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

Víctor M. Rodríguez1,*, Pablo Velasco1, José L. Garrido2, Pedro Revilla1, Amando Ordás1 and Ana Butrón1

1 Misión Biológica de Galicia (MBG-CSIC), Apartado 28, E-36080 Pontevedra, Spain
2 Instituto de Investigaciones Marinas (IIM-CSIC), c/ Eduardo Cabello 6, E-36208 Vigo, Spain

* To whom correspondence should be addressed. E-mail: vmrodriguez@mbg.csic.es

Received 11 December 2012; Revised 24 May 2013; Accepted 2 June 2013

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 F485 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 Mision 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 μmol m⁻² 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 out-crossed to the inbred line EP42 and plants were self-pollinated for two generations. A linkage map was constructed using 393 F₂,₃ 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 100μg 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 (ΦPSII) 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 μmol m⁻² 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 3 h. 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 suspended in 500 μl of acid water (pH 1.4 with HCl). Chromatographic analyses were carried out on a Symmetry Shield
Cold-induced albinism in maize | 3659

C18 column (150 mm × 4.6 mm, 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 phenylmethylsulfonyl 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 ºC. 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 ºC for 45 min, whereas controls were kept at 4 ºC. The reaction was stopped by adding 1 ml of hexane followed by vigorous shaking, and centrifuged at 16000 g for 10 min at 4 ºC. 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-FI1 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).

Gene expression was calculated using the LingRegPCR 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 false discovery rate filter. 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 the later steps of the biosynthetic pathway were analysed. Chlorophyll precursors remain at low levels in plant leaves

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 RQI 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 Universal 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 ºC (Fig. 1B). Between 20 ºC and 13 ºC, the reference inbred B73 showed a gradual reduction in the chlorophyll content, whereas at 15 ºC the inbred line A661 showed a 96% reduction compared with that quantified at 20 ºC (Fig. 1B). Both inbred lines reach the minimum chlorophyll levels at 13 ºC.

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...
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.

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 a/b 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 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 (Geadelmann 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*×*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
The QTL was located in a 16.8 Mb 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 7.3 Mb 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 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. Subchloroplast localization was predicted using the SubChlo software. (D) Schematic representation of significant changes in transcript levels of genes involved in the tetrapyrrole pathway.
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; protoporphyrinogen oxidase (por) 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 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 (Meskauskiene, 2001). Likewise, two transcripts (Chalb-6A and PSI-D1) involved in the conformation of the 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 microarray 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 observed in A661 compared with B73 under cold conditions (Fig. 4B).

**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    | Log2 (FC)* | P-valueb | FDRc |
|-------------------|--------------|------------|----------|------|
| Up-regulated      |              |            |          |      |
| feche             | BU060248     | 1.26       | 0.00013  | 0.0038 |
| elip              | CB350631     | 1.78       | 0.00026  | 0.0046 |
| toc34             | CD996491     | 1.00       | 0.00073  | 0.0079 |
| toc35             | BM073485     | 1.19       | 0.00042  | 0.0061 |
| toc75             | CA402647     | 1.20       | 0.00022  | 0.0047 |
| flu               | CA399555     | 1.08       | 0.00095  | 0.0091 |
| Down-regulated    |              |            |          |      |
| cao               | BG316973     | −1.22      | 0.00008  | 0.00324 |
| pora              | CO529824     | −1.08      | 0.00037  | 0.001897 |
| Cha/b-6A          | LOC100281791 | −1.40      | 0.00002  | 0.00198 |
| PSI-D1            | 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, protoporphyrilide reductase A; Cha/b-6A, chlorophyll a/b-binding protein 6A; PSI-D1, photosystem I subunit D1.

* FC, fold change.

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

c False discovery rate.
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; Bu-Trôn 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 7.3 Mb 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 chlorophylase 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 (Hoober, 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; Ihnatowicz 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 showed 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) F₂₃ segregating population was confirmed. In agreement with the present data, Tzvetkiva-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 (GRMZF5G8703422) 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.

