Transcriptional responses indicate maintenance of photosynthetic proteins as key to the exceptional chilling tolerance of C_4 photosynthesis in *Miscanthus × giganteus*

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Abstract

*Miscanthus × giganteus* is exceptional among C_4 plants in its ability to acclimate to chilling (≤14 °C) and maintain a high photosynthetic capacity, in sharp contrast to maize, leading to very high productivity even in cool temperate climates. To identify the mechanisms that underlie this acclimation, RNA was isolated from *M × giganteus* leaves in chilling and nonchilling conditions and hybridized to microarrays developed for its close relative *Zea mays*. Among 21 000 array probes that yielded robust signals, 723 showed significant expression change under chilling. Approximately half of these were for annotated genes. Thirty genes associated with chloroplast membrane function were all upregulated. Increases in transcripts for the *lhcb5* (chlorophyll a/b-binding protein CP26), *ndhF* (NADH dehydrogenase F, chloroplast), *atpA* (ATP synthase alpha subunit), *psbA* (D1), *petA* (cytochrome f), and *lhcb4* (chlorophyll a/b-binding protein CP29), relative to housekeeping genes in *M. × giganteus*, were confirmed by quantitative reverse-transcription PCR. In contrast, *psbO1*, *lhcb5*, *psbA*, and *lhcb4* were all significantly decreased in *Z. mays* after 14 days of chilling. Western blot analysis of the D1 protein and LHCII type II chlorophyll a/b-binding protein also showed significant increases in *M. × giganteus* during chilling and significant decreases in *Z. mays*. Compared to other C_4 species, *M. × giganteus* grown in chilling conditions appears to counteract the loss of photosynthetic proteins and proteins protecting photosystem II typically observed in other species by increasing mRNA levels for their synthesis.

Key words: C_4 photosynthesis, chilling, chlorophyll a/b-binding protein, cold, D1 protein, LHCII, low temperature, maize, *Miscanthus*, transcription.

Introduction

*Miscanthus × giganteus* (Greef & Deuter ex Hodkinson & Renvoize; Greef and Deuter, 1993; Hodkinson and Renvoize, 2001), a rhizomatous perennial grass and bioenergy crop, appears unique among C_4 species in its productivity and maintenance of photosynthetic capacity during chilling (≤14 °C) (Beale and Long, 1995; Beale et al., 1996; Naidu and Long, 2004; Long and Spence, 2013). In the closely related C_4 crop *Zea mays* L., photosynthetic capacity declines rapidly in response to chilling (Long et al., 1983; Naidu et al., 2003; Wang et al., 2008a). This limits the growth of *Z. mays* in the US corn belt to the chilling-free period of the year, despite intensive breeding efforts to improve chilling tolerance.
(Rodriguez et al., 2010). In a side-by-side comparison, M. × giganteus proved 59% more productive than Z. mays within the US corn belt even though both crops converted intercepted solar radiation into biomass with equal efficiency (Dohleman and Long, 2009). The higher productivity of M. × giganteus results from its ability to produce photosynthetically active leaves and maintain them earlier and to retain photosynthetically competent leaves later in the growing season—times when chilling temperatures damage photosynthesis in Z. mays (Dohleman and Long, 2009; Long and Spence, 2013).

Chilling during daylight reduces the maximum quantum yield of photosynthetic CO₂ assimilation (ΦCO₂-max) in Z. mays due to a combination of chilling-dependent photoinhibition and impaired synthesis of key proteins of the PSII and the light-harvesting complex (LHC) (Nie and Baker, 1991; Long et al., 1994; Fryer et al., 1995). M. × giganteus shows little depression in ΦCO₂-max and photosynthetic rate during chilling (Beale et al., 1996; Naidu and Long, 2004; Farage et al., 2006; Wang et al., 2008a; Purdy et al., 2013). When grown side-by-side in controlled environments at 14 °C, ΦCO₂-max was decreased significantly, relative to 25 °C, by 50% in Z. mays, but was not significantly decreased in M. × giganteus. In the same study, photosystem II (PSII) maximum dark-adapted efficiency (Fv/Fm) and operating efficiency (ΦPSII) in saturating light were reduced c.20 and 80%, respectively, in Z. mays yet were hardly affected in M. × giganteus (Naidu and Long, 1994). Previous work has also compared the photosynthetic capacity of M. × giganteus under chilling conditions to a population of the C₄ sedge Cyperus longus native to southern England at 52° N—an exceptionally high latitude for any C₄ species (Farage et al., 2006; Long and Spence, 2013). Growth of M. × giganteus at chilling temperatures ≥8 °C did not alter the number of carbon dioxide molecules fixed per electron transferred through PSII (A/JPSII). In contrast, A/JPSII declined significantly in Z. mays when transferred to 14 °C (Naidu et al., 2003; Wang et al., 2008a). While these differences likely account for maintenance of Aₚ during chilling acclimation, they do not explain the parallel maintenance of a high maximum quantum yield of leaf ΦCO₂-max (Naidu and Long, 1994). The initial slope of the response of leaf CO₂ assimilation (A) to photon flux (i.e. ΦCO₂-max) provides an important measure of capacity for light-limited photosynthesis. In Z. mays, a decline in ΦCO₂-max is attributed to loss of efficiency of PSII, which is associated with impairment in the synthesis of key PSII and LHC proteins (Nie and Baker, 1991; Fryer et al., 1995; Caffarri et al., 2005).

