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EXPRESSION OF A SESAME GERANYLGERANYL REDUCTASE cDNA IS INDUCED BY LIGHT BUT REPRESSED BY ABSCISIC ACID AND ETHYLENE

MYOUNG RYOUL PARK[1], EUN AE CHO[2], SHAFIQ REHMAN[3] AND SONG JOONG YUN[1,2*]

[1]*Institute of Agricultural Science and Technology, Chonbuk National University, Jeonju, 561-756, South Korea*
[2]*Department of Crop Science, Chonbuk National University, Jeonju, 561-756, South Korea*
[3]*Department of Botany, Kohat University of Science & Technology (KUST), Kohat 26000, Pakistan*
[*]*Corresponding author: E-mail: sjyun@chonbuk.ac.kr; Fax: +82-63-270-2640*

Abstract

Geranylgeranyl reductase (CHL P) is localized in plastids and catalyzes the reduction of geranylgeranyl diphosphate to phytyl diphosphate, a molecule required for both tocopherol and chlorophyll biosyntheses. To understand the regulation of *Chl P* expression in sesame, a *Chl P* cDNA (*SiChlP*) was isolated and the effects of light and phytohormones on *SiChl P* expression was investigated. The putative *SiChl P* cDNA was 1,633 bp and included a 1,394 bp open reading frame encoding a polypeptide of 465 amino acid residues. *SiChl P* mRNA was expressed most abundantly in leaves and cotyledons, moderately in developing capsules and seeds, but little in flowers, hypocotyls and roots of healthy seedlings and plants. Expression of *SiChl P* in a cotyledon was induced by light, but the level of induction was higher in red light than in far-red or blue light. However, *SiChl P* expression was diminished by dark, ethylene and abscisic acid.

Introduction

Diverse groups of prenyl lipids including chlorophylls and tocopherols are synthesized in the plastids of plant cells. Prenylation of these compounds with the C_{20}-intermediate geranylgeranyl diphosphate (GGPP) is required in order to form their hydrophobic tails for integration into plastid membranes. GGPP, an isoprenoid present in plastid, is synthesized from four molecules of isopentenyl pyrophosphate, which originate in the cytosol (Liu *et al*., 2005). In tocopherol synthesis in chloroplasts, phytyl diphosphate is transferred to homogentisic acid to form the first tocopherol intermediate (Hirschberg, 1999). The phytyl diphosphate is formed through stepwise reduction of GGPP before condensation with homogentisic acid. In chlorophyll synthesis in etioplasts, however, prenylation of chlorophyllide with GGPP occurs first and is followed by the reduction of GGPP to form phytyl chlorophyll (Rüdiger *et al*., 1980; Soll *et al*., 1983). In either case, reduction of GGPP to phytyl diphosphate is catalyzed by GGPP reductase (CHL P) (Tanaka *et al*., 1999).

Tocopherols are lipid soluble antioxidants known collectively as vitamin E. Four isoforms of tocopherols, α-, β-, γ- and δ-tocopherol, are synthesized in plants and other photosynthetic organisms. The isoforms differ by the numbers and positions of methyl substituents on the aromatic rings of the molecules (Hess, 1993). The major tocopherols found in human diets are γ- and α-tocopherol (Sheppard & Pennington *et al*., 1993). Natural sources of α-tocopherol (*R,R,R*-α-tocopherol) are the most bio-potent form of

vitamin E (Machilin, 1991). Clinical and epidemiological evidence suggest that vitamin E decreases the risk of cardiovascular disease and cancer, strengthens the immune system, and prevents or slows various chronic degenerative diseases and aging (Pryor, 2000; Venkateswaran *et al*., 2002). Sesame oil contains about 528 µg/g of total tocopherols (Cooney *et al*., 2001). Thus, consumption of tocopherol through sesame seeds and oils could provide health promoting effects (Cooney *et al*., 2001).

Seed oils of oilseed crops such as sesame and soybean are important sources of vitamin E in the human diet. Due to the beneficial effects of vitamin E, tocopherol content in seeds is becoming one of the major traits for improvement through conventional breeding and metabolic engineering (Shintani & DellaPenna, 1998). The immediate tocopherol intermediate is formed through a condensation of homogentisic acid (HGA) and a phytol sidechain. Therefore, to increase total tocopherol content in crops through genetic manipulation, genes for the key enzymes in the tocopherol biosynthesis pathway such as p-hydroxyphenyl pyruvate dioxygenase (HPPDase), CHL P, and phytyl/prenyl transferase should be identified and characterized. Recent cloning of tocopherol phytyltransferases in *Synechocystis* and *Arabidopsis* will promote cloning of the homologues in other species (Schledz, 2001; Savidge *et al*., 2002).

