

RESEARCH PAPER

Genetic regulation of cold-induced albinism in the maize inbred line A661

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Abstract

In spite of multiple studies elucidating the regulatory pathways controlling chlorophyll biosynthesis and photosynthetic activity, little is known about the molecular mechanism regulating cold-induced chlorosis in higher plants. Herein the characterization of the maize inbred line A661 which shows a cold-induced albino phenotype is reported. The data show that exposure of seedlings to low temperatures during early leaf biogenesis led to chlorophyll losses in this inbred. A661 shows a high plasticity, recovering resting levels of photosynthesis activity when exposed to optimal temperatures. Biochemical and transcriptome data indicate that at suboptimal temperatures chlorophyll could not be fully accommodated in the photosynthetic antenna in A661, remaining free in the chloroplast. The accumulation of free chlorophyll activates the expression of an *early light inducible protein (elip)* gene which binds chlorophyll to avoid cross-reactions that could lead to the generation of harmful reactive oxygen species. Higher levels of the *elip* transcript were observed in plants showing a cold-induced albino phenotype. Forward genetic analysis reveals that a gene located on the short arm of chromosome 2 regulates this protective mechanism.

Key words: Albinism, chlorophyll biosynthesis, cold, *early light inducible protein (elip)*, photosystem, *Zea mays*.

Introduction

Phototroph organisms constitute the primary trophic level due to their capacity to transform energy from the sunlight into chemical energy. In higher plants, this process initiates when a photon is absorbed by the photosynthetic antenna in the thylakoid membrane and the subsequent transfer of energy to the reaction centre (RC), which catalyses the photoinduced oxidation of water (Ferreira *et al.*, 2004). The primary pigment responsible for light harvesting is chlorophyll. Chlorophyll biosynthesis is a complex and highly coordinated process which constitutes a branch of the tetrapyrrole pathway, which results in the synthesis of two major classes of products, haem-related compounds and chlorophylls (for reviews, see Tanaka and Tanaka, 2007; Mochizuki *et al.*, 2010). Early reactions of the pathway are localized in the chloroplast stroma where glutamate is converted into 5-aminolaevulinic acid (5-ALA), the first committed precursor of

all tetrapyrroles (Beale, 1999; Joyard *et al.*, 2009). Once synthesized, 5-ALA is subject to a sequential series of condensation reactions to produce protoporphyrin IX (ProtoIX). At this point, ProtoIX is diverted toward either chlorophyll or haem biosynthesis by the chelation of magnesium or ferrous ions, respectively. In the chlorophyll branch, Mg-ProtoIX is subsequently modified to produce chlorophyll *a*. The immediate precursor of chlorophyll *a*, chlorophyllide *a*, can be reversibly converted to chlorophyll *b* via the so-called 'chlorophyll cycle' (Mochizuki *et al.*, 2010). Chlorophyll *b* acts as an accessory pigment located only in peripheral light-harvesting complexes (LHCs).

The final steady state of the leaf chlorophyll content is the result of a tight equilibrium between anabolism and catabolism. Chlorophyll turnover is a continuous process resulting in a chlorophyll half-life of ~50 h in resting tissues (Stobart

and Henry, 1984; Beisel *et al.*, 2010). The first step of chlorophyll degradation is catalysed by the enzyme chlorophyllase, which hydrolyses chlorophyll into chlorophyllide and phytol (Holden, 1961). Subsequent reactions lead to the conversion of chlorophyllide into a group of non-fluorescent-related compounds (Kräutler, 2008).

The chlorophyll biosynthetic pathway must be tightly regulated, not only due to its pre-eminent role in photosynthesis but also to avoid the accumulation of photo-toxic chlorophyll intermediates (op den Camp *et al.*, 2003; Aarti *et al.*, 2007). Environmental changes that promote photooxidative stress trigger regulatory mechanisms which lead to a readjustment of the chlorophyll biosynthesis rate and the photosynthetic antennae size (Tewari and Tripathy, 1998; Klenell *et al.*, 2005). During the last decades, considerable efforts have been made to elucidate regulatory pathways affecting chlorophyll biosynthesis driving such acclimation. Though transcriptome regulation has been considered marginal under resting conditions, several studies reported changes in transcript expression of genes involved in chlorophyll biosynthesis in response to changing environmental conditions (Tewari and Tripathy, 1998; Matsumoto *et al.*, 2004; Liu *et al.*, 2012). Interestingly, based on their expression profile at the onset of greening, these genes could be grouped into four categories, suggesting coordinate regulatory mechanisms of gene expression to control chlorophyll biosynthesis (Matsumoto *et al.*, 2004). Early steps of the tetrapyrrole pathway have been reported to play a key role in the regulation of chlorophyll biosynthesis (Kumar and Soll, 2000; Cornah *et al.*, 2003; Kim *et al.*, 2005).

Temperature is one of the major abiotic stresses reported to affect chlorophyll content in angiosperms dramatically. Up to 90% of chlorophyll content reduction has been quantified in both dicot and monocot plants grown under chilling temperatures (Tewari and Tripathy, 1998). Early and later steps of the chlorophyll biosynthetic pathway have been suggested to be key regulatory steps of this reduction in the chlorophyll content. For instance, Tewari and Tripathy (1998) stated that chilling temperatures inhibited protochlorophyllide biosynthesis by inactivation of early committed enzymes of the chlorophyll biosynthetic pathway. These authors did not report changes in the expression of later enzymes in the pathway, whereas Liu *et al.* (2012) propose that the cold-induced albino phenotype observed in the line of wheat *FA85* is due to the transcriptional down-regulation of the protochlorophyllide oxidoreductase and chlorophyll synthase activities. These apparently contradictory results suggest that cold regulation of chlorophyll biosynthesis is a complex network that is just beginning to be understood.

Chlorophyll-less plants have been a very useful tool to identify those genes involved in the regulation of the chlorophyll biosynthetic pathway under resting conditions. The characterization of the cold-induced albino phenotype of the maize inbred line A661 is reported here. This inbred line shows a dramatic reduction of the chlorophyll content when grown at temperature below 15 °C. Biochemical and genetic approaches were used to characterize the performance of this inbred line under cold conditions. The results indicate that suboptimal temperatures activate the expression of an *early*

light inducible protein (elip) gene which could be responsible of chlorophyll losses in such conditions. Moreover, genetic analysis suggests that a gene located on the short arm of chromosome 2 regulates the expression of this gene.