While the comparative physiological aspects of chilling tolerance of photosynthesis in M. × giganteus relative to Z. mays have been studied extensively, the molecular basis of this difference is largely unexplored. Analysis of gene expression in chilling-intolerant Z. mays during acclimation to 14 °C showed significant downregulation in transcripts encoding chlorophyll a/b-binding proteins, Rubisco, and PPDK (Trzcinska-Danielewicz et al., 2009; Wang et al., 2008a,b). The current study asked whether the demonstrated physiological tolerance of M. × giganteus to chilling compared to Z. mays corresponds to maintenance or upregulation of a wider range of genes encoding key aspects of the photosynthetic apparatus, using the chilling treatments of previous physiological studies (Wang et al., 2008a,b). Particular attention is given to those proteins shown to become damaged or deficient in Z. mays during acclimation to chilling associated with the decline in ΦCO₂-max.

**Materials and methods**

**Biological materials**

Plant propagation, chilling treatment, and leaf sample collection were performed in two independent experiments for M. × giganteus. The leaf samples from the first experiment were used in the microarray analysis and quantitative reverse-transcription PCR (qPCR) validation, and the leaf samples from the second experiment, which included a side-by-side comparison with Z. mays, were used for the comparisons of transcripts by qPCR and amounts of specific proteins.

Experiments followed the treatment protocol of Wang et al. (2008a,b). Briefly, plants were first grown in warm conditions of 25 °C days and then transferred to 14 °C for 14 days. Prior studies have shown that this is sufficient time for completion of acclimation of photosynthesis in mature leaves of both species. In the first experiment, M. × giganteus rhizomes were propagated in soil-less potting media (Sunshine Mix LC12, Sun Gro Horticulture, Bellevue, WA, USA) in 1.2-l pots following the procedure of Naidu and Long (2004). Eight individual plants were grown in two identical controlled-environment growth chambers (Conviron E15, Controlled Environments, Winnipeg, Manitoba, Canada) under 500 μmol photons m⁻² s⁻¹ incident light provided by an equal mixture of
high-pressure mercury and sodium lamps, in a 14/10 light/dark cycle at either 14/12 °C (chilling) or 25/20 °C (control). Both chambers were maintained at 70% relative humidity. The eight plants were initially grown in the control chamber for 2 weeks, and then half of these plants, selected by fully randomized design, were transferred to the chilling conditions. After 2 weeks of chilling, and 1 month of total growth, leaf samples were taken 2–3 h after illumination began (i.e. mid-morning) from the midpoint of the youngest fully expanded leaf (Naidu and Long, 2004; Wang et al., 2008b). Full expansion was defined by emergence of the ligule. Shoots had approximately four leaves each at the time of sample collection. All leaf samples were immediately plunged into liquid nitrogen and then transferred to storage at –80 °C until RNA extraction for microarray and qPCR analysis.

The conditions for the second experiment were identical to the first apart from the following addition. Z. mays cv. B73 seeds were germinated in 1.2 liter pots in soil-less potting media following the procedure of Naidu and Long (2004) and grown alongside M. × giganteus following the sampling and conditions described above.

4 × 44K Agilent oligonucleotide two-colour cDNA maize microarray

RNA extraction Total RNA was extracted from the leaf samples. Samples were homogenized in 1 ml TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) using a glass-Teflon homogenizer. The homogenized samples were then incubated for 5 min at room temperature to permit the complete dissociation of the nucleoprotein complexes. Each 1 ml sample had 0.2ml chloroform added, was incubated at room temperature for 150 s, and was centrifuged at 12 000 g for 15 min at 4 °C. The RNA in the supernatant was precipitated with 0.5 ml isopropyl alcohol and was isolated by centrifugation at 12 000 g for 10 min at 4 °C. The RNA pellet was dried and redissolved in 50-μl RNase-free water for 10 min at 55 °C and then further purified with the RNeasy Kit (Qiagen, USA). Final RNA quantity was determined spectrophotometrically at λ 260nm (NanoDrop ND1000, NanoDrop Technologies, Wilmington, DE, USA). The purity of RNA was assessed by the 260/280 absorbance ratio. Only those RNA samples with ratios of ≥2.0, indicating no significant presence of contaminants, were used.

