 563 bp *Eco*R1–*Pvu*II fragment of the *BNCBF17* coding region was used.

RT–PCR amplification of *GLK1* and *2* was carried out as described for *BNCBF*s in Gao et al. (2002) with the 3′ primer CTAAT-GCGGCGTCTATGCTCTC for both *GLK1* and *2* and the 5′ primers GGTGTGGCCGGAGATGTGCT and TGTCGCCACTTGTCGTCA for *GLK1* and *GLK2,* respectively.

Microarray analyses

Leaves of wild-type and *BNCBF17*-overexpressing lines, grown under non-acclimating conditions as described above, were collected 3 h after the resumption of daylight for RNA isolation. Isolation and labeling of poly$(A)^+$ RNA with Cy3-UTP and Cy5-UTP were carried out as described in Seki et al. (2002). Labeling, hybridization and analyses of the 7K RAFL cDNA microarray were carried out as described by Seki et al. (2002). A 2K *Brassica* array was assembled from unigenes derived from previously isolated clones of a cDNA library (GenBank accession nos BQ704162–705078 and CB686048–686453 as well as 900 unreleased ESTs) constructed from mRNA isolated from leaves of *BNCBF17*-overexpressing plants grown at room temperature as described above. DNAs were amplified directly from the stock cultures using M13 forward and reverse primers and spotted (0.2 ng per spot) using the BioRobotics Microgrid. The *Brassica* arrays were hybridized with 20 µg each of wild-type, cold-acclimated, *BNCBF5* and *17* overexpressing leaf poly$(A)^+$ RNA indirectly labeled with Cy3 and Cy5. Array scanning was carried out with a Perkin Elmer Scan Express and data analyzed using Acuity 4.

Leaf electrolyte leakage freeze test

Leaf electrolyte leakage measurements were carried essentially as described (Gilmour et al. 1988) with the following modifications. The fourth leaf from non-acclimated (N) and acclimated plants (C) was collected on the day of the freeze tests and placed in 14 ml Falcon tubes. The tubes were capped and submerged in a Lauda RM20 low temperature bath set at 0°C for 1 h. The temperature was then lowered to –2°C and held for 30 min. A small piece of ice was then added to each tube to initiate nucleation. The temperature was further held at –2°C for 1 h and then lowered by 2°C h^{-1}. Samples were removed at –2°C intervals and immediately allowed to thaw overnight in a dark cold room set at 4°C. Subsequently, 6 ml of sterile demineralized water was added to each tube and the tubes were shaken for 4 h. Leaves were removed from the tubes and the conductivities of the supernatants were measured. The leaves were frozen at –80°C overnight in an ultra-low temperature freezer and were returned to their respective tubes containing the initial 6 ml of water (which were kept cold in the meantime to prevent microbial growth) and shaken for another 4 h. Leaves were then discarded and conductivity measurements taken with a YSI 32 Conductance Meter (Yellow Springs, OH, USA).

Chl a fluorescence measurements

Chl *a* fluorescence measurements were made using a PAM modulated fluorescence system (Heinz Walz, Effletrich, Germany) as described in detail by Gray et al. (1996). Fo and Fm were determined after leaves were dark adapted for 30 min. All fluorescence parameters were calculated according Schreiber et al. (1994). Fv/Fm measurements were made on leaves of 3-week-old control plants (N leaves), 4- (3+1S) and 5- (3+2S) week-old cold-stressed plants (NS leaves) and 5-, 6- and 9- (3+2, 3+3, and 3+6) week-old cold acclimated plants (C leaves). First, fluorescence measurements were made 8 h after the onset of the photoperiod on leaves of plants grown at a light irradiance of 250 PFD and 20 or 4°C, respectively (Fig. 6A). Secondly, susceptibility to low temperature-induced photoinhibition was quantified

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by monitoring changes in Fv/Fm after exposure of leaves from control as well as from cold-stressed/cold-acclimated plants to 4°C at 1,000 µmol m^{-2} s^{-1} for 6 h (Fig. 6D). In the case of the MV treatment, leaf discs of 1.3 cm^2 collected from the central part of fully developed leaves were first infiltrated under a mild vacuum for 30 min and then soaked for 2 h in the dark with 4 ml of 0 and 10 µM MV solutions. The MV-dependent inhibition of PSII photochemistry was estimated by monitoring the changes in Fv/Fm after the exposure of leaf discs for 6 h to 20°C and 500 PFD (Fig. 6E). To assess the maximum photosynthetic efficiency of photosynthesis, CO_2 assimilation rates and fluorescence parameters were measured on non-acclimated leaves (N) at 20°C and p(CO_2) of 35 Pa to determine the light response curves, and 20°C and 1,500 PFD to determine the CO_2 response curves at p(CO_2) of 35, 50, 75 and 100 Pa using an LI-6400 system with a 6400–40 leaf chamber fluorometer (LI-COR, Lincoln, NE, USA) (Fig. 4).

Pigment analysis

Photosynthetic pigments were extracted and analyzed by HPLC according to Gray et al. (1996). The EPS of the xanthophyll pool was calculated as: EPS = (V + 0.5A)/(V + A + Z), where V, A and Z correspond to the concentrations of the xanthophyll carotenoids violaxanthin, antheraxanthin and zeaxanthin, respectively. The xanthophyll pool size was calculated as the sum of violaxanthin, antheraxanthin and zeaxanthin (V + A + Z). Anthocyanins were extracted from leaf discs (1 cm in diameter) in ice-cold acidified methanol (1%, v/v HCl). Samples were kept on ice in the dark for 1 h and absorption spectra of the extracts were determined after centrifugation using a Hitachi U2000 spectrophotometer (Hitachi, Ltd, Tokyo, Japan). The total anthocyanin content was estimated as A_{530}–0.24A_{653} (Murray and Hackett 1991). Leaf samples for pigment analysis were collected 8 h after the onset of the photoperiod at prevailing growth conditions of 20°C/250 PFD for control grown plants and 4°C/250 PFD for cold-stressed and cold-acclimated plants.

Enzyme assays

The mid-portion of the fully expanded leaf was sampled in the growth chamber at the prevailing growth condition and quickly frozen in liquid N_2 for subsequent biochemical analysis. The activity of Rubisco was determined using the photometric method described by Sharkey et al. (1991a). Carbamylation was determined as the ratio of the initial Rubisco activity/total Rubisco activity. The NADP-MDH and NAD-MDH activity was assayed as described by Pammenter et al. (1993). The NADP-MDH activation state was determined as the ratio of the initial activity/total activity after pre-incubation of leaf extracts with 100 mM dithiothreitol (DTT). Cytosolic and stromal FBPase activity was assayed according to Sharkey et al. (1991b). The activity of AGPase and SPS was assayed as described by Savitch et al. (1997) and Savitch et al. (2000a), respectively. AGPase was measured in the reverse reaction (Glc 1-P synthesis) using a continuous spectrophotometric assay at 340 nm. AGPase activation state was determined as the ratio of the AGPase initial activity/AGPase activity after pre-incubation of leaf extracts with 5 mM 3-phosphoglyceric acid (PGA). SPS activity was assayed under V_{max} and V_{lim} substrate concentrations without Pi and with Pi, respectively, as the time-dependent formation of sucrose plus sucrose-P from uridine diphosphoglucose (UDP-Glc) and fructose 6-phosphate (Fru 6-P) using the anthrone method described by Guy et al. (1992). SPS activation state was determined as the ratio of the V_{lim}/V_{max} activities.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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