Materials and methods

Growth conditions and seed genetic stocks

A661 and B73 seeds were obtained from the Misión Biológica de Galicia (CSIC; Spain) stock collection. The US stock of A661 was ordered from the Maize Genetic Cooperation Stock Center (Urbana, IL, USA). Maize seeds were grown on sterilized peat in a phytotron under fluorescent light ($228 \mu\text{mol m}^{-2} \text{s}^{-1}$) in a 14h light/10h dark light regime and watered as needed. A constant day/night temperature regime was set up. Control temperature was established at 25 °C and cold temperature at 15 °C. For recovery experiments, A661 seeds were planted at three different sowing dates with a 1 week interval between them. Ten days after the last sowing, the temperature was increased to 25 °C under the same conditions.

Genetic mapping

To obtain a mapping population, the inbred line A661 was outcrossed to the inbred line EP42 and plants were self-pollinated for two generations. A linkage map was constructed using 393 F_{2,3} families genotyped with 98 polymorphic simple sequence repeat (SSR) markers with the software MAPMAKER 3.0b (Lander *et al.*, 1987). Quantitative trait locus (QTL) analysis was performed using the PLABQTL software (Utz and Melchinger, 1996). A likelihood odds (LOD) threshold of 3.0 was chosen for declaring a putative QTL significant. The LOD score threshold was obtained by the permutation test method (Churchill and Doerge, 1994), yielding an experiment-wise error rate of 0.25. The analysis and cofactor election were carried out using an 'F-to-enter' and 'F-to-delete' value of 7. Chlorophyll content was recorded from 10 plants of each family following Sims *et al.* (2002). After positioning the gene on the short arm of chromosome 2, fine mapping was performed using 690 chromosomes from two different F₂ mapping populations obtained by outcrossing the inbred line A661 to the inbreds EP42 and EP74. Information about markers is available at <http://www.maizegdb.org/>.

Leaf pigments measurement

Samples were collected in liquid nitrogen and conserved at -80 °C until extraction. Chlorophyll precursors were extracted from etiolated tissue collected under a dim green safe light. A 100mg aliquot of frozen leaf tissue was ground using a mortar and pestle in liquid nitrogen. Pigments were extracted in 1 ml of acetone overnight at 4 °C and centrifuged at 3200 g for 10 min. The supernatant was transferred into auto-sample vials for HPLC analysis. Analyses were performed as described in Zapata *et al.* (2000).

Non-destructive determination of chlorophyll content was performed on the fully expanded second leaf using a chlorophyll content meter (ADC: OSI CCM 200; ADC BioScientific). The quantum yield of photosystem II (Fv/Fm) was recorded using a portable fluorometer (OS-30p Chlorophyll Fluorometer, OptiScience, Inc.) in light-adapted plants (plants were allowed to adapt to light conditions for at least 1 h at $228 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Extraction and detection of anthocyanins

Total anthocyanins were extracted from lyophilized leaf tissue in 3 ml of MeOH:HCl 0.1 N (95:5 v/v) at 4 °C for 3h. Thereafter, samples were centrifuged at 4000 g at 4 °C for 5 min. The supernatant was recovered and evaporated to dryness under a gentle nitrogen flow. The residue was resuspended in 500 μl of acid water (pH 1.4 with HCl). Chromatographic analyses were carried out on a Symmetry Shield

C18 column (150 mm×4.6mm, 5 µm particle size; Waters, Milford, MA, USA). The mobile phase was a mixture of (A) ultrapure methanol and (B) formic acid/ultrapure water (10:90). The flow rate was 0.55 ml min⁻¹ in a linear gradient starting with 95% B for 1 min, reaching 50% B after 25 min, 5% B after 3 min, and 95% B after 3 min. The injection volume was 20 µl and chromatograms were recorded at 520 nm in a model 2690 HPLC instrument (Waters), equipped with a model 996 UV absorbance detector (Waters). Compounds were quantified by using cyanidin chloride (Sigma) as standard.

Chlorophyllase activity measurement

Leaf samples (0.5 g) were extracted on ice with 15 ml of extraction buffer [100 mM phosphate buffer and 10 µM phenylmethylsulphonyl fluoride (PMSF)] using a Heidolph Diax 900 homogenizer (Sigma). The homogenate was filtered through four layers of cheese-cloth and centrifuged at 16 000 g for 10 min at 4 °C. Enzymatic assay was performed in a 2 ml reaction mixture containing 1 ml of protein extract and 100 µM chlorophyll *a* and *b* extracted from spinach. Samples were incubated at 40 °C for 45 min, whereas controls were kept at 4 °C. The reaction was stopped by adding 1 ml of hexane followed by vigorous shaking, and centrifuged at 16000 g for 10 min at 4 °C. Chlorophyll was quantified in the organic phase spectrophotometrically (Rassadina *et al.*, 2004) and compared with controls.

Microscopy

Transverse free-hand sections were obtained by placing the maize leaf between two pieces of Parafilm, folding the specimen in half through the distal–proximal axis, and cutting with a razor blade. Observations were made on a Nikon Eclipse E200 microscope equipped with a Nikon C-SHG1 Super High Pressure Power Supply provided with a 100 W mercury lamp. Images were captured using a Nikon DS-F11 camera under bright field and subsequently under UV using a 330–380 nm excitation filter and a 420 long-pass emission filter.

Microarray hybridization and data analysis

A661 plants were grown under cold conditions for 3 weeks and then exposed to control temperature for one extra week. After this extra week at control temperature, the third leaf showed two well-defined sections: distal (chlorophyll-less; CL) and proximal (chlorophyll-containing; CC) sections. Two centimetres of the distal (CL) and proximal (CC) sections closest to the transition zone were collected from three biological replicates. Sample tissues were ground in liquid nitrogen and the RNA was extracted using the RiboPure™ kit (Ambion, Foster City, CA, USA). Standard Affymetrix procedures were used to perform microarray hybridization and analysis. A 250 ng aliquot of total RNA was labelled with the GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. A 15 µg aliquot of cRNA per sample was hybridized on a GeneChip® Maize Genome Array (Affymetrix) which interrogates 13393 maize genes, and fluorescence signalling scanned using a GeneChip® 3000 scanner (Affymetrix). Data analysis was performed using the Robust Multichip Average (RMA) tool included in the Affymetrix Expression Console software. The complete microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE41956 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41956>).

