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Effects on both the roots and shoots of soybean during dark chilling determine the nature and extent of photosynthesis inhibition

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ABSTRACT

The relative contribution of low soil and air temperatures towards the overall inhibition of photosynthesis in soybean is still unclear. The mechanisms involved in the dark chilling-induced inhibition of photosynthesis were explored further in a chilling tolerant (Highveld Top) and sensitive (PAN809) soybean genotype in experiments where low soil temperatures were present (whole plant chilling, WPC) or absent (shoot-localised chilling, SC). Initially (after three nights of chilling) both the WPC and SC treatments induced the same symptoms in PAN809. These symptoms could thus be ascribed to chilling stress effects on the shoots. Typical symptoms included reduced CO_2 assimilation capacity, inhibition of photosystem II function and lower chloroplast fructose-1,6-bisphosphatase (cFBPase) and sucrose-phosphate-synthase (SPS) activity. When the nodulated root systems of PAN809 were also exposed to low temperatures (WPC treatment), additional constraints gradually developed, which were not observed in Highveld Top. Novel evidence is provided showing that the response in PAN809 is influenced by whether whole-plant or shoot-localised dark chilling occurs and that cFBPase is specifically targeted resulting in severe inhibition of CO_2 assimilation capacity.

1. Introduction

Low night temperatures (dark chilling) during critical stages of soybean [*Glycine max* (L.) Merr] growth and development often result in reduced production (Holmberg, 1973), mainly through the inhibition of key processes such as growth, photosynthesis and symbiotic nitrogen fixation (SNF) (Caulfield and Bunce, 1988; Zhang et al., 1995). Although soybean is a major source of protein its dark chilling sensitivity poses a significant problem, which should be addressed to ensure that future production requirements are met.

Plants experience varied degrees of stress, which are usually determined by its immediate environmental conditions. Response of plants to the environment usually manifest as changes in physiological and biochemical processes. For example, where root systems experience low temperatures, such as in temperate agricultural regions, this could lead to reduced water uptake because of lower root hydraulic conductivity, resulting in a chill-induced state of drought stress (Melkonian et al., 2004). To the contrary, when chilling is localized to the shoots it is unlikely that these chill-induced drought symptoms will occur (Allen and Ort, 2001).

Although many investigators have studied the effects of chilling temperatures on plants (e.g. Guy et al., 1997; Aroca et al., 2005; Bertamini et al., 2005), most employed an entire exposure of the potted plants to low temperatures, i.e. both roots and shoots. Furthermore, these experimental plants often received continued exposure to chilling temperatures (full diurnal cycle) either in the dark (Bertamini et al., 2005, 2006) or in combination with irradiance of various intensities (Guy et al., 1997). In many soybean-producing regions, however, plants are frequently exposed to normal day temperatures, with low temperatures only occurring at night. Although soil temperatures often do not cool down to the same extent as air temperatures, there are examples where low soil temperatures are problematic (e.g. Walsh and Layzell, 1986; Legros and Smith, 1994; Zhang and Smith, 1994), especially in temperate production areas. In soybean, a cluster of nitrogen-fixing root nodules are situated just below the soil surface at the base of the primary root where the risk of exposure to

Abbreviations: A, CO_2 assimilation rate at ambient CO_2 concentration; A_{max}, maximal CO_2 assimilation rate at saturating CO_2 concentration; cFBPase, chloroplastic fructose-1,6-bisphosphatase; F_V/F_M, maximum quantum yield of primary photochemistry; PI, plastochron index; PSII, photosystem II; SBPase, sedoheptulose-1,7-bisphosphatase; SC, shoot-localised chilling; SNF, symbiotic nitrogen fixation; SPS, sucrose-phosphate synthase; WPC, whole plant chilling.

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low temperatures is quite high. This could lead to additional constraints because besides the inhibition of photosynthesis by low air temperatures, low temperatures just below the soil surface can also inhibit symbiotic nitrogen fixation (Guy et al., 1997), which might indirectly aggravate effects on photosynthesis. In soybean this makes it impossible to dissect the relative contribution of low soil and air temperatures towards the overall inhibition of photosynthesis in the case of experiments where entire (whole) plants are chilled.

Strauss et al. (2007) investigated the effect of chilling temperatures on two soybeans genotypes (PAN809 and Highveld Top) of known but contrasting chilling tolerance (Strauss et al., 2006) by exposing potted plants to low night temperatures of 6 °C (9 h) and normal day temperatures 26 °C (15 h). Furthermore, this study also compared the response of plants where both the roots and shoots were chilled with plants where only the shoots were chilled. It was shown that in the presence of low soil-temperatures, leaf ureide (products of symbiotic nitrogen fixation (SNF) in warm-climate legumes such as soybean) content decreased so that the shoot of the chilling sensitive genotype (PAN809) gradually became chlorotic. The same symptoms were absent from the chilling tolerant genotype (Highveld Top), which suggested large genotypic differences in the chilling tolerance of SNF. Further work showed that nitrogenase activity in PAN809 is highly susceptible to chilling whereas Highveld Top is much more tolerant (Van Heerden et al., 2008). However, the mechanistic basis underpinning the more severe inhibition of photosynthesis that was observed in whole-chilled plants on the one hand and the large genotypic difference between the two genotypes on the other hand, was not explored in any detail.

Chilling temperatures are known to often inhibit the photosynthetic carbon reduction (Calvin-Benson) cycle reactions proportionately more than the energy-transducing light reactions (Sassenrath et al., 1990). Furthermore, since the Calvin-Benson cycle provides precursors for the formation of carbohydrates, it also influences the activity of enzymes such as sucrose-phosphate synthase (SPS), which is known to be sensitive towards unfavorable shifts in temperature (Labate and Leegood, 1990). Dark chilling, however, may also influence SPS activity directly in species such as tomato, for example through disruption of the enzyme's circadian rhythm (Jones et al., 1998). Generally, chilling stress also causes inhibition of CO_2 assimilation through reduced stomatal conductance (Melkonian et al., 2004; Singh et al., 2005; Kościelniak and Biesaga-Kościelniak, 2006) indicating a complex interplay between both diffusive and metabolic factors in the inhibition of photosynthesis.

Previous experiments in whole-chilled (roots and shoots) potted soybean plants of tropical (cv. Java 29) and temperate (cv. Maple Arrow) origin revealed that chloroplastic fructose-1,6-bisphosphatase (cFBPase) appears to be particularly sensitive to dark chilling in the genotype from the tropics (Van Heerden et al., 2004). However, as mentioned above, such an experimental design makes it impossible to assess to what extent this sensitivity is related to chilling of the roots or the shoots.

In the present study two soybean genotypes of contrasting dark chilling tolerance (Strauss et al., 2006, 2007; Van Heerden et al., 2008) were used in experiments where the effects induced by whole-plant chilling were compared with the effects induced by shoot-localised chilling. Experiments were designed in order to advance insight into the mechanistic basis underpinning the more severe inhibition of photosynthesis previously observed in whole-chilled plants, as well as the large difference in response between the genotypes. We hypothesised that such an experimental approach would make it possible to dissect the relative contribution of low soil and air temperatures towards the inhibition of photosynthesis in experiments or environments where entire (whole) plants are exposed to chilling.