Microarray fluorescent cDNA synthesis and hybridization cDNA was synthesized from total RNA using a Agilent Low RNA Input Linear Amplification Kit, with spike-in controls (5184-3523), following the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA, USA). Two of the four control samples and two of the four treatment samples were labelled with Cy3 dye and the other samples were labelled with Cy5 dye, to account for any possible dye-bias that may occur during hybridization or fluorescence detection during scanning of the slides. Agilent 4 × 44K oligonucleotide two-colour cDNA Maize microarray slides were used. Hybridization and washing was performed following the Agilent Two-Colour Microarray-Based Gene Expression Analysis protocol (Agilent Technologies). A total of four 4 × 44K slides (i.e. four technical replicates) were used for each of the four biological replicates. Hybridized slides were scanned with a GenePix4000B microarray scanner (Axon Instruments, Concord, ON, Canada) and features were extracted and analysed using GenePix Pro 6.1 (Axon Instruments). Each array was inspected individually and low-quality spots (i.e. those with fluorescence levels that were not significantly distinguishable from the background fluorescence in the red-to-green channel intensity ratio histograms of the array) were manually flagged and eliminated from further analysis.

Microarray statistical analysis All calculations were performed using the R computing environment (Venables et al., 2013). Normalization and statistical analysis was done using the R package Limma provided by the Bioconductor repository (http://bioconductor.org/; Wittenhall and Smyth, 2004). No background correction was performed, a common practice for spotted arrays with low background (Smyth et al., 2003, 2005). The global loess function was employed for within-array normalization and the scale method was applied for between-array normalization (Smyth and Speed, 2003). Test-statistics were determined using eBayes fit and topTable functions (Smyth, 2004). Use of the duplicate correlation function accounted for duplicated probe spots on the Agilent array and for technical replication (Smyth et al., 2005). The llimFit function was applied to fit the data to a linear model using quantitative nonnegative weights for flagged spots, and a design matrix was used to compare expression values in the control vs. treatment arrays. Genes that were differentially expressed at 14 °C versus 25 °C were defined on the basis of a significant P-value, adjusted for multiple testing, using FDR (adj. P-value 0.00001) and a log, fold-change cut off of 0.70 or –0.70. These criteria were validated by the ability to also detect significant expression differences by qPCR, as defined previously (Wurmbach et al., 2003).

Gene annotation and MapMan application

Sequences for the FASTA 60-mer probes on the Agilent 4 × 44K oligonucleotide two-colour cDNA Maize microarray slides were provided by Dr. Virginia Walbot at Stanford University. A gene annotation file for the Agilent 60-mer probe sequences was generated by BLASTN of probe sequences to the Z. mays B73 reference genome sequence, build 2 (Schnable et al., 2009). For genes found to change significantly between control and treatment, but lacking any published annotation at the time of the experiment, annotation was attempted by performing a tblastn BLAST query (http://www.ncbi.nlm.nih.gov/) on the corresponding 60-mer probe FASTA sequence.

To visualize the differentially expressed genes, the MapMan software tool was used (Usadel et al., 2009). The MapMan software utilizes the Affymetrix platform to generate hierarchical categories of gene function and pathway placement. In order to use this software, to visualize results obtained here on the Agilent microarray, a ‘mapping file’ was created based upon the pre-existing Z. mays ontology/ mapping file with Affymetrix-assigned probe identifiers. The Agilent annotations were cross-referenced with the Z. mays mapping file and gene annotations matching those in the Z. mays mapping file BIN or sub-BIN for functional categories. The Affymetrix identifier was replaced with the Agilent identifier. The Agilent identifier corresponding to the assigned BIN/category was then used to create an experiment file containing the corresponding down- or upregulated log2 fold-change value. This experiment file and the new mapping file was then uploaded into the MapMan Image Annotator module (Usadel et al., 2009).

Quantitative reverse-transcription PCR

Quantitative reverse-transcription PCR was used to validate the up- and downregulation of transcripts of particular interest that were found to be differentially expressed in the microarray experiment. Genes for validation with known Z. mays sequences (http://www.ncbi.nlm.nih.gov/) were compared against available M. × giganteus genome sequence surveys obtained by 454 sequencing (Swaminathan et al., 2010) and transcripts assembled from Illumina short-read sequencing (Barling et al., 2013). Sequences with more than 98% identity were candidates for qPCR validation. Six transcripts were chosen: ndtF (NADH dehydrogenase F, chloroplast), atpA (ATP synthase alpha subunit), lhcB5 (chlorophyll a/b-binding protein CP26), psbA (oxygen-evolving enhancer protein 1), aps (ATP synthase), and psb (terpene synthase). The primers for the transcripts were designed based on M. × giganteus sequences (Table 1). Four candidate reference genes for qPCR were chosen because they are common housekeeping genes that have stable expression levels between the control and treatment samples in the microarray experiment: tuba6 (alpha-tubulin 6), tubb3 and tubb6 (beta-tubulin 3 and 6), and tubg1 (gamma-tubulin 1).