Genes with an expression ratio (CL versus CC) ≤ 0.5 or ≥ 2 were considered down- and up-regulated, respectively. To identify genes differentially expressed between treatments, a Benjamini–Hochberg multitest-adjusted false discovery rate (FDR) was calculated between log₂-transformed expression ratios from CL and CC sections using the LIMMA software (Smyth, 2004). Genes with an FDR ≤ 0.05 were considered as differentially expressed. To identify functional gene categories differentially expressed in both leaf sections, a

Gene Ontology (GO) enrichment analysis was performed with the Blast2Go bioinformatics tool (Conesa *et al.*, 2005) using Fisher's exact test with an FDR ≤ 0.05 filter value. Transcripts were categorized according to their predicted cellular localization based on GO. When no GO information was available, subcellular localization was predicted using WoLF PSORT (Horton *et al.*, 2007). Subchloroplast localization was predicted using SubChlo (Du *et al.*, 2009).

Quantitative reverse transcription–PCR (RT–qPCR)

Leaf RNA from three biological replicates of B73 and A661 seedlings grown under cold and control temperatures was extracted with the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St Louis, MO, USA). To remove any traces of genomic DNA from RNA extractions, the RNA was treated with RQ1 RNase-Free DNase (Promega, CA, USA) following the manufacturer's instructions. A 1 µg aliquot of total RNA was reverse transcribed using the GoScript™ reverse transcription system and oligo(dT20) (Promega). RT–qPCR was performed in a 20 µl reaction with the Fast Start Universe SYBR Green Master (ROX) mix (Roche, IN, USA). The glyceraldehyde-3-phosphate dehydrogenase (*GAP*) transcript was used as reference gene. Primer efficiency was calculated using the LingRegPCR software (Ramakers *et al.*, 2003). RT–qPCRs were carried out on a 7500 Real Time PCR System (Applied Biosystem, Foster City, CA, USA). The primers used for RT–qPCR are listed in Supplementary Table S4 available at *JXB* online.

Results

The maize inbred line A661 shows a cold-induced albino phenotype

When grown at optimal temperatures, A661 leaves show a dark green colour, similar to that observed in the inbred line B73 (this inbred line was used as a reference since it shows an intermediate cold tolerance). However, at cold temperatures, A661 undergoes a dramatic reduction of chlorophyll content, showing a light pink leaf colour (Fig. 1A). To characterize the performance of this inbred line under cold conditions in more depth, the leaf chlorophyll content was quantified in plants grown at different temperatures. Spectrophotometric quantifications confirmed that the total chlorophyll content was similar in the A661 and B73 leaves when plants were grown at temperatures >20 °C (Fig. 1B). Between 20 °C and 13 °C, the reference inbred B73 showed a gradual reduction in the chlorophyll content, whereas at 15 °C the inbred line A661 showed a 96% reduction compared with that quantified at 20 °C (Fig. 1B). Both inbred lines reach the minimum chlorophyll levels at 13 °C.

To test whether the low chlorophyll content in A661 is due to a higher degradation rate than that observed in B73, the *in vitro* chlorophyllase activity was quantified. Chlorophyllase is the enzyme that catalyses the first step of chlorophyll degradation (Holden, 1961). Both inbred lines show a similar chlorophyllase activity under control and cold conditions (Fig. 1C).

This result prompted the study of the possibility that the observed albino phenotype is due to an impairment of chlorophyll biosynthesis in A661 under cold conditions. To test this hypothesis, the levels of chlorophyll intermediates at the later steps of the biosynthetic pathway were analysed. Chlorophyll precursors remain at low levels in plant leaves (in

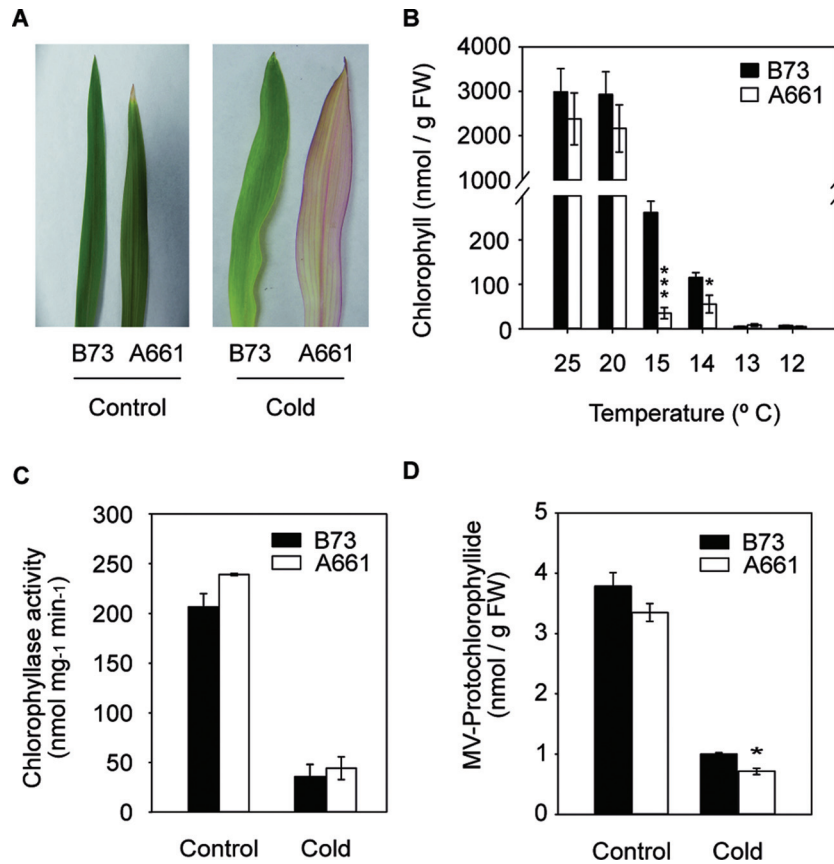


Fig. 1. The maize inbred line A661 shows a cold-induced albino phenotype. (A) Detail of B73 and A661 leaves grown under control (25 °C) and cold (15 °C) conditions. (B) Spectrophotometric quantification of the chlorophyll content of the second leaves from maize plants grown under different temperatures. * $P < 0.05$; *** $P < 0.001$ (t -test). (C) Chlorophyllase activity in extracts from B73 and A661 seedlings grown under control and cold conditions. (D) HPLC quantification of monovinyl-protochlorophyllide in maize leaves grown in darkness under control and cold conditions. * $P < 0.05$ (t -test). (B–D) Data are representative of at least two independent experiments \pm SE of at least three replicates.