2. Materials and methods

2.1. Plant materials and growth conditions

Seedlings of the chilling sensitive (PAN809) and chilling tolerant (Highveld Top) soybean [*Glycine max* (L.) Merr] genotype were grown in 2 dm^3 plastic pots containing a mixture of commercial potting soil low in water-soluble nitrogen, river sand and vermiculite (mixture of 4:2:1). The total water soluble N-content of this mixture was 4.9 mg l^{-1}. The low nitrogen content of the growth medium, coupled with the inoculation of seeds with *Bradyrhizobium japonicum* (bacterial strain WB 74) at the time of sowing, ensured optimal root nodule development and symbiotic nitrogen fixation. Seedlings were grown in a Conviron PGW 36 growth chamber (Controlled Environments Ltd., Winnipeg, MB, Canada, R3H 0R9) under rigorously controlled growth conditions: 15 h/9 h and 26 °C/20 °C light/dark cycle with a light intensity of 800 μmol m^{-2} s^{-1} at the level of the plant canopy. Illumination was provided by a combination of very high output fluorescent (Sylvania Cool White VHO, 215 W) and incandescent lamps (Sylvania, 100 W). Seedlings were watered daily and supplied with nitrogen-free (to prevent inhibition of nodulation) full-strength Hoagland nutrient solution (Hoagland and Arnon, 1950) three times a week.

2.2. Dark chilling treatment

The vegetative development of plants prior to induction of dark chilling stress was quantified by measuring the plastochron index (PI) (Erickson and Michelini, 1957). Dark chilling stress was initiated in each genotype when a PI value of five was obtained thus ensuring that all plants were at a comparable stage of vegetative development. A group of plants of each genotype were transferred to a refrigerated chamber controlled at 6 °C for a full dark period (9 h). The remaining plants were kept under normal conditions in the growth chamber at 20 °C for the same dark period and represented the control treatment. Two chilling regimes were used to compare the effects of whole plant chilling (WPC, roots and shoots) with that of shoot-localised chilling (SC, shoots only). For the SC treatment, shoots and leaves of plants of each genotype were exposed to 6 °C, with the roots being kept at 20 °C by inserting pots into custom designed pot-incubators (Analytical Scientific Instruments, PO Box 5832, Weltevreden Park, 1715, South Africa), circulating warm air around the pots. By maintaining normal root temperatures in the SC treatment, direct chill-induced effects on the root systems and nodules were prevented. For the WPC treatment, both shoots and roots of plants of each genotype were exposed to 6 °C for the entire dark period. At the end of the dark period the experimental plants were returned to the growth chamber containing the control plants for the subsequent light period at 26 °C (15 h). This temperature regime and chilling stress treatments were repeated for twelve consecutive light/dark cycles on the same set of plants. All experiments were repeated at least twice.

2.3. CO_2 gas exchange and fluorescence measurements

CO_2 gas exchange was measured before the onset of chilling stress, and again after every third night of exposure to dark chilling, using a portable photosynthesis system (CIRAS-1, PP Systems, Hertz, UK). Measurements were conducted on four plants of the

control, SC and WPC treatments. A 2.5 cm^2 section of the central leaflet of the fourth trifoliate leaf was clamped in a photosynthetic leaf chamber with light and temperature control. Humidity was maintained close to ambient conditions. Measurements were conducted 3 h after the end of the dark period at a leaf temperature of 26 °C, an irradiance of 800 $\mu mol\ m^{-2}\ s^{-1}$ and at CO_2 concentrations of 350 (ambient, C_a) and 2000 $\mu mol\ mol^{-1}$ in order to measure ambient (A) and CO_2 saturated (A_{max}) rates of photosynthesis.

Chlorophyll *a* fluorescence measurements were conducted with a Plant Efficiency Analyser (PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk, PE 30 4NE, UK) on the same leaves as those used for photosynthesis measurements. All measurements were conducted on fully dark-adapted attached leaves. For all measurements the same leaves were repeatedly used over the period of twelve light/dark cycles.

2.4. Sampling of leaf material

Before the onset of dark chilling, and again after every third night of chilling, at the time of day when CO_2 assimilation measurements were conducted, leaf discs (3.14 cm^2) were sampled using a freeze clamp (pre-cooled in liquid nitrogen). Samples were taken from the fourth trifoliate leaf of four control, WPC treated and SC treated plants during each sampling. Shading of the respective leaf was avoided during the sampling procedure. Leaf discs were stored at −80 °C for biochemical procedures.

2.5. Determining of enzyme activities

2.5.1. Sedoheptulose-1,7-bisphosphatase (SBPase)

The phosphate-release method of Harrison et al. (1998) was used to determine SBPase (E.C. 3.1.3.37) activity. Leaf discs were ground with liquid nitrogen and extracted with 1 ml extraction buffer, containing 50 mM Hepes-KOH (pH 8.2), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.1% Triton X-100, 10 mM DTT, 0.5 mM PMSF and 2 mM benzamidine. The extract was centrifuged at 14,000 × *g* and 4 °C for 1 min. The supernatant (400 μl) was desalted by centrifugation according to the method of Helmerhorst and Stokes (1980) using Sephadex G-25 (superfine) and desalting buffer containing 50 mM Hepes-KOH (pH 7.4), 4 mM $MgCl_2$ and 1 mM EDTA. A volume of 20 μl of desalted extract was added to 66 μl assay buffer containing 50 mM Tris-HCl (pH 8.2), 15 mM $MgCl_2$, 1.5 mM EDTA and 10 mM DTT. The reaction was started by addition of 2 mM sedoheptulose-1,7-bisphosphate (SBP) (kindly provided by Prof Christine Raines, University of Essex, UK) and left to run at room temperature for 5 min. The reaction was terminated by addition of 50 μl 1 M perchloric acid, followed by centrifugation at 14,000 × *g* and 4 °C for 10 min. For each reaction, a control was included by adding desalted extract to assay buffer already containing SBP and perchloric acid. Samples, controls and phosphate standards (0.125–4 nmol PO_4^{3-}) were incubated for 25 min at room temperature in the presence of Biomol Green reagent (BIOMOL Research Laboratories, Inc., Plymouth Meeting, Philadelphia, USA) and the absorbance measured at 620 nm using a microplate reader (Power Wave Select, Bio-Tek Instruments, Inc. Highland Park, USA).