SuperScript-III RT (Invitrogen) was used to synthesize cDNA from the same M. × giganteus samples used for the microarray experiment. SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was used in reverse transcription reaction and the conditions were a pre-incubation step of 10 min at 50 °C followed by 15 min at 95 °C, a denaturation step of 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 15 s at 55 °C, and 15 s at 72 °C, and a final extension step of 5 min at 72 °C. The calibration curve was generated using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the plots were generated with the R package (Venables et al., 2013). The R package geesM (GWI Biometrics, 2007) was used to calculate the half-life of transcripts (Lütke-Hofmann et al., 2004).
City, CA, USA) was used for qPCR and samples were amplified on a high-throughput microowell-plate-based thermo-cycler (LightCycler 480 System, Hoffmann-La Roche, Indianapolis, IN, USA). Results were normalized against tubb6 and analysed using the ΔΔCT method (Schmittgen and Livak, 2008). tubb6 was chosen for standardization as it exhibited the lowest variance across all samples included in the qPCR experiment. One-tailed Student’s t-test was used to determine a significant increase in transcript level at α=0.05 due to chilling for each gene.

The second experiment comparing *M. × giganteus* to *Z. mays* grown side by side in control and chilling conditions used the same protocol. Three additional transcripts were added with known involvement in chilling response in *Z. mays*: *psbA* (D1), *petA* (cytochrome f), and *lhcb4* (chlorophyll a/b-binding protein CP29) (Trzcinska-Danielowicz et al., 2009; Table 1). *Act* was used as the reference gene for normalization of qPCR results because it shows stable expression in both species and has been previously shown to have stable expression during chilling in *Z. mays* (Trzcinska-Danielowicz et al., 2009). Results were normalized against *Act* and analysed using the ΔΔCT method (Schmittgen and Livak, 2008).

Student’s t-test was used to determine significant differences at α≤0.05 between treatments for each species and gene.

### Western blot analysis

Total leaf protein was extracted from leaf discs of area 1.27 cm² punched from the centre of the lamina of each biological replicates (n=4) of each species. Proteins were extracted by grinding frozen samples in individual tubes with stainless steel grinding balls (GBSS n=4) of each species. Proteins were extracted by grinding frozen tissue and buffer was homogenized and centrifuged for 1 min at 15 000 g and then the resulting supernatant was stored at –80 °C until Western blot analysis.

Total leaf protein, on an equal leaf area basis, was loaded onto the lane of the Agilent 44K maize oligonucleotide microarray with 48% efficiency. Approximately 21 000 probes showed a median fluorescence intensity that could be clearly distinguished from the background. Chilling changed the relative signal intensity for 723 probes relative to the control; based on a significance threshold of a log2 fold-change of ≥0.7 and false-discovery-rate-adjusted probability of α<0.00001. These thresholds were related to changes in photosynthetic capacity, which is measured on a leaf area basis (Naidu and Long, 1994; Wang et al., 2008b). Polyproteins were separated by SDS-PAGE, as described previously (Wang et al., 2008b). Each gel contained four biological replicates for the control and of the chilling treatment for both *M. × giganteus* and *Z. mays*. Protein-pretained standards were used to provide a molecular weight ladder (Precision Plus Protein Kaleidoscope Standards 161–0375, Bio-Rad Life Science). Separated proteins were blotted onto polyvinylidene fluoride membranes in transfer buffer (0.025 M Tris base, 0.192 M glycine, and 20%, v/v, methanol) at 100 V for 1 h at 4 °C. Membranes were then incubated with primary polyclonal antibodies in Tris-buffered saline (170–6435, Bio-Rad Life Science) with 0.05% Tween 20 (P5927, Sigma-Aldrich, St. Louis, MO, USA). LHCI chlorophyll a/b-binding proteins and the D1 protein and its degradation products were detected with rabbit polyclonal antibodies (AS01-003 and AS05-084, Agrisera Antibodies, Vännäs, Sweden). Membranes were then incubated with anti-rabbit IgG (whole molecule) alkaline phosphatase antibody produced in goat (A7539, Sigma-Aldrich). Digital images were made of each membrane and the relative volume and intensity for each band was quantified using the 2D image analysis software ImageQuant TL 7.0 (GE Healthcare Biosciences, Little Chalfont, UK).