most cases undetectable under conventional methodology) due to their phototoxicity. However, mutations in structural genes of the chlorophyll biosynthetic pathway could lead to accumulation of chlorophyll intermediates (Supplemental material in Tanaka and Tanaka, 2007). No chlorophyll precursors were detected in A661 or B73 seedling extracts grown upon illumination, under either cold or control conditions, indicating that the chlorophyll biosynthetic pathway is not blocked in the inbred line A661 at the final steps of the biosynthetic pathway. To test whether the chlorophyll pathway could be blocked at earlier stages, chlorophyll precursors were analysed in seedlings grown in darkness. In such conditions, angiosperms accumulate monovinyl-protochlorophyllide (MV-Pchide) due to the lack of the light-independent isoform of the POR enzyme (Lancer *et al.*, 1976; op den Camp *et al.*, 2003). An accumulation of MV-Pchide was observed in both genotypes when grown in darkness (Fig. 1D), indicating that A661 has a functional chlorophyll pathway even under cold conditions. Interestingly, A661 accumulates significantly less MV-Pchide under cold conditions than under control conditions, indicating that the chlorophyll biosynthetic pathway is somehow repressed in this inbred line by low temperatures.

Minor changes in the accumulation of accessory pigments were observed in A661 and B73 leaves

Accessory pigments play an essential role in extending the range of wavelengths over which light can drive photosynthesis or dissipating excess light, essentially under stressful conditions (Bode *et al.*, 2009). To test if the accumulation of accessory pigments is also affected in the inbred line A661 by cold temperatures, these pigments were quantified in leaves grown under cold and control conditions. Maize seedlings showed a drastic reduction in chlorophyll *b* and carotenoid content under cold conditions (Fig. 2A). The inbred line A661 accumulates significantly less chlorophyll *b* under cold conditions than the inbred line B73 (Fig. 2A). No significant differences were observed in the chlorophyll *alb* ratio between genotypes or temperature conditions (Supplementary Fig. S1A at JXB online). The content of β -carotene and two xanthophylls (lutein and antheraxanthin) was significantly reduced in the inbred line A661 compared with that observed in B73.

Concerning anthocyanins, derivatives of two major anthocyanidins, cyanidin and pelargonidin, were identified (Fig. 2B). In general terms, leaves from both genotypes

accumulated similar levels of these derivatives which are not apparently affected by temperature (Fig. 2B); however, the inbred line A661 accumulates more cyanidin-glucoside content under cold conditions than does B73.

A661 leaves recover chlorophyll content when exposed to control conditions

The cold-induced albino phenotype was observed in A661 maize seedlings germinated under cold conditions. To study whether the chlorophyll content in developed leaves of A661 is affected by changes in temperature, two different experiments

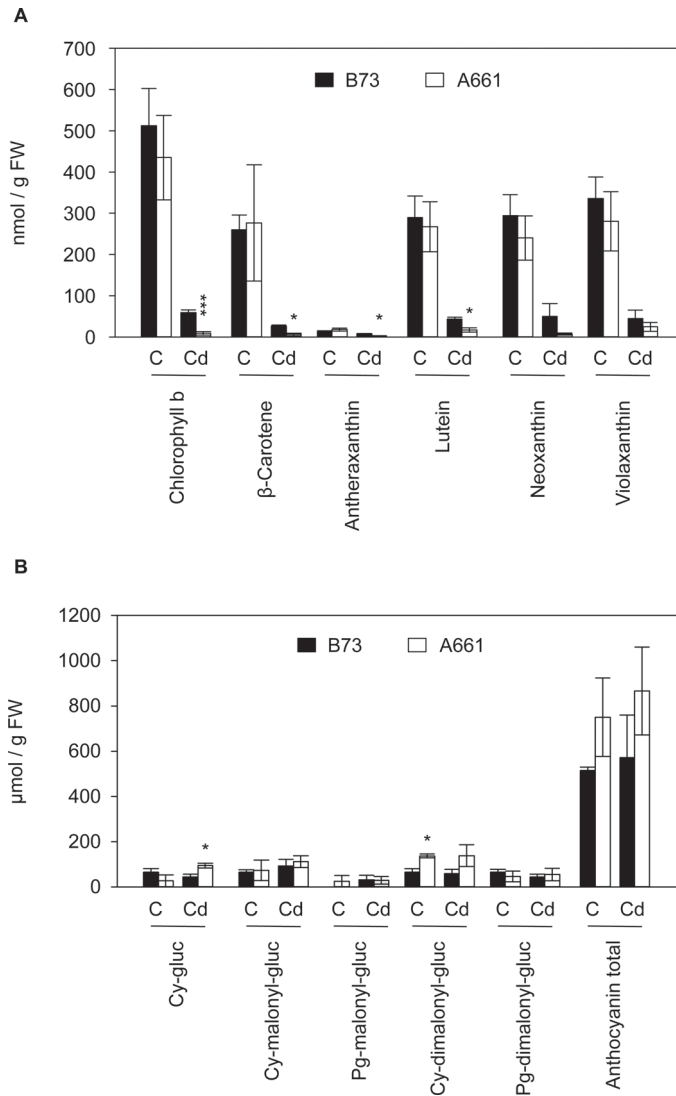


Fig. 2. The inbred lines B73 and A661 accumulate similar levels of accessory pigments. (A) HPLC quantification of chlorophyll *b* and carotenoids in A661 and B73 seedlings grown under cold (Cd) and control (C) conditions. * $P < 0.05$; *** $P < 0.001$ (*t*-test). (B) HPLC quantification of anthocyanins in A661 and B73 seedlings grown under cold (Cd) and control (C) conditions. * $P < 0.05$; *** $P < 0.001$ (*t*-test). Abbreviations: Cy; cyanidin, Gluc; glucoside, Pg; pelargonidin. Data are representative of at least two independent experiments \pm SE of three replicates.