2.5.2. Chloroplast fructose-1,6-bisphosphatase (cFBPase)

The method of Leegood (1993) was employed to determine the initial and total activity of cFBPase (E.C. 3.1.3.11). Total activity was measured after incubation of the enzyme at 25 °C in the presence of dithiothreitol (DTT), to ensure full activation of cFBPase *in vitro*. Leaf discs were ground with liquid nitrogen and extracted with 1 ml extraction buffer, containing 100 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 5 mM PMSF, 2 mM benzamidine, 1 mM DTT, 0.1% Triton X-100 and PVPP. The crude extract was centrifuged at 10,000 × *g* and 4 °C for 1 min, and the supernatant immediately used to determine initial cFBPase activity. Initial activity was measured in a total volume of 1 ml, containing 50 μl supernatant, 40 mM NAD, 5 mM DTT, 3 units glucose-6-phosphate-dehydrogenase (G6PDH), 4 units phosphoglucose isomerase (PGI) and 900 μl assay buffer. The assay buffer consisted of 100 mM Hepes-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.05% Triton X-100. The reaction was started by addition of 10 μl 100 mM FBP and the rate of NADP formation was measured at 340 nm. Total activity was measured after a 15 min incubation of 50 μl supernatant at 25 °C with 10 μl 0.5 M DTT as described above.

2.5.3. Sucrose-phosphate-synthase (SPS)

Leaf discs were ground with liquid nitrogen and extracted with 800 μl extraction buffer, containing 50 mM Hepes-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 6 mM PMSF, 2 mM benzamidine and PVPP (Stitt et al., 1988). Desalted supernatant was used immediately to assay SPS (E.C. 2.4.1.14) activity according to the method of Guy et al. (1992), using limiting substrates containing inorganic phosphate (Pi) (V_{lim} assay) or saturating substrates without Pi (V_{max} assay). Reaction mixtures for the V_{lim} assay contained 35 μl desalted supernatant, 6 mM fructose-6-phosphate (F-6-P), 24 mM glucose-6-phosphate (G-6-P), 10 mM uridine 5′-diphosphoglucose (UDPG), 15 mM K_2HPO_4, 50 mM Hepes-KOH (pH 7.4), 4 mM $MgCl_2$. and 1 mM EDTA, in a final volume of 70 μl. K_2HPO_4 was excluded from the V_{max} assay, with the concentrations of F-6-P and G-6-P increased to 20 mM and 80 mM respectively. Included as a control for each sample was desalted supernatant in reaction mixture containing 10 mM UDPG, but in the absence of F-6-P and G-6-P. The reaction was performed at 25 °C for 15 min and terminated by the addition of 70 μl 30% (w/v) KOH. Samples were then incubated at 100 °C for 10 min and briefly cooled on ice, after which 1 ml of resorcinol reagent was added (1%, w/v Resorcinol in ethanol, added to 30% HCl in a 1:3 ratio) (Huber and Israel, 1982). After incubation for 10 min at 80 °C, the formation of sucrose-phosphate was measured at 520 nm.

2.6. Detection of proteins by western blot analysis

Leaf discs were ground with liquid nitrogen and extracted with 1.5 ml ice-cold extraction buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 3 mM DTT, 30 mg insoluble PVPP and 15 μl protease inhibitor cocktail (for plant cell extracts, containing AEBSF, Pepstatin A, Leupeptin, bestatin and E-64 in DMSO (Sigma–Aldrich Chemie GmbH, 89552 Steinheim, Germany). The crude extracts were centrifuged at 10,000 × *g* at 4 °C for 15 min. After addition of SDS-PAGE sample buffer samples were boiled for 5 min.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the separation of proteins and protein subunits. Proteins were separated using a mini-gel system (Mini-PROTEAN 3 Electrophoresis Module, Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, California, USA). Equal amounts of soluble protein were loaded, together with 10 μl of a broad range pre-stained SDS-PAGE protein standard (Bio-Rad Laboratories). Proteins were separated for 120 min at 75 V and 4 °C. After separation proteins were transferred to a Hybond C-extra nitrocellulose membrane (Amersham Biosciences UK Limited, Amersham Place, Little Chalfont, Bucks HP 79NA, UK).

For immunoblotting, the Hybond C-extra nitrocellulose membranes containing the transferred proteins were incubated in

blocking solution containing 25 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1 mM EDTA and 1% skimmed milk powder. The membranes were probed overnight with primary anti-rabbit antibodies specific for FBPase and SBPase (kindly provided by Prof Bob Buchanan, University of California, Berkley, USA). The membrane was probed with anti-rabbit IgG secondary Horse Radish Peroxidase linked antibody for 2 h (1:1000 dilution). Labeled proteins were detected by chemiluminescence (ECL Western blotting detection kit, Amersham).

2.7. Data analysis

Statistical analysis was conducted with the software package Statistica for Windows version 6 (StatSoft, Inc. 2300 East 14th Street, Tulsa OK 74104, USA). Normal distribution of data was determined using the Shapiro-Wilk W test (Shapiro et al., 1968). In data sets with parametric distribution, significant differences between treatment means were determined using Student's t-test. In data sets with non-parametric distribution, significant differences between treatment means were determined with the Mann–Whitney U-test (Mann and Whitney, 1947).

3. Results

3.1. Dark chilling reduced ambient CO_2 assimilation rates (A) irrespective of genotype and type of treatment

In general dark chilling reduced *A* in both genotypes and under both conditions (WPC and SC) of chilling stress. In Highveld Top only WPC treated plants showed a significant ($P < 0.05$) negative response after three nights of chilling, with an inhibition of 73% in *A* compared to control plants (Fig. 1A). However, with further nights of exposure to chilling, both WPC and SC treated plants reacted very similarly with inhibition of between 43% and 54% ($P < 0.05$) in *A*. In PAN809 on the other hand, *A* was significantly inhibited by 57% ($P < 0.01$) in both WPC and SC treated plants after only three nights of chilling (Fig. 1B). With continued nightly exposure to chilling temperatures, WPC treated plants of PAN809 experienced very large inhibitions of up to 75% ($P < 0.01$) in *A*, while the inhibition in SC treated plants remained stable as after 3 nights (Fig. 1B).

3.2. Dark chilling inhibited CO_2 saturated rates of photosynthesis (A_{max}) in a genotype and treatment dependent fashion

After three nights of chilling, A_{max} was inhibited by 46% ($P < 0.01$) in both the WPC and SC treated plants of PAN809 (Fig. 2B). Upon continuation of chilling treatment a clear treatment difference developed in PAN809. In the WPC treatment the inhibition of A_{max} increased to 73% ($P < 0.01$) after six nights of dark chilling and was maintained at this low level for the remainder of the treatment period. In SC plants of PAN809 the inhibition of A_{max} was less, being only 41%, 33% and 21% after six, nine and twelve nights of dark chilling respectively. In the case of Highveld Top, no significant ($P > 0.05$) change in A_{max} as a result of dark chilling could be measured in either WPC or SC treated plants (Fig. 2A).

3.3. Decreased PSII function is an early symptom of dark chilling

Dark chilling led to a decrease in PSII function after only three nights of chilling as indicated by the lower quantum yield of primary photochemistry (F_v/F_m) values recorded in both genotypes and treatments (Fig. 3). However, these values were lowered considerably more in PAN809 than in Highveld Top, but the initial effect induced by the WPC and SC treatments was similar. Only beyond the third night of chilling did a clear treatment difference developed in PAN809, with F_v/F_m values lowered to a much greater extent in the WPC than SC treatment.