Mixed-model analysis of variance followed by least significant difference tests of differences between means determined the significance of the effect of chilling on the content of each of LHClI chlorophyll a/b-binding protein, D1 protein, and D1 degradation products in *M. × giganteus* and *Z. mays* (PROC MIXED SAS version 9.2, SAS Institute, Cary, NC, USA). Results were considered significant at α≤0.05 (p=4).

### Results

#### *M. × giganteus* leaf cDNA hybridized to the Agilent 44K maize oligonucleotide microarray with 48% efficiency.

Approximately 21 000 probes showed a median fluorescence intensity that could be clearly distinguished from the background. Chilling changed the relative signal intensity for 723 probes relative to the control; based on a significance threshold of a log2 fold-change of ≥0.7 and false-discovery-rate-adjusted probability of α<0.00001. These thresholds were related to changes in photosynthetic capacity, which is measured on a leaf area basis (Naidu and Long, 1994; Wang et al., 2008b).

**Table 1. Primer sequences for qPCR validation of microarray results and comparison with Z. mays**

| Transcript | Forward (5′–3′) | Reverse (5′–3′) | Expected product size (bp) |
|------------|----------------|----------------|---------------------------|
| tubb6      | AAGACGGCACTATCAACCTTGCCC | CACCACACACAGCATTGAACACCA | 140 |
| tubb3      | ATGGCAAGATGAGTGTCAGGCAAA | TGTGTAAGCAGACGGGAGACT | 174 |
| tubb5      | CTGTTTTCACCTGACCCCGCTGQA | AGGAAGGAACCATCAGAGGAGCAA | 98  |
| tubb6      | TTGTACCCCTGATTGGAGCGGTTGT | TGGCCAAAACAAAGTGGTGAGG | 164 |
| tubg1      | ATGAGCTTGAATGGGCGAGAACCG | ATGAGGGACTGTTTCCGGAAGCA | 81  |
| act        | TGAGGACCACTGACACCATCTGCTAT | CTTCTGACGGTGGCAGAACCACACTT | 180 |
| ndhF       | ACCCACTCCCATTTGGGTCTTAT | GAGCAAGAGCTAAGTGGCCCTCTA | 166 |
| atpA       | ATGGGCGTACCCACCCGCTACATTA | TGGGATGAAAGGGTGGTGGTGGT | 134 |
| lhcb4      | AGTAGAAGATGAGCATGGGAGACA | AGAACTGTCGCAACCTCACCGAG | 120 |
| lhcb5      | TGCCATGCTCTCCATGTTGCGAGAT | TAGGCCAAGGGTGGTGTGGA | 114 |
| psbo1      | ATCCAGACAGACGGTCAGGACAA | GCAGACCTTTCCTAGCCTGATTCTT | 134 |
| aps        | TGGATGAGCCAGAGAGAACAGAGGTT | TGGCGATTGCTGCTCCTACTTG | 120 |
| tps        | ATGGCAAGATGACAAGACCTGAGG | CAGCTACATTGCGGCAGGAAGAA | 88  |
| psbA       | AACTTATTCCTGGTCTGATGAGGG | TAAAGGATTTGCTGCTCCTGAG | 185 |
| petA       | CGCATCTTATCTTAAAAGGATCA | TACAAATTGTCGACGTGCTTTCG | 81  |

#### Transcript Forward (5′–3′) Reverse (5′–3′) Expected product size (bp)

- tubb6: AAGACGGCACTATCAACCTTGCCC CACCACACACAGCATTGAACACCA 140
- tubb3: ATGGCAAGATGAGTGTCAGGCAAA TGTGTAAGCAGACGGGAGACT 174
- tubb5: CTGTTTTCACCTGACCCCGCTGQA AGGAAGGAACCATCAGAGGAGCAA 98
- tubb6: TTGTACCCCTGATTGGAGCGGTTGT TGGCCAAAACAAAGTGGTGAGG 164
- tubg1: ATGAGCTTGAATGGGCGAGAACCG ATGAGGGACTGTTTCCGGAAGCA 81
- act: TGAGGACCACTGACACCATCTGCTAT CTTCTGACGGTGGCAGAACCACACTT 180
- ndhF: ACCCACTCCCATTTGGGTCTTAT GAGCAAGAGCTAAGTGGCCCTCTA 166
- atpA: ATGGGCGTACCCACCCGCTACATTA TGGGATGAAAGGGTGGTGGTGGT 134
- lhcb4: AGTAGAAGATGAGCATGGGAGACA AGAACTGTCGCAACCTCACCGAG 120
- lhcb5: TGCCATGCTCTCCATGTTGCGAGAT TAGGCCAAGGGTGGTGTGGA 114
- psbo1: ATCCAGACAGACGGTCAGGACAA GCAGACCTTTCCTAGCCTGATTCTT 134
- aps: TGGATGAGCCAGAGAGAACAGAGGTT TGGCGATTGCTGCTCCTACTTG 120
- tps: ATGGCAAGATGACAAGACCTGAGG CAGCTACATTGCGGCAGGAAGAA 88
- psbA: AACTTATTCCTGGTCTGATGAGGG TAAAGGATTTGCTGCTCCTGAG 185
- petA: CGCATCTTATCTTAAAAGGATCA TACAAATTGTCGACGTGCTTTCG 81
selected to include candidate genes with small but reproducible changes in expression (Wurmbach et al., 2003). Chilling caused significant increase in signal intensity for 410 probes and a decrease in 313. Approximately half of the probes showing significant changes corresponded to sequences annotated for predicted or putative functions in Z. mays, and these were categorized accordingly in MapMan (Fig. 1). A complete list of putative chilling-responsive genes identified here is given in the supporting information (Supplementary Tables S1 and S2 available at JXB online). Functional categories that were significantly represented among chilling-responsive genes included photosynthesis, stress response, RNA processing, protein synthesis, targeting and degradation, transport, secondary metabolism, and hormone metabolism.