were performed. In the first one, plants were grown under control conditions for 3 weeks and then exposed to low temperatures for 15 d. The chlorophyll content was monitored using a non-destructive method. No significant reduction in the chlorophyll content was observed in developed A661 or B73 leaves under control conditions during the 15 d period of cold treatment (Supplementary Fig. S1B at *JXB* online). The second experiment was performed in the opposite way. Plants were grown under cold conditions and then exposed to control temperatures at different developmental stages. In this case, a sequential greening of leaves was observed in A661 leaves. The recovery of chlorophyll is translated into an increase in activity of PSII which reached resting levels after 8 d of exposure to control temperatures (Fig. 3A). A differential response was observed when albino plants were exposed to control conditions at different developmental stages. Younger leaves respond earlier than more developed ones, reaching the maximum chlorophyll content within 5 d after exposure at control temperatures (Supplementary Fig. S1C). On more developed leaves, the recovery of the chlorophyll content follows a proximal to distal pattern which eventually extends to the whole leaf tissue. During this process, developed leaves showed a characteristic coloration pattern with two well-defined sections: a proximal CC section and a distal CL section (Fig. 3B). To study the morphological differences between these two leaf sections further, transverse free-hand leaf sections were visualized using bright field and UV illumination (Supplementary Fig. S2A–F). Upon UV illumination, chlorophyll autofluoresces a red colour, whereas cell walls autofluoresce blue (Braun *et al.*, 2006). Mesophyll cells in the CC section showed intense chlorophyll autofluorescence, which, as expected, was more intense than that observed in the CL section, where just traces of chlorophyll could be observed (Supplementary Fig. S2B). The observation of sections in the boundary region between CC and CL reveals that chlorophyll starts to accumulate in mesophyll cells in this region, especially in the bundle sheath cells (Supplementary Fig. S2D).

Genetic characterization of the cold-induced albino phenotype of A661

The inbred line A661 was developed from the synthetic Minnesota AS-A (derived from 13 Corn Belt lines) in a research programme conducted by the Minnesota Agriculture Experimental Station (Gaedelmann and Peterson, 1976). The A661 cold-induced phenotype was not observed in seedlings from the original AS-A synthetic (data not shown). The possibility that a mutation occurred during the *ex situ* conservation was ruled out by comparing the performance of the seed stock with that obtained from the Maize Stock Center (USA) (Supplementary Fig. S3 at *JXB* online).

To identify those chromosome regions regulating the cold-induced albino phenotype, a QTL analysis was performed on a (A661 \times EP42) $F_{2,3}$ segregating population. High significant thresholds were used in the analysis to reduce the possibility of false-positive QTLs. A single QTL with a LOD of 6.56 was identified which explains 14.2% of the phenotypic