3.4. Chloroplast fructose-1,6-bisphosphatase (cFBPase), but not sedoheptulose-1,7-bisphospahatase (SBPase) was targeted by dark chilling in a treatment dependent fashion

Initial and total cFBPase activity of Highveld Top remained unchanged by dark chilling in both WPC and SC treated plants until the twelfth night of treatment, where significant ($P < 0.01$) inhibition of 34% and 29% occurred in initial cFBPase activity and 23% and 31% in total cFBPase activity of WPC and SC treated plants respectively (Fig. 4A and C). PAN809 on the other hand was more sensitive and experienced earlier and larger inhibition of both initial and total cFBPase activity (Fig. 4B and D). After six nights of chilling a

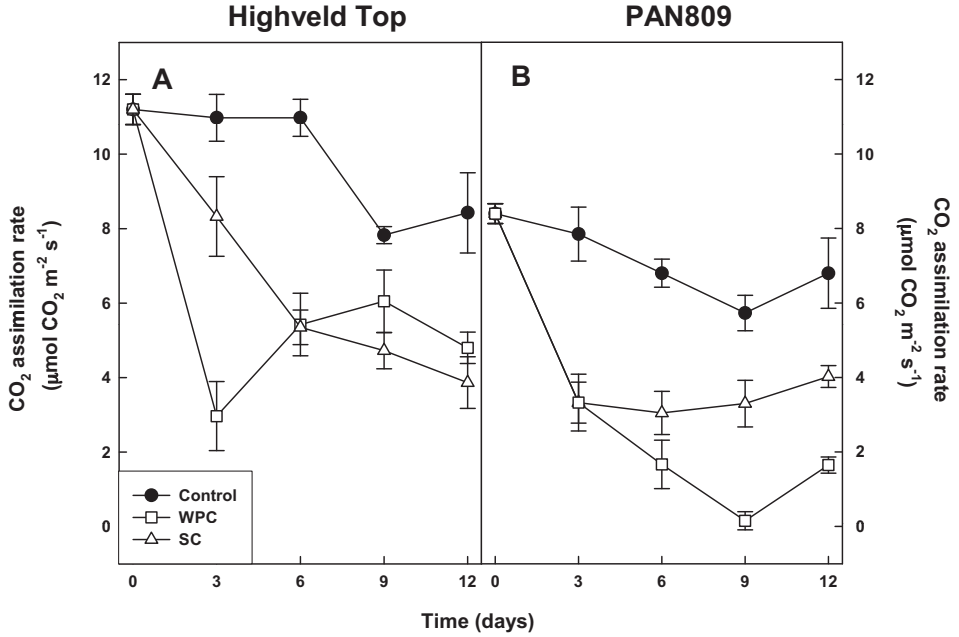

Fig. 1. Effects of dark chilling on CO_2 assimilation rate in Highveld Top (A) and PAN809 (B) over a period of 12 consecutive nights of treatment, measured at 800 µmol m^{-2} s^{-1} and ambient CO_2. Each data point (Control ●, whole plant chilling (WPC) treatment □ and shoot chilling (SC) treatment △) represents the mean of four replicates ± SE.

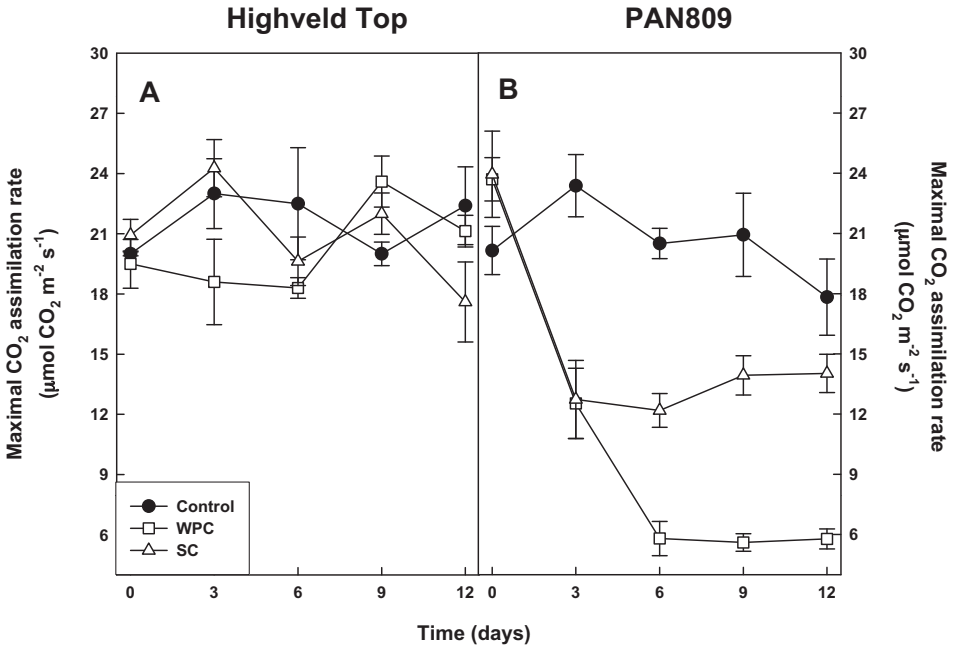

Fig. 2. Effects of dark chilling on CO_2 saturated rate of photosynthesis (A_{max}) in Highveld Top (A) and PAN809 (B) over a period of 12 consecutive nights of treatment, measured at 800 μmol m^{-2} s^{-1} light and 2000 μmol mol^{-1} CO_2. Each data point (Control ●, WPC-treatment □ and SC-treatment △) represents the mean of four replicates ± SE.

significant inhibition of initial and total cFBPase activity was already observed in both the WPC and SC treated plants. Upon further chilling, a clear treatment effect developed with both initial and total cFBPase activity being inhibited more in the WPC treated plants. During the treatment period the difference between the SC/WPC treatments and control became larger not only because of chilling stress, but also because of a steady increase in cFBPase activity in the control over time. However, the large effects of dark chilling on cFBPase activity were not obscured by this. For example, in the case of Highveld Top this gradual increase in activity over time was also seen for the most part in the SC and WPC treatments. However, in the WPC treatment of PAN809, both initial and total FBPase activity measured after 12 nights was still at a similar level as night 0, emphasising the large effect of whole-plant chilling on these plants.