Most striking among these categories were genes associated with the light reactions. Among the probes binned by MapMan to this category, 30 were significantly upregulated and none were significantly downregulated (Table 2). Particularly prominent were transcripts for PSII-associated proteins (Fig. 2 and Table 2). Within the carbon metabolism pathway, the probes for PPDK and Rubisco were also maintained and showed high expression values (Supplementary Table S3). Probes for rca1 and rca2, the genes for Rubisco activase, an enzyme that is critical to the activation and stability of Rubisco (Portis, 2003), were upregulated 145%, although just below the fold-change cut off (log2 ≤ 0.7).

Six of the probes found to change on the microarray were selected for validation by qPCR in the first experiment. Each showed the same directional change as well as similar quantitative changes in expression to that observed from the microarray (α ≤ 0.05; Fig. 3). These included four upregulated probes representing key proteins of photosynthetic light reactions: psbo1 (oxygen-evolving enhancer protein 1), lhcb5 (chlorophyll a/b-binding protein CP26), ndhF (chloroplast NADH dehydrogenase subunit 5), and atpA (ATP synthase alpha subunit), as well as two separate downregulated probes, one for aps (ATP sulphurylase) and the other for tps (terpene synthase). Five probes representing genes with likely housekeeping functions were also tested for constancy of expression between the treatment and control. tubb6 had the most stable expression among these genes and was therefore used as a reference gene for each of the other seven transcripts in estimating relative fold-changes by the ΔΔCT method (Schmittgen and Livak, 2008).

In a second qPCR experiment comparing M. × giganteus to Z. mays, using five transcripts validated from the microarray experiment and three additional genes with known regulation during chilling, significant differences in gene expression between the two species were found during chilling. Changes in transcripts for M. × giganteus were consistent with those found in the first experiment, although psbo1 expression change was not significantly different (P=0.14). In Z. mays, transcripts for psbA (D1), lhcb4 (chlorophyll a/b-binding protein CP29), psbo1, and lhcb5 all showed significant downregulation (α ≤ 0.05), while the same transcripts, with the exception of psbo1, in M. × giganteus were significantly upregulated (α ≤ 0.05) (Fig. 4). petA (cytochrome F) indicated downregulation in Z. mays but it was not significantly different from control (P=0.14). Similar expression patterns were seen in ndhF for both species (Fig. 4). These two qPCR experiments were performed on RNA samples from completely independent chilling treatments and showed the apparent reproducibility of these results in M. × giganteus.
on the photosynthetic membrane. Decline in the maximum quantum yield of $\Phi_{CO_{2\max}}$ in maize with chilling has been associated with the loss of key proteins of the photosynthetic membrane, particularly those of PSII and those involved in transfer of excitation energy to PSII (Fryer et al., 1995).

In contrast, a small but not statistically significant loss of $\Phi_{CO_{2\max}}$ was indicated when $M. \times$ giganteus was subjected to the same chilling conditions (Naidu and Long, 2004). This raises the question of how this species avoids this loss. Here, 30 genes annotated as encoding proteins with key functions in the light reactions of photosynthesis showed significantly increased expression, and none of this category showed a decrease. This suggests that $M. \times$ giganteus may avoid the losses observed in maize, at least in part, by increasing synthesis of these key proteins. Across all of the MapMan categories, photosynthesis was the only one that did not show any down-regulated probes (Tables 2 and 3 and Fig. 2). Functions of the proteins encoded by upregulated transcripts include electron transport, light harvesting, photosystem reaction centres, ATP synthesis, and the xanthophyll cycle which protects