Acknowledgements

We thank the staff of the Unidad de Genómica (UCM) for microarray hybridization, Ana Carballeda, Raquel Diaz, and Noelia Sanz for technical support, and the Maize Genetic Cooperation Stock Center (Urbana, IL) for kindly providing the A661 US stock. We are grateful to Pilar Soengas, Elena Zubiaurre, and Virginia Alonso for assistance with microscopy. This work was supported by the Spanish National Plan for Research and Development (grant no. AGL2010-22254).
VMR acknowledges a contract from the Spanish National Research Council (JAEDoc program, European Social Fund, ESF).

References

Aarti D, Tanaka R, Ito H, Tanaka A. 2007. High light inhibits chlorophyll biosynthesis at the level of 5-aminolevulinate synthesis during de-etiolation in cucumber (Cucumis sativus) cotyledons. Photochemistry and Photobiology 83, 171–176.

Adamska I, Kloppstech K, Ohad I. 1993. Early light-inducible protein in pea is stable during light stress but is degraded during recovery at low light intensity. Journal of Biological Chemistry 268, 5438–5444.

Agne B, Kessler F. 2009. Protein transport in organelles: the trans-complex way of preprotein import. FEBS Journal 276, 1156–1165.

Beale SL. 1999. Enzymes of chlorophyll biosynthesis. Photosynthesis Research 60, 43–73.

Beisel KG, Jahne S, Hofmann D, Köppchen S, Schurr U, Matsubara S. 2010. Continuous turnover of carotenoids and chlorophyll a in mature leaves of Arabidopsis revealed by 14CO2 pulse-chase labeling. Plant Physiology 152, 2188–2199.

Bode S, Quentmeier CC, Liao P-N, Hafi N, Barros T, Wilk L, Bittner F, Wajsa P. 2009. On the regulation of photosynthesis by excitonic interactions between carotenoids and chlorophylls. Proceedings of the National Academy of Sciences, USA 106, 12311–12316.

Braun DM, Ma Y, Inada N, Muszynski MG, Baker RF. 2006. Tied-dy1 regulates carbohydrate accumulation in maize leaves. Plant Physiology 142, 1511–1522.

Butrón A, Malvar RA, Velasco P, Cartea ME, Ordás A. 1998. Combining abilities and reciprocal effects for maize ear resistance to pink stem borer. Maydica 43, 117–122.

Conesa A, Gotz S, García-Gomez JM, Terol J, Talon M, Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676.

Cornah JE, Terry MJ, Smith AG. 2003. Green or red: what stops the traffic in the tetrapyrrole pathway? Trends in Plant Science 8, 224–230.

Chang YM, Liu WY, Shih ACC, et al. 2012. Characterizing regulatory and functional differentiation between maize mesophyll and bundle sheath cells by transcriptomic analysis. Plant Physiology 160, 165–177.

Churchill GA, Doerge RW. 1994. Empirical threshold values for quantitative trait mapping. Genetics 138, 963–971.

Dolstra O, Jongmans MA, de Jong K. 1988. Improvement and significance of resistance to low-temperature damage in maize (Zea mays L.). I. Chlorosis resistance. Euphytica 39, 117–123.

Du P, Cao S, Li Y. 2009. SubChlo: predicting protein subchloroplast locations with pseudo-amino acid composition and the evidence-theoretic K-nearest neighbor (ET-KNN) algorithm. Journal of Theoretical Biology 261, 330–335.

Eagles HA, Hardacre AK, Brooking IR, Cameron AJ, Smillie RM, Hetherington SM. 1983. Evaluation of a high altitude tropical population of maize for agronomic performance and seedling growth at low temperature. New Zealand Journal of Agricultural Research 26, 281–287.

Ferreira KN, Iverson TM, Maghlouei K, Barber J, Iwata S. 2004. Architecture of the photosynthetic oxygen-evolving center. Science 303, 1831–1838.