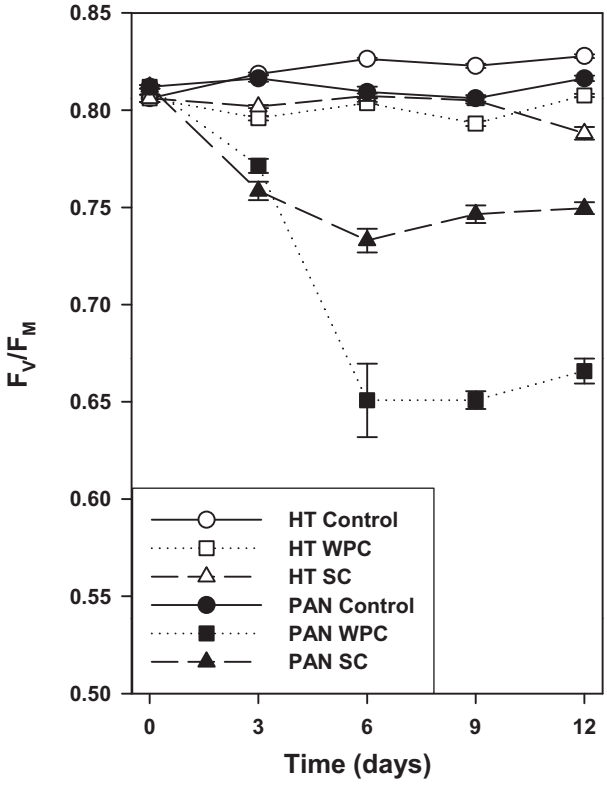

Fig. 3. Effects of dark chilling on the maximum quantum yield of primary photochemistry (F_v/F_m) of Highveld Top (open symbols) and PAN809 (closed symbols) over a period of 12 consecutive nights of chilling. Each data point (Control ●, WPC-treatment ■ and SC-treatment ▲) represents the mean of 24 measurements ± SE.

Although the regulation of cFBPase activity is complex, the decrease in total FBPase activity in response to chilling stress pointed, at least in part, towards a decrease in the steady state content of this enzyme. This was investigated further through immunoblotting, which revealed a clear decrease in cFBPase protein abundance, but only in the WPC treated plants of PAN809. This effect became visible after 9 nights of chilling and was also clearly evident after twelve nights (Fig. 4E). The time-series showing how the chilling-induced effects on cFBPase protein content developed during the entire treatment period of 12 nights is also provided (Supplemental Fig. S1). The largest effect on both initial and total cFBPase activity in WPC plants of PAN809 developed beyond 6 nights of chilling (Fig. 4B and D), suggesting strongly that the intensification of inhibition was linked to a chilling-induced decrease in cFBPase protein content.

Since the two stromal bisphosphatases, cFBPase and SBPase, operate in tandem in the Calvin cycle and share similar regulatory properties, the effects on total SBPase activity was also assessed. It was found that chilling did not affect total SBPase activity significantly ($P > 0.05$) (Fig. 5A and B). Although there was a tendency of lower total SBPase activity in WPC and SC treated plants of PAN809 (Fig. 5B), these differences were not significant. In addition, contrary to cFBPase activity, no differentiation in response between WPC and SC treated plants could be discerned. Confirming this lack in response further, no decline in the steady state protein content of SBPase was detected either (Fig. 5C).

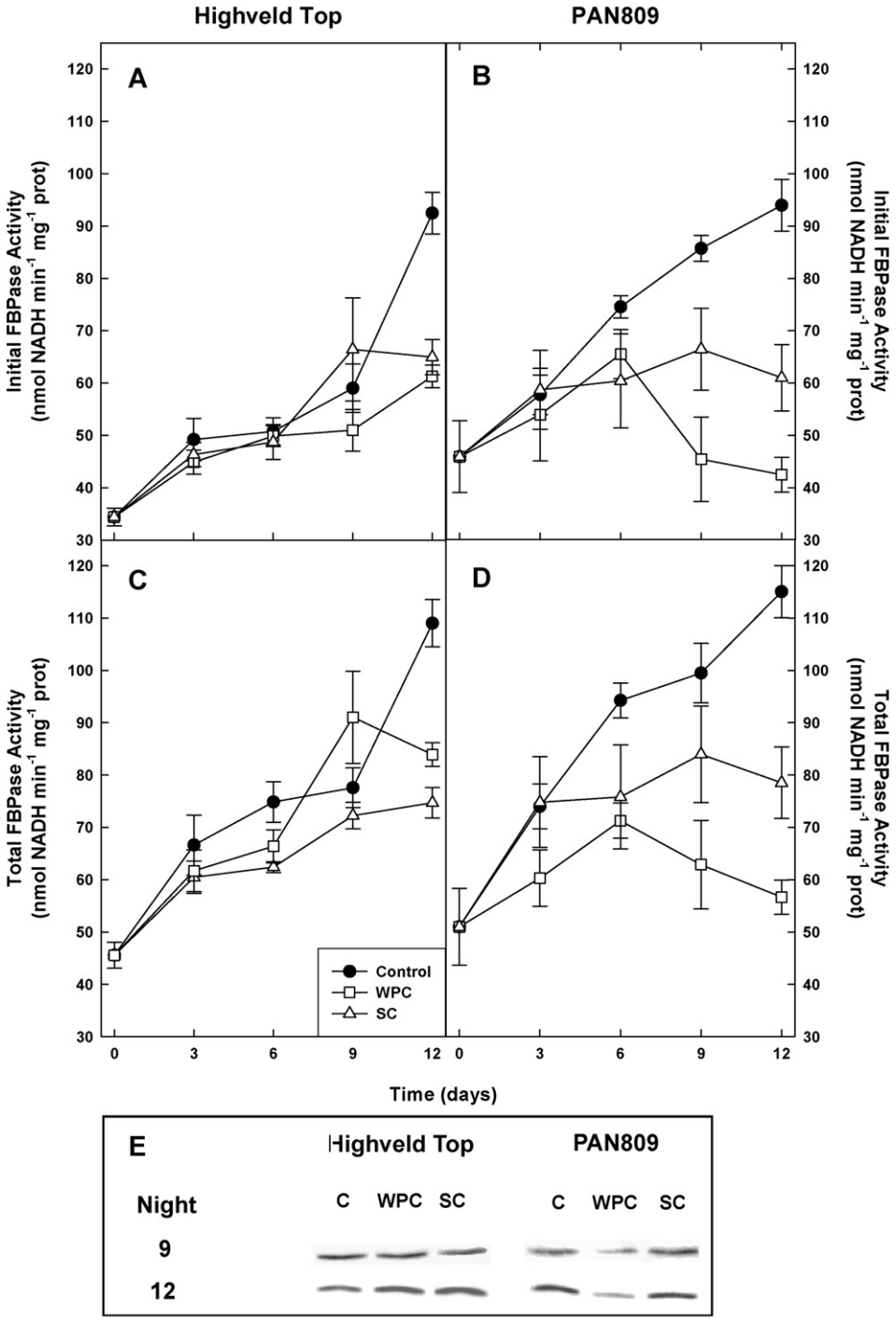

Fig. 4. Effects of dark chilling on initial and total cFBPase activity in leaves of Highveld Top (A, C) and PAN809 (B, D) as well as on cFBPase protein content (E), sampled at 800 μmol m^{-2} s^{-1} and ambient CO_2. Each data point (Control ●, WPC-treatment □ and SC-treatment △) represents the mean of five replicates ± SE.