### Discussion

The present study asked whether the physiological chilling tolerance of photosynthesis in $M. \times$ giganteus corresponded to maintenance or upregulation of genes encoding key aspects of the photosynthetic apparatus, specifically those proteins shown to be damaged or deficient in $Z. mays$ during acclimation to chilling that relate to energy transduction on the photosynthetic membrane. Decline in the maximum...
PSII against photoinhibitory damage (Demmig-Adams and Adams, 1992; Fryer et al., 1995; Farage et al., 2006). In the current study, in Z. mays following 28 h of 14 °C chilling, four genes associated with the light reactions of photosynthesis were significantly downregulated. These encode the chlorophyll a/b-binding protein precursor, chlorophyll a/b-binding apoprotein and minor antenna complex CP24 precursor, chlorophyll a/b-binding and minor antenna complex CP26, and NAD kinase chloroplast precursor (Trzcinska-Danielewicz et al., 2009). The current study, which used a longer chilling treatment, similarly showed that genes encoding key proteins of the photosynthetic light reactions (i.e. D1, cytochrome f, PSII CP29, oxygen-evolving enhance protein 1, and chlorophyll a/b-binding proteins) were significantly downregulated in maize but upregulated in M. × giganteus (Fig. 4).

Parallel to these transcript studies, proteomic analysis of Z. mays leaves developed at 13 °C show large decreases in the minor antenna complexes CP24, CP26, and CP29 (Caffarri et al., 2005). In a similar experiment, Nie and Baker (1991) showed large decreases at 12 °C also in the 26-kDa D1 reaction centre protein, cytochrome f (33 kDa), cytochrome b6/f subunit IV (17 kDa), and the α and β subunits of the coupling factor (58 and 57 kDa). Consistent with these prior studies, Western blotting in the current study similarly showed significant decreases in both D1 reaction centre protein and LHCII chlorophyll a/b-binding protein in Z. mays after 14 days of chilling. In contrast, these same proteins increased in M. × giganteus (Fig. 5). For plants that acclimate to chilling conditions, increased overall leaf protein contents are commonly observed. This study group has previously shown that acclimation to the chilling conditions used here lowers total protein per unit leaf area by 17% in Z. mays and increases content by 2% in M. × giganteus (Naidu et al., 2003). This suggests that the much larger increases observed here in LHCII chlorophyll a/b-binding protein and D1 are more than the result of an overall increase in protein content. This difference in levels of these light reaction-related transcripts and protein contents is likely to be a key underlying mechanism, explaining an unusual but effective acclimation of photosynthesis to chilling.

A critical function necessary to the maintenance of photosynthesis during chilling in C3 and C4 plants is the ability of the chloroplast to degrade damaged D1, a component of PSII, and to then synthesize and assemble D1 back into PSII (Bredenkamp and Baker, 1994; Fryer et al., 1995; Ruelland et al., 2009). This repair function is inhibited in Z. mays during chilling and this has been attributed to decreased expression of psbA, the plastid gene that encodes D1 (Bredenkamp and Baker, 1994; Allen and Ort, 2001). The current results show that the transcripts for the gene for D1 protease,
Spence et al. involved in the degradation of damaged D1, and for psbA, the gene encoding D1, are strongly upregulated in M. × giganteus grown under chilling conditions, with fold-changes of 3.37 (Table 2) and 2.06 (Fig. 4), respectively. Damage to D1 is a major cause of loss of PSII efficiency, since absorbed light energy will be channelled to inactive centres that, under light-limiting conditions, will lower $\Phi_{CO_{2max}}$ (reviewed by Long et al., 1994). Previously a high expression of xanthophyll cycle pigments and accumulation of zeaxanthin during chilling of M. × giganteus has been shown (Farage et al., 2006). The present study provides an explanation for this: a significant increase in expression of the transcript for a violaxanthin de-epoxidase, which converts violaxanthin to zeaxanthin ($\log_2$ fold-change 0.855, Table 2). Accumulation of zeaxanthin would serve to lessen damage to D1 by dissipating excess excitation energy by thermal de-excitation (Farage et al., 2006).
This, combined with increased capacity for processing damaged D1 should greatly decrease the number of centres with damaged D1 at any point in time and could explain the maintenance of high $\Phi_{CO_2}^{max}$ during chilling, as observed in $M. \times giganteus$ in chilling in contrast to maize (Naidu and Long, 1994; Beale et al., 1996).