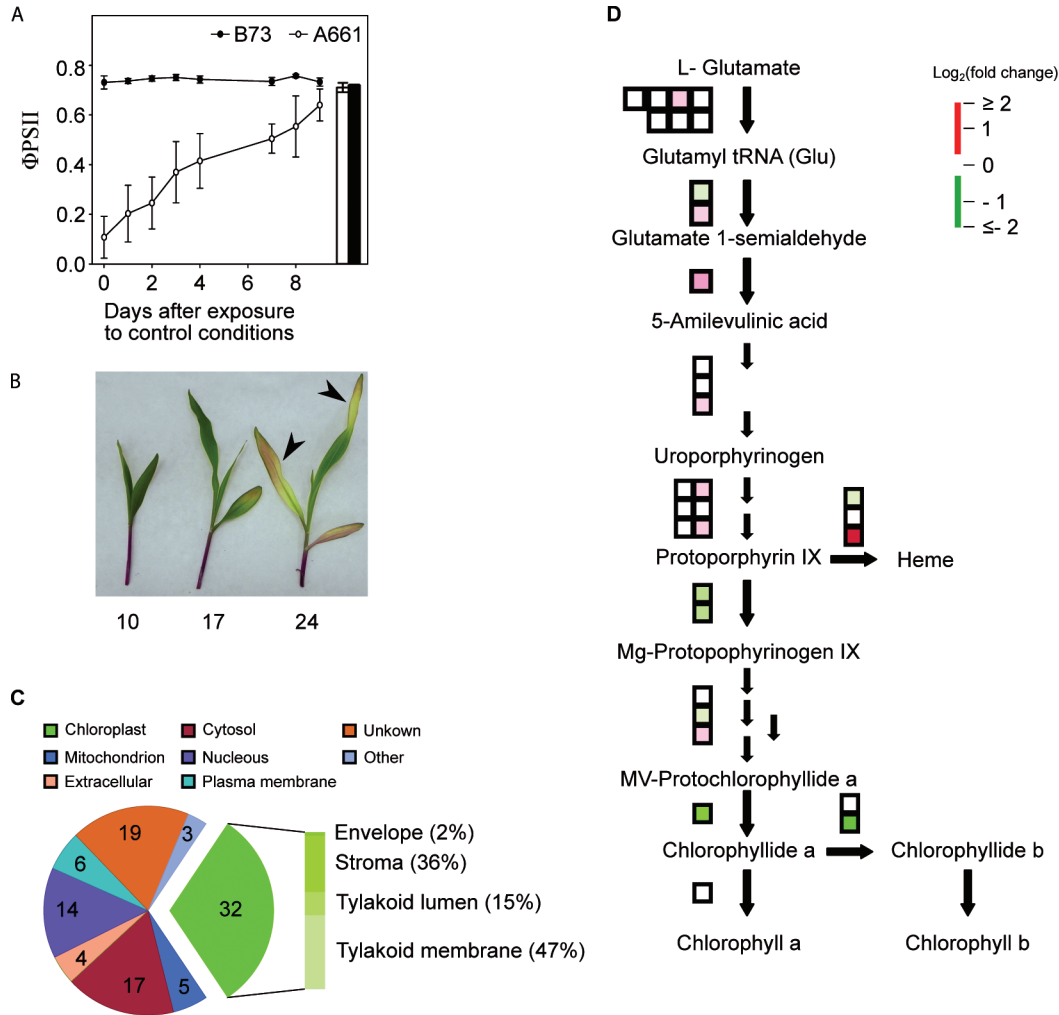


Fig. 3. Cold-induced albino A661 plants recover chlorophyll content when exposed to control temperatures. (A) Quantum yield of photosystem II (Φ PSII) recorded using a portable fluorometer (OS-30P) in plants grown during 24 d under cold conditions and then exposed to control temperatures. Bars denote levels of Φ PSII in plants grown under control conditions (white, A661; black, B73). Data are representative of at least two independent experiments \pm SE of five replicates. (B) Seedlings of A661 exposed to control conditions at different developmental stages. A661 seeds were planted during three different periods with a 1 week interval between sowings. Ten days after the last sowing, the temperature was increased to 25 °C under the same conditions. (C) Subcellular and subchloroplastic localization of genes differentially expressed in the CL versus CC sections of A661 leaves. The number within each sector represents the percentage of genes with that localization. Subcellular localization was predicted using the WoLF PSORT software. Subchloroplastic localization was predicted using the SubChlo software. (D) Schematic representation of significant changes in transcript levels of genes involved in the tetrapyrrole pathway.

variance. The QTL was located in a 16.8Mb confidence interval of the short arm of chromosome 2. Fine mapping using two independent F₂ populations (A661×EP42 and A661 EP74) narrowed the region to 7.3Mb between markers *umc1265* and *bnlg125* (Supplementary Fig. S4 at JXB online), which encompasses >450 genes. The lack of polymorphic molecular markers makes it difficult to reduce this region further.

Transcriptome analysis of A661 leaves

To investigate further the performance of the inbred line A661 under cold conditions, a gene expression profiling analysis was conducted using large-scale maize

microarrays. This experiment was performed with RNA extracted from CL and CC leaf sections of A661 (see the Materials and methods for details), in order to reduce background noise due to differences in development. A total of 1002 transcripts were identified as differentially expressed in the CL section compared with the CC section. Transcripts corresponding to 546 genes were up-regulated, whereas 456 were down-regulated (Supplementary Tables S1, S2 at JXB online). Enrichment statistical analysis (Conesa *et al.*, 2005) did not reveal any significant functional category for the up- or down-regulated sets of genes. The genes from up- and down-regulated data sets were classified into 23 categories using the MapMan software (Masuda and Fujita, 2008). In both sets of transcripts,

the most abundant categories were: miscellaneous (which encompasses all those categories with fewer than two transcripts) and RNA and protein metabolism, which suggests extensive metabolism reprogramming in the CL versus CC section (Supplementary Fig. S5).

As expected, the subcellular localization analysis yields a significant percentage of transcripts encoding proteins located in the chloroplast (Fig. 3C). More than 80% of these genes encoded proteins located either in the stroma or in the thylakoid membrane, where chlorophyll biosynthesis takes place. Transcripts encoding differentially expressed chloroplastic proteins are listed in Supplementary Table S3 at JXB online.

Among these transcripts, just a few genes involved in the tetrapyrrole biosynthetic pathway or photosynthesis were differentially expressed. An overview of the tetrapyrrole pathway indicates that regulation at the transcript level could take place at the later steps of the pathway (Fig. 3D). Three genes involved in these later steps were differentially expressed in the two leaf sections; *protochlorophyllide reductase A (pora)* and *chlorophyllide a oxygenase (cao)* were down-regulated in the CL section whereas *ferrochelatase-2 (feche2)* was up-regulated (Fig. 3D, Table 1).

Two major regulators of the chlorophyll biosynthetic pathway were up-regulated in the CL leaf section. The *elip* gene is associated with LHC proteins and its expression has been associated with high light and cold stresses (Montané *et al.*, 1997). The *fluorescent (flu)* protein has been reported to play a role in a regulatory feedback in the biosynthesis of chlorophyll (Meskauskienė, 2001).

Likewise, two transcripts (*Chalb-6A* and *PSI-D1*) involved in the conformation of the LHC were down-regulated (Table 1). *Photosystem I subunit D1 (PSI-D1)* forms a compact structure in the core of PSI, along with subunits C and E (Ihnatowicz *et al.*, 2004), whereas *chlorophyll a-b binding protein 6A (Chalb-6A)* is a constituent of the outer

light-harvesting antenna system associated with PSI (LHCI) (Zolla *et al.*, 2002).

Related to chloroplast biogenesis, three genes encoding proteins involved in peptide translocation through the outer chloroplast membrane (*toc34*, *toc35*, and *toc75*) (Sveshnikova *et al.*, 2000; Agne and Kessler, 2009) were up-regulated. These proteins may play an essential role in the assembly of the photosynthetic RCs (Table 1).

The expression of an early inducible protein correlates with the cold-induced albino phenotype

To confirm the microarrays results, the expression of key genes in the chlorophyll biosynthetic pathway was quantified (Fig. 4A). Among the genes involved in the initial steps of chlorophyll biosynthesis, a significant reduction in the expression of *Mgche* was observed in A661 compared with B73, but this was also observed under control conditions. Likewise, RT-qPCR results indicate that the *feche2* transcript is not affected by cold temperature in either A661 or B73 inbred lines, in contrast to results obtained in the microarray experiment. Concerning genes involved in the later steps of the pathway, a remarkable reduction of the expression of the *cao* and *pora* genes was observed under cold conditions, albeit that both inbred lines showed equal expression of these genes under control and cold conditions (Fig. 4A).

The *elip* gene stands out among the transcripts differentially regulated in the microarray experiment. The accumulation of this protein in thylakoid membranes correlates with the inactivation of photosystems and changes in the level of photosynthetic pigments (Adamska *et al.*, 1993; Tzvetkova-Chevolleau *et al.*, 2007). The expression of this gene is significantly higher in A661 compared with B73 under cold conditions (Fig. 4B). Moreover, a negative and significant correlation between the expression of the *elip* gene and the chlorophyll content was

Table 1. List of genes differentially expressed in the chlorophyll-less (CL) versus the chlorophyll-containing (CC) A661 leaf sections and involved in tetrapyrrole biosynthesis and chloroplast biogenesis

Gene description	Public ID	Log ₂ (FC) ^a	P-value ^b	FDR ^c
Up-regulated				
<i>feche</i>	BU050248	1.26	0.00013	0.0038
<i>elip</i>	CB350631	1.78	0.00226	0.0146
<i>toc34</i>	CD996491	1.00	0.00073	0.0079
<i>toc35</i>	BM073485	1.19	0.00042	0.0061
<i>toc75</i>	CA402647	1.20	0.00022	0.0047
<i>flu</i>	CA399585	1.08	0.00095	0.0091
Down-regulated				
<i>cao</i>	BG316973	-1.22	0.00008	0.00324
<i>pora</i>	CO528824	-1.08	0.00347	0.01897
<i>Chalb-6A</i>	LOC100281791	-1.40	0.00002	0.00198
<i>PSI-D1</i>	AF147726.1	-1.72	0.00009	0.00329

Feche, ferrochelatase-2; *elip*, early light inducible protein; *toc*, translocon at outer membrane of chloroplast; *flu*, fluorescence protein; *cao*, chlorophyllide a oxygenase; *pora*, protochlorophyllide reductase A; *Chalb-6A*, chlorophyll a/b-binding protein 6A; *PSI-D1*, photosystem I subunit D1.