Geadelmann JL, Peterson RH. 1976. Registration of five maize parental lines (Reg. No. PL 43 to 47). Crop Science 16, 747.

Goslings D, Meskauskiene R, Kim C, Lee KP, Nater M, Apel K. 2004. Concurrent interactions of heme and FLU with Glu tRNA reductase (HEMA1), the target of metabolic feedback inhibition of tetrapyrrole biosynthesis, in dark- and light-grown Arabidopsis plants. The Plant Journal 40, 957–967.

Greaves JA. 1996. Improving suboptimal temperature tolerance in maize: the search for variation. Journal of Experimental Botany 47, 307–323.

Harari-Steinberg O, Ohad I, Chamovitz DA. 2001. Dissection of the light signal transduction pathways regulating the two early light-induced protein genes in Arabidopsis. Plant Physiology 127, 986–997.

Heddam M, Adamska I. 2000. Light stress-regulated two-helix proteins in Arabidopsis thaliana related to the chlorophyll a/b-binding gene family. Proceedings of the National Academy of Sciences, USA 97, 3741–3746.

Hofa D, Kokova M, Rothova O, Wilhelmova N, Benesova M. 2007. Recovery of maize (Zea mays L.) inbreds and hybrids from chilling stress of various duration: photosynthesis and antioxidant enzymes. Journal of Plant Physiology 164, 868–877.

Holden M. 1961. The breakdown of chlorophyll by chlorophyllase. Biochemical Journal 78, 359–364.

Hoober JK. 2007. Chloroplast development: whence and whither. In: Wise RR, Hoober JK, eds. The structure and function of plastids. Dordrecht: Springer, 27–51.

Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K. 2007. WolF PSORT: protein localization predictor. Nucleic Acids Research 35, W585–W587.

Ihnatowicz A, Pesaresi P, Varotto C, Richly E, Schneider A, Jahn P, Salmami F, Leister D. 2004. Mutants for plastidial isoprenoid biosynthetic pathways I subunit D of Arabidopsis thaliana: effects on photosynthesis, photosystem I stability and expression of nuclear genes for chloroplast functions. The Plant Journal 37, 839–852.

Joyard J, Ferro M, Masselon C, Seigneurin-Berny D, Salvi D, Garin J, Rolland N. 2016. Photosynthetic oxygen-evolving center. Science 354, 1154–1180.

Kim Y-K, Lee J-Y, Cho HS, Lee SS, Ha HJ, Kim S, Choi D, Pai H-S. 2005. Inactivation of organellar glutamyl- and seryl-tRNA synthetases leads to developmental arrest of chloroplasts and mitochondria in higher plants. Journal of Biological Chemistry 280, 37098–37106.

Klenell M, Morita S, Tiembo-Olmo M, Muhlenbock P, Karpinski S, Karpinska B. 2005. Involvement of the chloroplast signal recognition particle cpSRP43 in acclimation to conditions promoting photooxidative stress in Arabidopsis. Plant and Cell Physiology 46, 118–129.
Kräutler B. 2008. Chlorophyll breakdown and chlorophyll catabolites in leaves and fruit. *Photochemical and Photobiological Sciences* **7**, 1114.

Kumar AM, Soll D. 2000. Antisense HEMA1 RNA expression inhibits heme and chlorophyll biosynthesis in Arabidopsis. *Plant Physiology* **122**, 49–55.

Lancer HA, Cohen CE, Schiff JA. 1976. Changing ratios of phototransformable protophylochlorophyll and protochlorophyllide of bean seedlings developing in dark. *Plant Physiology* **57**, 369–374.

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newberg LA. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* **1**, 174–181.

Liu XG, Xu H, Zhang JY, Liang GW, Liu YT, Guo AG. 2012. Effect of low temperature on chlorophyll metabolism in albinism line of wheat (*Triticum aestivum*) FAS5. *Physiologia Plantarum* **145**, 384–394.

Masuda T, Fujiyama Y. 2008. Regulation and evolution of chlorophyll metabolism. *Photochemical and Photobiological Sciences* **7**, 1131.