3.5. Dark chilling inhibited sucrose-phosphate-synthase (SPS) activity but without clear correlation with treatment-dependent effects on A_{max}

Published results indicate that SPS activity is closely related to RuBP regeneration capacity (Galtier et al., 1995; Strand et al., 2000). Because chilling stress inhibited A_{max} in a genotype and treatment-dependent fashion the effects on V_{lim} and V_{max} SPS activity were determined. In contrast to the absence of inhibition of A_{max}, and the general insensitivity or delayed inhibition of the other measured enzymes in Highveld Top, V_{lim} SPS activity was inhibited ($P < 0.01$) by 36% and 30% after nine and twelve nights respectively of WPC treatment (Fig. 6A). PAN809 was more severely affected with significant reductions visible after six nights of dark chilling in both V_{lim} and V_{max} SPS activity of WPC and SC treated plants (Fig. 6B and D). Although V_{lim} SPS activity of SC treated plants of PAN809 was inhibited by 33% ($P < 0.05$) after six nights of chilling, this inhibition was not statistically maintained for the remainder of the experimental period, in contrast to the sustained inhibition of A_{max} (Fig. 2B). After nine and twelve nights of chilling the V_{lim} SPS activity of WPC treated plants of PAN809 was inhibited to a larger extent than in SC treated plants. However, this

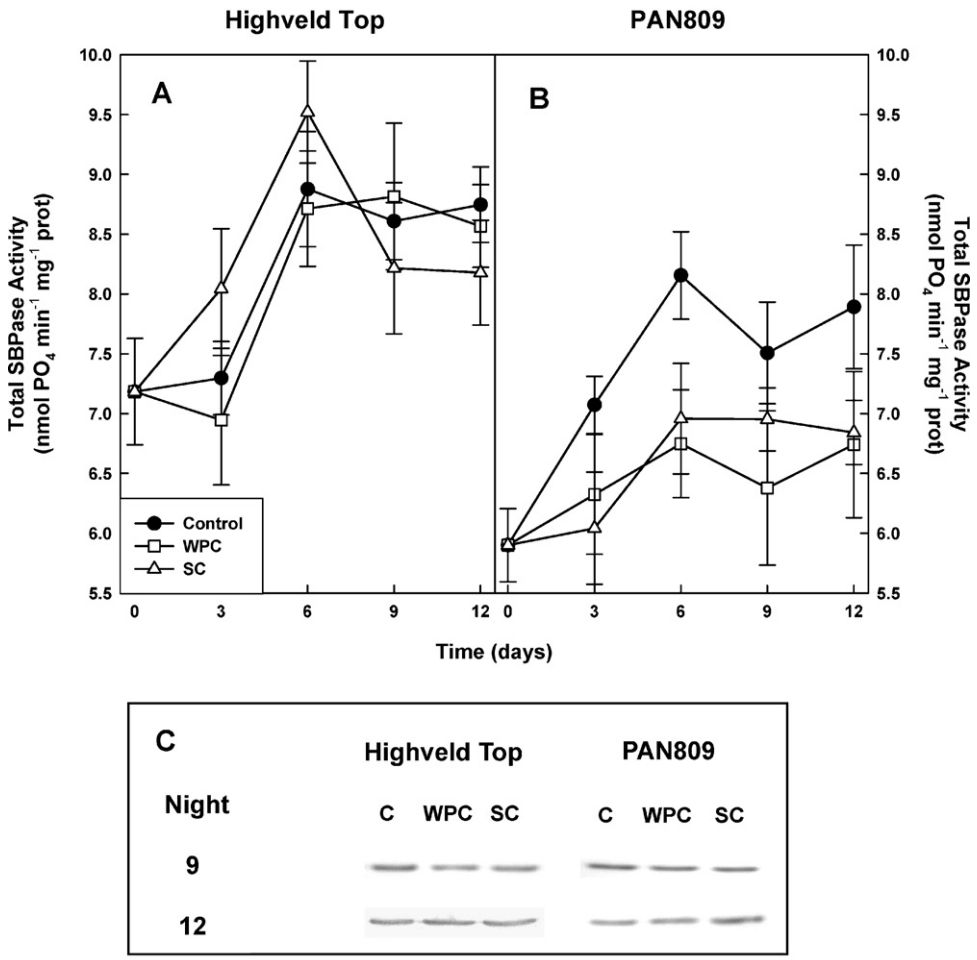

Fig. 5. Effects of dark chilling on total SBPase activity in leaves of Highveld Top (A) and PAN809 (B) as well as on SBPase protein content (C), sampled at 800 ± mol m^{-2} s^{-1} and ambient CO_2. Each data point (Control ●, WPC-treatment □ and SC-treatment △) represents the mean of five replicates ± SE.

treatment difference occurred later than for A_{max}, where the difference between WPC and SC treated plants was already fully established after six nights of chilling (Fig. 2B). Although V_{max} SPS activity of WPC and SC treated plants of PAN809 was clearly lower than the control, a minor treatment difference developed very slowly and was only significant after twelve nights of chilling (Fig. 6D). This corresponded poorly with the large difference in A_{max} between these two groups of plants that was already fully established after the sixth night of chilling. In Highveld Top no significant inhibition in V_{max} SPS activity occurred in WPC or SC treated plants (Fig. 6C). Taken together above results showed that dark chilling inhibited V_{lim} SPS activity in both genotypes and V_{max} SPS activity in PAN809 only, but that these effects were either inconsistent or delayed relative to the clear treatment and genotype differences in the response of A_{max} to dark chilling.

4. Discussion

Because chilling of entire (roots and shoots) plants makes it impossible to dissect the relative contribution of low soil and air temperatures towards the inhibition of photosynthesis, experiments were designed so that chilling responses in WPC and SC treated plants could be compared. In the case of PAN809, the inhibition of photosynthesis (A) could not be reversed through exposure to high CO_2 concentrations (i.e. measuring of A_{max}), contrary to the observations in Highveld Top, where diffusive limitations clearly dominated the inhibition of A. Measurements of A_{max} showed large reductions in chilled plants of PAN809 but not in Highveld Top. This indicates that mesophyll constraints were the main factor involved in the dark chilling-induced inhibition of photosynthesis in PAN809, especially in the WPC treatment.

Two key role-players involved in RuBP regeneration are the stromal bisphosphatases cFBPase and SBPase (Kossmann et al., 1994; Ashton, 1998; Raines, 2006). In contrast to chilling in the light (Sassenrath et al., 1990), very little information exists on the effects of dark chilling on these enzymes, in particular SBPase. Until now, similar effects of chilling on these two stromal bisphosphatases have also been assumed. The current investigation provides new insights into the effects of dark chilling on these two enzymes. Firstly, in the chilling tolerant genotype Highveld Top, cFBPase activity was only moderately inhibited after extended (12 nights) exposure to dark chilling and without any effects on A_{max}, while in PAN809 the decrease in activity occurred already at a much earlier stage. Secondly, in Highveld Top, both the WPC and SC treatments elicited a similar response, while in PAN809 a clear treatment effect was seen with activities reduced severely in WPC-treated plants. Thirdly, SBPase did not exhibit similar chilling sensitivity as cFBPase and its activity was not significantly affected.