^a FC, fold change.

^b The P-values from the Student's *t*-test are shown.

^c False discovery rate.

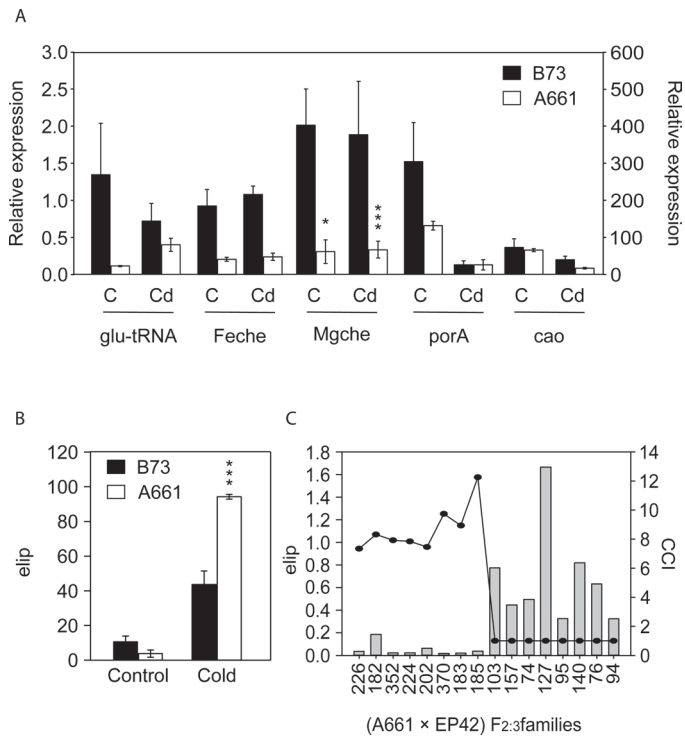


Fig. 4. Quantitative RT-PCR expression analysis of genes involved in the chlorophyll biosynthetic pathway. (A) Relative expression of key genes involved in the chlorophyll biosynthetic pathway. Expression of *glu-tRNA* (glutamyl-tRNA synthetase), *feche* (ferrochelatase), and *MgChe* (magnesium chelatase) is shown on the left axis. Values of *pora* (protochlorophyllide reductase A) and *cao* (chlorophyllide *a* oxygenase) are shown on the right axis. The glyceraldehyde-3-phosphate dehydrogenase (*gap*) was used to normalize test gene transcript levels. * $P < 0.05$; *** $P < 0.001$ (*t*-test). (B) Relative expression of the *elip* (early light inducible protein) gene. The *gap* gene was used to normalize test gene transcript levels. *** $P < 0.001$ (*t*-test). (C) Bars represent the relative expression of the *elip* gene of 16 (A661×EP42) F_{2:3} families grown under cold conditions. The *gap* gene was used to normalize test gene transcript levels. Samples were collected from eight plants from each family and combined for mRNA extraction. The line graph represents the chlorophyll content index (CCI) of the same families. Values are the mean of eight plants from each family. (A and B) Data are representative of at least two independent experiments ±SE of three replicates.

observed in 16 F_{2:3} families from the cross A661×EP42 that showed extreme phenotypes for chlorophyll content. Those families with higher expression of the *elip* gene show the lowest values of chlorophyll content (Fig. 4C).

Discussion

A thorough genetic and biochemical characterization of the maize inbred line A661, which shows a cold-induced albino phenotype when grown at suboptimal temperatures, was performed. In maize, classical studies have established a thermal threshold for the development of chlorotic leaves between

10 °C and 15 °C (Dolstra *et al.*, 1988; Greaves, 1996). In agreement with that, the present data show that maize seedlings undergo chlorophyll losses when the temperature drops below 15 °C. Both A661 and the reference inbred line B73 reach the minimum chlorophyll content at 13 °C. However, while B73 shows a gradual reduction at temperatures between 20 °C and 13 °C, the A661 inbred line loses >96% of the resting chlorophyll content at 15 °C, indicating that the inbred lines differ in the thermal threshold for leaf chlorosis. The obvious consequence of such a reduction of chlorophyll content is a dramatic inhibition of the photosynthesis rate which compromises seedling survival beyond the heterotrophic phase. Even so, the A661 inbred line has been cultivated in temperate regions where it has been exposed to cold temperatures during early development (Eagles *et al.*, 1983; Butrón *et al.*, 1998). The present data reveal that the exposure of A661 leaves grown under control conditions to suboptimal temperatures has a limited impact on chlorophyll content which would allow plant survival in the field, where the temperature is fluctuating. The results also indicate that just a prolonged exposure to suboptimal temperatures during early leaf biogenesis could lead to the development of albino leaves in A661.

Interestingly, resting levels of PSII activity are restored in A661 leaves within a few days after exposure to optimal temperatures. The recovery of the PSII activity after the onset of favourable conditions has been previously reported (Nie *et al.*, 1995; Hola *et al.*, 2007). Sowiński *et al.* (2005) showed a differential recovery rate between younger and more developed leaves. A similar pattern was observed in A661, with the youngest leaves being able to restore resting PSII activity within 5 d after the onset of control conditions. Nonetheless, more developed leaves show, during this period, a characteristic phenotype with two well-defined regions: a CC proximal section and a distal CL section. The boundary between these two sections displaces towards the distal part of the leaf, eventually extending the CC section to the whole leaf tissue. The microscopic observation of these sections confirms the lack of chlorophyll accumulation in mesophyll cells of the CL section. Curiously, even in this section, traces of chlorophyll could be observed in bundle sheath cells, which is more prominent in the boundary section. Photosynthesis in C₄ plants requires the coordination of both cell types, mesophyll and bundle sheath, which show cell-specific regulatory mechanisms (Chang *et al.*, 2012). Whether bundle sheath cells play an essential role in A661 chlorophyll recovery must be further investigated.