Matsumoto F, Obayashi T, Sasaki-Sekimoto Y, Ohta H, Takamiya KI, Masuda T. 2004. Gene expression profiling of the tetrapyrrole metabolic pathway in Arabidopsis with a mini-array system. *Plant Physiology* **135**, 2379–2391.

Meskauskiene R. 2001. FLU: a negative regulator of chlorophyll biosynthesis in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences, USA* **98**, 12826–12831.

Mochizuki N, Tanaka R, Grimm B, Masuda T, Moulin M, Smith AG, Tanaka A, Terry MJ. 2010. The cell biology of tetrapyrroles: a life and death struggle. *Trends in Plant Science* **15**, 488–498.

Montané MH, Dreyer S, Triantaphylides C, Kloppstech K. 1997. Early light-inducible proteins during long-term acclimation of barley to photooxidative stress caused by light and cold: high level of accumulation by posttranscriptional regulation. *Planta* **202**, 293–302.

Nie GY, Robertson EJ, Fryer MJ, Leech RM, Baker NR. 1995. Response of the photosynthetic apparatus in maize leaves grown at low temperature on transfer to normal growth temperature. *Plant, Cell and Environment* **18**, 1–12.

op den Camp RGL, Przybyla D, et al. 2003. Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. *The Plant Cell* **15**, 2320–2332.

Rassadina V, Domanskii V, Averina NG, Schoch S, Rudiger W. 2004. Correlation between chlorophyllide esterification, Shibata shift and regeneration of protochlorophyllide 650 in flash-irradiated etiolated barley leaves. *Physiologia Plantarum* **121**, 556–567.

Ramakers C, Ruijter JM, Deprez RH, Moorman AF. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters* **339**, 62–66.

Shimosaka E, Sasanuma T, Handa H. 1999. A wheat cold-regulated cDNA encoding and early light-inducible protein (ELIP): its structure, expression and chromosomal location. *Plant and Cell Physiology* **40**, 319–325.

Sims DA, Gamon JA. 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment* **81**, 337–354.

Szymkowiak P, Rudzinska-Langwald A, Adamczyk J, Kubica I, Fronk J. 2005. Recovery of maize seedling growth, development and photosynthetic efficiency after initial growth at low temperature. *Journal of Plant Physiology* **162**, 67–80.

Stobart AK, Henry GAF. 1984. The turnover of chlorophyll in greening wheat leaves. *Phytochemistry* **23**, 27–30.

Sveshnikova N, Soll J, Schleiff E. 2000. ToC34 is a preprotein receptor regulated by GTP and phosphorylation. *Proceedings of the National Academy of Sciences, USA* **97**, 4973–4978.

Tanaka R, Tanaka A. 2007. Tetrapyrrole biosynthesis in higher plants. *Annual Review of Plant Biology* **58**, 321–346.

Tewari AK, Tripathy BC. 1998. Temperature-stress-induced impairment of chlorophyll biosynthetic reactions in cucumber and wheat. *Plant Physiology* **117**, 851–858.

Tzvetkova-Chevilleau T, Franck F, Alawady AE, Dall’Osto L, Carriere F, Bassi R, Grimm B, Nussaume L, Havaux M. 2007. The light stress-induced protein ELIP2 is a regulator of chlorophyll synthesis in Arabidopsis thaliana. *The Plant Journal* **50**, 795–809.

Utz HF, Melchinger AE. 1996. PLABQTL: a program for composite interval mapping of QTL. *Journal of Agricultural Genomics* **2**, 1–5.

Zapata M, Rodriguez F, Garrido JL. 2000. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. *Marine Ecology Progress Series* **185**, 29–45.

Zolla L, Rinalducci S, Maria Timperio A, Huber CG. 2002. Proteinomics of light-harvesting proteins in different plant species. Analysis and comparison by liquid chromatography-electrospray ionization mass spectrometry. *Photosystem I* *Plant Physiology* **130**, 1938–1950.