Since cFBPase and SBPase operate in tandem in the Calvin cycle and share similar regulatory properties (Nishizawa and Buchanan, 1981; Kelly, 2001), the finding that they responded differently to dark chilling is intriguing. Since both cFBPase and SBPase are activated through the Fd/thioredoxin system, the likelihood that decreased electron transport could have resulted in lower

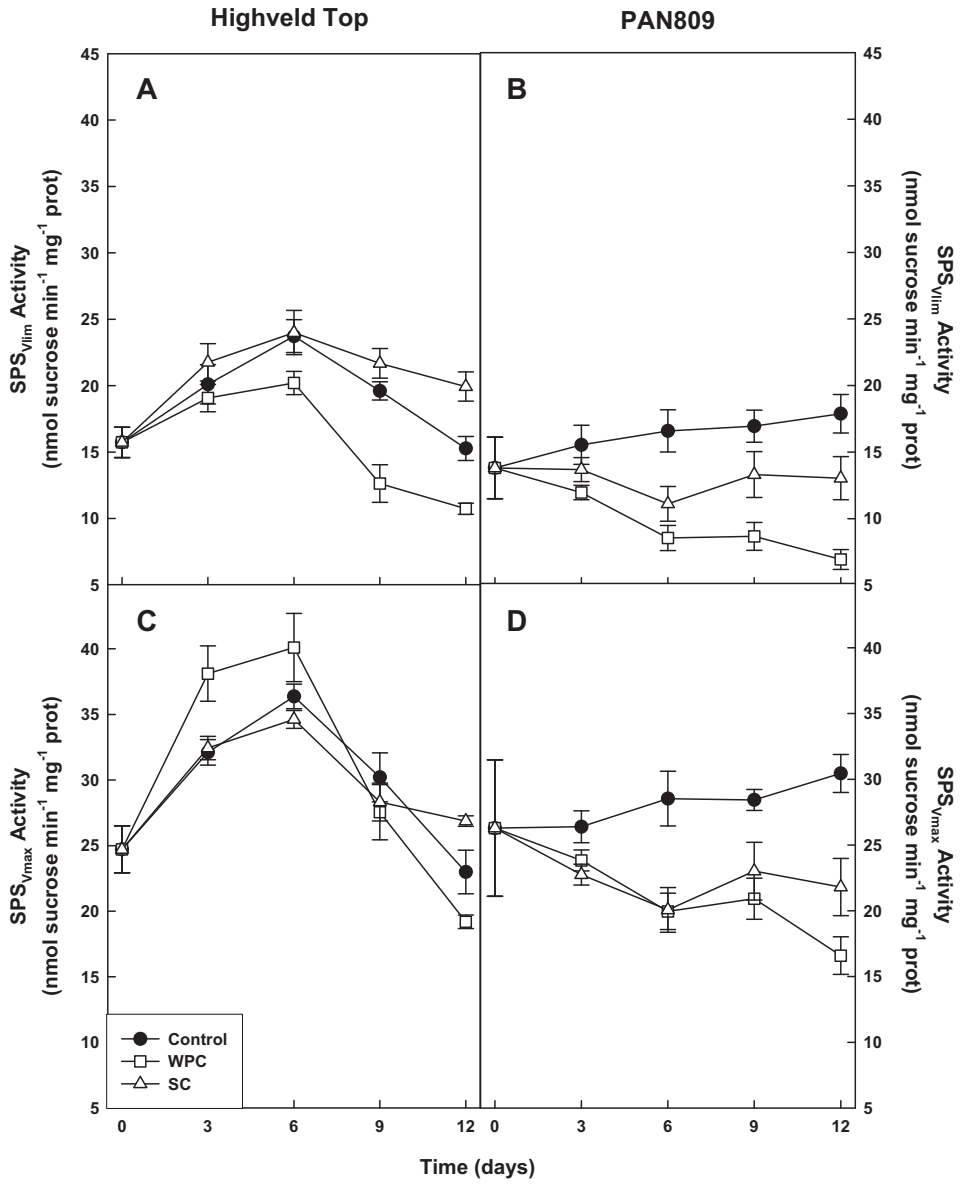

Fig. 6. Effects of dark chilling on V_{lim} and V_{max} SPS activity in leaves of Highveld Top (A, C) and PAN809 (B, D) over a period of 12 consecutive nights of treatment, sampled at 800 μmol m^{-2} s^{-1} and ambient CO_2. Each data point (Control ●, WPC-treatment □ and SC-treatment △) represents the mean of five replicates ± SE.

activation state should be considered. The results presented indicated that PSII function was already inhibited early on during dark chilling in both WPC and SC-treated plants of PAN809 (Fig. 3), before any effects on initial cFBPase activity could be demonstrated. The initial effects of dark chilling on photosynthetic rate in PAN809 therefore most likely involved lowered PSII activity. In addition, if reduced light activation was the main cause for the large inhibition of cFBPase activity, it is reasonable to assume that it would also have affected SBPase activity significantly. Instead, the current investigation provided evidence that prolonged dark chilling specifically targeted cFBPase and that the degree of inhibition, which depended on the presence or absence of low root temperatures, influenced the magnitude of reduction in A_{max}. This was supported further by the western blot analysis, which showed that the content of cFBPase, but not SBPase, was reduced in the WPC-treated plants PAN809.

Aggravation of chilling stress effects on cFBPase in the WPC treatment, compared to the SC treatment, suggest that it is not caused by chilling effects on the shoots/leaves *per se*, but rather that chilling of the roots and nodules must be involved. Although chilling of entire soybean plants do result in mild perturbations in leaf water status (Van Heerden et al., 2003), these effects are transient with any signs of leaf wilting disappearing soon (within 2 h) after return to normal daytime temperatures. It is unlikely that such mild and transient effects on leaf water status would have played a significant role, especially since the biochemical reactions of photosynthesis are known to be rather tolerant to moderate decreases in leaf water status (Medrano et al., 2002). After 6 nights of chilling no changes in cFBPase protein content were visible on western blots (supplemental Fig. S1). The differential effects of the WPC and SC treatments on total cFBPase activity started after 6 nights of chilling (Fig. 4D) and were associated with lower cFBPase protein contents (Fig. 4E) that developed sometime between 6 and 9 nights of chilling. Consideration of the onset of these responses is important, because it was recently shown that leaf ureide content severely decreased in WPC-treated plants of PAN809 between 6 and 9 nights of chilling, predisposing these plants to N-limitation, which ultimately caused the characteristic chlorotic shoot

phenotype (Strauss et al., 2007). Atkins et al. (1988) demonstrated the role of low ureide content in the progressive development of chlorosis in another ureide-exporting legume, cowpea. The large decrease in leaf ureide content was absent from SC-treated plants, suggesting that direct chilling effects on root nodules and inhibition of SNF was causing the response in WPC-treated plants (Strauss et al., 2007). Indeed, recent evidence has shown that SNF is highly sensitive to dark chilling in PAN809, mainly because of the inability of nodule respiration to acclimate to chilling and the lack of a counter-response against elevated internal O_2 concentrations by the variable oxygen diffusion barrier within the nodules (Van Heerden et al., 2008). In Highveld Top, nodule respiration undergoes acclimation to dark chilling and, most importantly, electron dense occlusions form within the intercellular spaces in the nodule cortex and infected zone, restricting flux of O_2 thought the nodule, thereby protecting nitrogenase from O_2-induced deactivation (Van Heerden et al., 2008).