CHL P has been investigated in detail in a few plant species such as tobacco (Tanaka *et al*., 1999) and *Arabidopsis* (Keller *et al*., 1988). Soybean, ice plant and several photosynthetic bacterial *Chl P* sequences have also been identified. A few studies in tobacco and peaches have reported that expression of *Chl P* was induced by light (Tanaka *et al*., 1999; Giannino *et al*., 1988). However, there has been little research conducted on the effects of light quality and phytohormones on *Chl P* expression. Thus, this study was conducted to characterize the effects of light and phytohormones on the expression of *Chl P* from sesame.

Materials and Methods

Bacterial strains and plant materials: *Escherichia coli* strains XL1 Blue (Stratagene, USA), JM109 (Promega, USA), and Qiagen EZ (Qiagen, USA) were used for cloning according to standard techniques (Sambrook & Russell, 2001) homologues in other species (Schledz, 2001; Savidge *et al*., 2002).

Sesame (*Sesamum indicum* L. cv. Dasak) and soybean (*Glycine max* (L.) Merrill. cv. Iksan) plants were grown in a greenhouse and in the experimental field at Chonbuk National University, Jeonju, Korea. Leaves and flowers of sesame were collected from healthy plants grown in the field. Developing capsules were collected 14 and 28 days after flowering (DAF), and developing seeds were separated from the capsules. Sesame seeds were germinated for seven days, and the seedlings were dissected into cotyledons, hypocotyls and roots. Soybean leaves were collected from healthy plants grown in a greenhouse. All the samples were ground into fine powder in liquid nitrogen and kept at -70°C until use.

Cloning and DNA sequence analysis: A soybean *Chl P* probe was generated by amplification using bioinformatics on the known soybean *Chl P* sequence. The sequence of soybean *Chl P* was obtained from the GenBank database (NIH, USA), and forward (5'-GGCTCGACATGAACTCCATAGCC-3') and reverse (5'-TAAGATCTTCATACGT TAAGTTTGTTCATCATCTCCC-3') primers targeted to the 5' and 3' ends of the ORF were synthesized. A soybean *Chl P* sequence was amplified by polymerase chain reaction (PCR) with the above primer pair and soybean genomic DNA in a 25 µl reaction mixture consisting of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM primer, 200

μM dNTP, 1 unit *Taq* DNA polymerase (TaKaRa, Japan), and 10 ng template DNA. Amplifications were performed in a thermal cycler (Hybaid, UK) with an initial denaturation at 95ºC for five min, 35 cycles of 50 sec at 95ºC, one min at 50ºC, one min at 72ºC, and a final extension at 72ºC for five min. PCR products were separated by electrophoresis on 1.5% w/v agarose gels in 1 × TAE buffer (Sambrook & Russell, 2001). The specifically amplified 1.5 kb DNA fragment was cloned into pGEM-T Easy vector (Promega, USA). The nucleotide sequence of the resulting clone, p*GmChl P*, was determined as described (Sambrook & Russell, 2001) and confirmed by comparing against the sequence deposited in GenBank (NCBI, USA). The sequence of p*GmChl P* showed 99% identity to that of soybean *Chl P* (Accession no. AAD28640) and the clone was used as a probe for screening a sesame cDNA library. A cDNA library of sesame seed was constructed and screened in order to isolate a *Chl P* clone. About 2×10^5 *pfu* of the cDNA library was plated onto NZY agar plates and the plaques were transferred to nylon membranes (Hybond-N^+, Amersham, UK). The DNA on the membrane was fixed using a UV crosslinker (GS Gene Linker, BioRad, USA). The membrane was used for hybridization with the *GmChl P* probe. Labeling, hybridization, washing and detection of sesame *Chl P* cDNA were conducted with AlkPhos Direct system (Amersham Pharmacia Biotech, UK) according to the manufacturer's recommendations. From the screening, one secondary positive clone was isolated and designated *SiChl P*. The insert DNA of *SiChl P* was subcloned into pBluescript SK (+), and the nucleotide sequences of both strands were determined by the dideoxy chain termination method (Sanger *et al*., 1977). Nucleotide and deduced amino acid sequence analyses were performed using DNASIS (HITACHI, USA) and the programs and databases offered by the National Center for Biotechnology Information (NCBI, USA) and European Bioinformatics Institute (EBI, UK). Multiple sequence analysis was performed using the program AliBee (GeneBee, SU).