Maximum quantum yield ($\Phi_{CO_2}^{max}$) and light-saturated rates ($A_{sat}$) of carbon assimilation are equally important in maintaining crop canopy carbon gain over a diurnal course, since this depends on both sunlit and shaded canopy leaves (Baker et al., 1988). While $\Phi_{CO_2}^{max}$ in C$_4$ photosynthesis depends on the efficiency of energy transduction on the photosynthetic membrane and the stability of key proteins, such as D1, $A_{sat}$ depends on the maximum capacity of the rate-limiting steps in electron flow and carbon metabolism beyond the reaction centres, and diversion of electrons to sinks other than CO$_2$ assimilation. During chilling, $M. \times giganteus$, in sharp contrast to $Z. mays$, maintains high levels of the enzymes of photosynthetic carbon metabolism that exert maximal metabolic control of $A_{sat}$, Rubisco, and PPDK. Rubisco protein contents are stable in $M. \times giganteus$ in response to chilling while they decline by more than 50% in $Z. mays$ during chilling (Naidu et al., 2003; Wang et al., 2008a). PPDK declines to an even greater extent in $Z. mays$ but increases in $M. \times giganteus$ (Naidu et al., 2003; Wang et al., 2008b). The current results correspond with these findings, with the expression levels of both Rubisco (log$_2$ fold-change 0.142) and PPDK (log$_2$ fold-change 0.015)
remaining stable during chilling. In addition, the transcripts for the two isoforms of Rubisco activase (rca1 and rca2) were upregulated during chilling (Supplementary Table S3). Rubisco activase is a catalytic chaperone that is critical to maintaining the stability and high catalytic activity of Rubisco. Specifically, Rubisco activase promotes the dissociation of a wide variety of inhibitory sugar phosphates from the Rubisco active site (Portis, 2003). More recently, Shan et al. (2011) found that downregulation of rca1 was a key and necessary component in the jasmonate-induced senescence that is associated with a wide range of abiotic and biotic stresses. It is intriguing that chilling in M. × giganteus induced the opposite response to that seen in induced senescence.

The Agilent 44K maize oligonucleotide microarray chip is a platform that was designed to include nearly all available maize genes (Skibbe and Walbot, 2009). It includes expressed sequence tags involved in flowering, fruit formation, root and shoot development, and germination, as well as developing and mature leaves (Skibbe and Walbot, 2009). A prior study utilizing this platform found that unstressed mature Z. mays leaves expressed approximately 26 000 transcripts under greenhouse growth conditions (Casati and Walbot, 2008). This indicates that approximately 59% of transcripts on the array are expressed in adult Z. mays leaves. A study on the diurnal rhythms of the maize leaf transcriptome, using a 105K Agilent maize array designed for all maize genomic and transcript sequences, found that approximately 44 000 transcripts were detectable in the tested maize leaves, equating to 42% of the total transcripts (Hayes et al., 2010). Since the majority of transcripts associated with photosynthesis are maximally expressed during daylight hours (Urbanczyk-Wochniak et al., 2005), samples were taken in this study 2–3 h into the photoperiod. At this time point, 48% of the probes were expressed in M. × giganteus leaves in the control (25 °C) and chilling (14 °C). This is comparable to other studies using Agilent maize arrays. This hybridization efficiency for M. × giganteus leaf mRNA to the maize array is consistent with the >90% sequence identity observed among exon sequences between M. × giganteus and Z. mays (Swaminathan et al., 2010). This is not surprising given that both are members of the same grass tribe, Andropogoneae. This grass tribe is exclusively of the NADP-ME C4 subtype (Giussani et al., 2001), suggesting that C4 photosynthesis in maize, Miscanthus, and other species of the tribe most likely evolved from a single common ancestor (Sage, 2004; Swaminathan et al., 2010).

While Z. mays is a crop of tropical origin, more than 60% of global production today is in the temperate zone (Long and Spence, 2013). It is therefore grown in colder climates more than any other C4 food crop, yet its inability to acclimate photosynthesis to chilling conditions is an Achilles heel (Baker et al., 1989; Dohleman et al., 2009). It is assumed that C4 photosynthesis evolved in hot climates and subsequently radiated to more temperate climates. The photosynthetic genes identified as upregulated in chilling-tolerant M. × giganteus in this comparative study indicate targets for upregulation, by selection or bioengineering, to improve the chilling tolerance of maize and to contribute to allowing a longer growing season for the crop in temperate climates.

**Supplementary material**

Supplementary data are available at JXB online.

**Supplementary Table S1.** All transcripts of M. × giganteus leaves found to be significantly upregulated by treatment with a log2 fold-change ≥0.70.

**Supplementary Table S2.** All transcripts of M. × giganteus leaves found to be significantly downregulated by treatment with a log2 fold-change ≥0.70.

**Supplementary Table S3.** Transcripts for Rubisco, Rubisco-interacting proteins, and PPDK transcripts showing no significant log2 fold-change and maintaining high expression during chilling.

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