The decrease in leaf ureide content might therefore trigger the onset of leaf senescence in WPC-treated plants of PAN809 (Strauss et al., 2007) and the results presented here suggests that cFBPase is an important target during this process, even before the chlorotic phenotype becomes visible (between 9 and 12 nights of chilling, Strauss et al., 2007). Crafts-Brandner et al. (1996) found that in bean, limited availability of N results in premature leaf senescence.

The question remains why cFBPase, but not SBPase, is selectively targeted in WPC-treated plants? Since the reduction in FBPase protein content seems to be related to the inhibition of SNF, rather than to chilling effects on the leaves *per se*, this is an important question because any environmental factor that leads to lower leaf ureide contents below a certain threshold could perhaps trigger similar effects. This would certainly be an interesting hypothesis to test, since recent evidence suggest that ureides cannot only be seen as a transportable N-metabolite, but that it also functions as a regulator of SNF (Ladrera et al., 2007) and cellular scavenger of reactive oxygen species (Brychkova et al., 2008). Exogenous application of ureides to senescing Arabidopsis leaf discs led to diminished H_2O_2 accumulation and delayed progression of chlorophyll degradation (Brychkova et al., 2008). Based on these and our previous findings (Strauss et al., 2007; Van Heerden et al., 2008) it is hypothesised that the reduction in leaf ureide levels in WPC-treated plants of PAN809 below a certain threshold possibly predisposes the leaf to senescence and associated selective protein degradation as indicated by the immunoblotting results shown in Fig. 4E. Kingston-Smith et al. (2005) has shown that foliar nitrogen limitation in white clover alters protease isoform composition in the leaves. Although speculatory, it is possible that cFBPase and SBPase are degraded by different proteolytic pathways, which show differential activation in responses to decreased ureide levels. Such a hypothesis would certainly merit further testing.

Based on the results reported here, and from our previous findings (Strauss et al., 2007; Van Heerden et al., 2008), a sequence of events are proposed that could help explain the severe chilling stress symptoms that developed in PAN809 (Fig. 7). In both SC and WPC-treated plants, photosynthesis was initially impaired in a similar way, with the rate of CO_2 assimilation (A) at least partially being inhibited due to increased resistance to CO_2 diffusion (Van Heerden et al., 2008) (Fig. 7, step 1). At this early stage of dark chilling, impairment of PSII function also occurred in both SC and WPC-treated plants (Fig. 3 and Strauss et al., 2007) (Fig. 7, step 2). These similarities in response indicate that the inhibition of photosynthesis at this early stage was caused by chilling of leaves *per se* and not by chilling of the roots and nodules. However, chilling of the nodules severely inhibits SNF in PAN809, even after only a single night of treatment (Van Heerden et al., 2008). Continuation of chilling exposure under these conditions causes the progressive development of N-limitation (low ureide content) in leaves of PAN809 plants (Strauss et al., 2007) (Fig. 7, step 3). In Highveld Top nitrogenase is protected from deactivation,

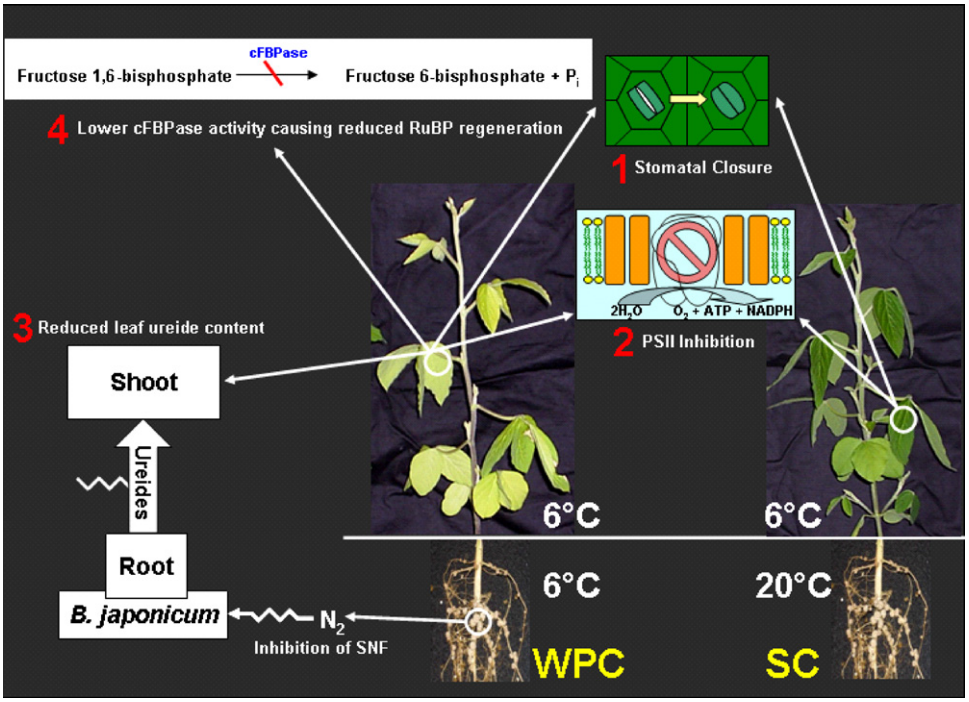

Fig. 7. Scheme illustrating the main target sites where whole plant chilling (WPC, left) and shoot chilling (SC, right) affects above and below-ground metabolism in the chilling sensitive soybean genotype PAN809. In both treatments chilling stress effects on the leaves leads to stomatal closure (1) and inhibition of PSII function (2), which initially causes similar inhibition of photosynthesis. Chilling however also leads to rapid inhibition of SNF in WPC-treated plants (Van Heerden et al., 2008). Reduced ureide production and export from the nodules to the shoot (indicated by resistance symbols), causes a reduction in leaf ureide content (3) and the development of a chlorotic phenotype (Strauss et al., 2007). When leaf ureide content drops below a certain level, cFBPase is specifically targeted (4), which considerably aggravates the inhibition of photosynthesis by reducing RuBP regeneration capacity.

which constitutes a main difference in the dark chilling response of these two genotypes (Van Heerden et al., 2008). The reduction in leaf ureide content (N-limitation) ultimately leads to the development of the chlorotic phenotype characteristic of WPC-treated plants of PAN808 (Fig. 7). When leaf ureide content drops below a certain level in these plants, additional constraints on photosynthesis appear to develops. Specific targeting of cFBPase (step 4 in Fig. 7) as demonstrated in this paper, leads to reduced cFBPase protein content and further reductions in its specific activity. As a result, RuBP regeneration capacity (indicated by A_{max}) is severely compromised.

These findings have ecological relevance in areas where low soil temperatures interfere with soybean cultivation and also demonstrate that root-chilling effects in laboratory studies with potted legumes could modulate chilling response to a large extent, a potential artefact that is often overlooked in these types of studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envexpbot.2011.06.008.

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