Light and phytohormone treatments: Sesame seeds were germinated either in the light or in the dark for one week. Phytohormone treatments were performed with light-germinated seedlings. One-week-old light-germinated seedlings were separated into the cotyledon and root and then they were immersed in sterile water, 100 μM abscisic acid (ABA) or 0.5 mM ethylene in the dark for 24 h at 25C^o. Light treatment was conducted using dark-germinated seedlings. The dissected cotyledons of one-week-old dark-germinated seedlings were immersed in sterile water and kept in the dark or treated with white light, red light (50μmol/m^2/s), far-red light (50μmol/m^2/s), or blue light (50μmol/m^2/s) for 24 h at 25^{o}C.

Southern and Northern blot analysis: Southern blot analysis was carried out with genomic DNA as described (Sambrook & Russell, 2001). Genomic DNA digested to completion with restriction enzymes (*Bgl*II, *Nde*I, or *Bgl*II + *Nde*I) was separated by agarose gel electrophoresis and transferred onto a nylon membrane (Hybond-N^+, Amersham, UK). The membrane was used for hybridization with the *SiChl P* probe. Labeling, hybridization, washing and detection of signals were conducted with the AlkPhos Direct system (Amersham Pharmacia Biotech, UK) according to the manufacturer's recommendations. Total RNA was extracted using the TRI reagent procedure (MRC, USA). For Northern blot analysis, total RNA (20 μg) was denatured, separated on a 1.2% formaldehyde gel, and transferred onto a nylon membrane (Hybond-N^+, Amersham, UK). The membrane was hybridized with the *SiChl P* probe labeled with alkaline phosphatase, and the signal was detected as for Southern blots.

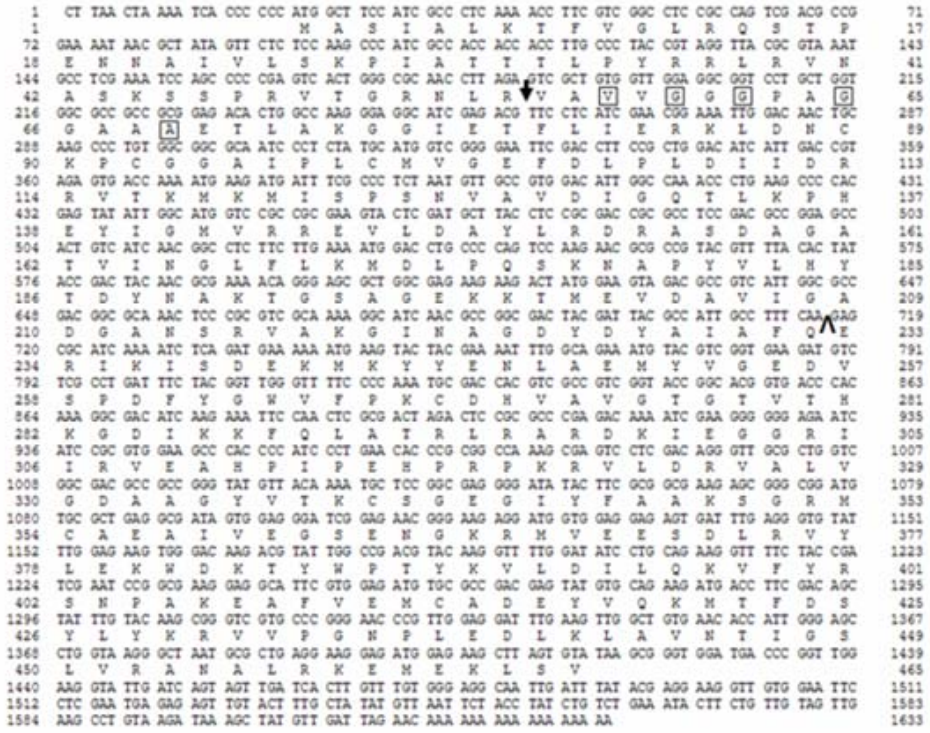

Fig. 1. Nucleotide and deduced amino acid sequences of the sesame *Chl P* cDNA clone, *SiChl P* (GenBank Accession Number GQ860303). The arrow indicates the predicted protease cleavage site of the predicted signal peptide. The boxed residues are in a consensus sequence of the V/IXGX$_{1-2}$GXXGXXXG/A motif that stabilizes binding of a nucleotide cofactor, FAD or NAD(P).