Using a forward genetic approach, a QTL sited on chromosome 2 that regulates the cold-induced albino phenotype in A661 was identified. This QTL explains 14% of the phenotypic variation, suggesting that other genes with minor effects could also be involved in the regulation of this trait. This chromosome region was reduced to a 7.3Mb contig which encompasses >450 genes. According to the latest maize genome annotation, one of these genes putatively encodes a structural protein of the chlorophyll biosynthetic pathway: *coproporphyrinogen III oxidase* (GRMZM5G870342). Quantification of the mRNA levels limits the possible role of

this gene in inducing the observed albino phenotype in A661 since its expression is not significantly affected by temperature (Supplementary Fig. S6 at *JXB* online).

The slight inhibition of the chlorophyll biosynthesis rate could be explained by the moderate up-regulation of the *flu* transcript in the CL leaf section. This protein has been proposed to play an essential role in the regulation of the chlorophyll biosynthetic pathway, in both dark- and light-adapted plants (Meskauskiene, 2001; Goslings *et al.*, 2004). The present data also suggest a post-transcriptional regulation of the pathway since the transcription profile of the tetrapyrrole biosynthetic pathway does not show a significant misregulation in CL versus CC sections. The transcriptome results were confirmed by RT-qPCR in plants grown under cold conditions. These results, however, do not explain the strong reduction of chlorophyll content in A661 grown under cold conditions. To complete the overview of chlorophyll metabolism in A661 leaves, the possibility that the cold-induced albino phenotype was due to higher chlorophyll degradation was discarded by quantifying the activity of the chlorophyllase enzyme, the first enzyme involved in chlorophyll breakdown (Holden, 1961).

How do suboptimal temperatures impair chlorophyll accumulation in A661? In photosynthetically active chloroplasts, chlorophyll exists mostly associated with proteins. These proteins form the PS complexes which are made up of two moieties, the RC and the LHC (Hooper, 2007). The present transcriptome-wide analysis reveals the down-regulation of two genes, *PSI-D1* and *Chalb-6A*, which code for structural subunits of the RC and LHCa of PSI, respectively. Subunit D of the PSI RC is essential for the stability of PSI and strongly correlates with the number of PSI centres (Harari-Steinberg *et al.*, 2001; Innatowicz *et al.*, 2004), suggesting that under cold conditions A661 contains fewer PSI RCs. The significant decrease in β -carotene, antheraxanthin, and lutein content in A661 under cold conditions also supports this hypothesis.

When the amount of chlorophyll-binding apoproteins decreases, excess chlorophyll cannot be accommodated in the PS and remains free in the chloroplast. Free chlorophyll is a highly reactive molecule which tends to form long-lived triplet species producing singlet oxygen (Masuda and Fujita, 2008). Accumulation of free chlorophyll would repress tetrapyrrole biosynthesis by binding sensor proteins. Two families of proteins [ELIPs and stress enhanced proteins (SEPs)] have been postulated as sensors of free chlorophyll in the cell (Heddad and Adamska, 2000; Tzvetkova-Chevolleau *et al.*, 2007). The present transcriptome data reveal that the expression of one *elip* transcript is induced in the CL leaf section. Two paralogues of the *elip* genes have been described in the maize genome, located on chromosomes 2 (*elip1*) and 7 (*elip2*). Only the *elip2* transcript is significantly up-regulated in the CL section. Two additional independent experiments strongly support the important role of the *elip2* gene in regulating chlorophyll accumulation in A661. On the one hand, A661 seedlings grown under cold conditions show a significant overexpression of the *elip2* transcript compared with that observed in B73; on the other hand, a negative correlation between the expression of *elip2* and the chlorophyll

content in the (A661×EP42) F_{2,3} segregating population was confirmed. In agreement with the present data, Tzvetkova-Chevolleau *et al.* (2007) reported decreased accumulation of the PSII-D1 subunit protein in the PSII RC and a concomitant decrease of proteins of the PSI RC and LHCa in transgenic *Arabidopsis* plants overexpressing the *elip2* gene. These plants also showed a reduced accumulation of several chlorophyll precursors.

Different studies have reported that cold and high light promote the accumulation of *elip* transcripts in higher plants (Montané *et al.*, 1997; Shimosaka *et al.*, 1999). Harari-Steinberg *et al.* (2001) established that the light induction of *elip* genes is mediated by phytochromes A and B and promoted by the transcription factor *hy5*. Nevertheless, these authors also showed that the heat shock induction of *elip* genes is independent of this pathway. Based on the present data, it is believed that a gene located on chromosome 2 could regulate directly or indirectly the expression of the *elip2* gene in maize. Neither *hy5* nor phytochrome-encoding genes is located in this region, supporting the hypothesis of an independent regulatory pathway of *elip* expression under cold stress.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Characterization of chlorophyll content in A661 leaves under different conditions.

Fig. S2. Light microscope images of free-hand sections showing the different sectors in A661 when albino leaves are exposed to control temperatures.

Fig. S3. Chlorophyll quantification in two different A661 seed stocks.

Fig. S4. Genetic characterization of cold-induced albino phenotype of A661.

Fig. S5. Microarray analysis of CL (chlorophyll-less) versus CC (chlorophyll-containing) sections of A661 leaves.

Fig. S6. Quantitative RT-PCR expression analysis of the coproporphyrinogen III oxidase (GRMZM5G8703422) gene.

Table S1. List of up-regulated genes in the chlorophyll-less (CL) versus chlorophyll-containing (CC) A661 leaf sections.

Table S2. List of down-regulated genes in the chlorophyll-less (CL) versus chlorophyll-containing (CC) A661 leaf sections.

Table S3. List of transcripts encoding chloroplastic proteins differentially expressed in the chlorophyll-less (CL) versus chlorophyll-containing (CC) A661 leaf sections.

Table S4. Primers used for RT-qPCR.

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