Results

Structural characteristics of SiChl P cDNA: The 1,633-bp nucleotide and deduced amino acid sequences of *SiChl P* (NCBI GenBank Accession Number GQ860303) are shown in Fig. 1. The nucleotide sequence of *SiChl P* includes a 1,394 bp ORF and untranslated 5' and 3' flanking sequences, including a poly (A) tail. The ORF encoded a 465-amino acid polypeptide with a predicted molecular mass of 51,379 Da and a theoretical pI of 9.01. In the signal peptide sequence, there were six residues each of serine and threonine, constituting 22% of the signal peptide residues, but there was only one glutamate and no aspartate (Fig. 1). The predicted molecular mass of the transit peptide was 6,036 Da. The resulting mature polypeptide consisted of 405 amino acid residues with a calculated molecular mass of 45 kDa.

The deduced 537-amino acid sequence of SiCHL P showed 91, 88, and 84% identities to tobacco (NtCHL P, CAA07683), soybean (GmCHL P, AAD28640) and Arabidopsis (AtCHL P, CAA74372), respectively. However, the SiCHL P sequence showed less than 65% identity to bacterial CHL P sequences. Among the bacterial CHL P polypeptides, the Synechocystis CHL P shared the highest identity, 65%, with SiCHL P (Fig. 2). A striking difference between plant and bacterial CHL Ps is the lack of a transit peptide sequence at the amino terminus in bacterial CHL Ps (Fig. 2A). The V/IXGX$_{1-2}$GXXGXXXG/A motif that binds NAD(P) or FAD is conserved in both plant and

bacterial CHL Ps. A phylogenetic tree based on multiple comparisons of CHL P polypeptide sequences clusters SiCHL P with CHL P of tobacco (Fig. 2B).

Southern blot was probed to estimate the number of sequences homologous to *SiChl P* cDNA present in the sesame genome. The blot was washed at high stringency and showed the presence of one or two major and minor bands (Fig. 3).

Tissue-specific expression of SiChl P: Expression of *SiChl P* was investigated in the different tissues of the sesame plant and germinating seedlings. A 1.6-kb *SiChl P* mRNA was detected most abundantly in leaves and cotyledons, moderately in developing capsules and seeds, but little in flowers, hypocotyls or roots of healthy plants and seedlings (Fig. 4).

Effect of light and phytohormone on SiChl expression: *SiChl P* mRNAs in the cotyledon were induced by light but repressed by dark. Red light was more effective in the induction of *SiChl P* expression than was blue or far-red light (Fig. 5A). Induction of *SiChl P* mRNA in the cotyledon by light was abolished by ABA and ethylene. However, *SiChl P* mRNAs in the root were not induced by light, ABA or ethylene (Fig. 5B).

Discussion

CHL P catalyzes the reduction of GGPP to phytyl diphosphate in the chloroplast. As plant CHL Ps are localized in chloroplasts, the precursor protein should contain a targeting sequence at the N-terminal region for proper localization of chloroplast proteins synthesized in the cytoplasm (Keegstra & Cline, 1999). The N-terminal sequence of the deduced SiChl P polypeptide contains a sequence characteristic of transit peptides found in precursor proteins targeted to the chloroplast. The neural network program ChloroP (Emanuelsson *et al*., 1999) predicts that the transit peptide consists of 55 amino acids with the cleavage site located between A55 and A56 in the sequence NLR $\rightarrow$ VAV. Despite their common function, transit peptide domains from various precursor proteins share minimal sequence identity, so no consensus sequences have been established. Similar to most chloroplast transit peptides, the transit peptide predicted from *SiChl P* is rich in serine and threonine but deficient in acidic amino acids (Keegstra *et al*., 1989).

Geranylgeranyl reductase belongs to the family of oxidoreductases. Oxidoreductases contain a nucleotide cofactor-binding domain stabilizing the interaction of the β-strand and α-helix connected by a short loop in which the ligand binding domain is located (Kleiger & Eisenberg, 2002). A typical motif commonly found in oxidoreductases, $V/IXGX_{1-2}GXXGXXXG/A$, is also present in the N-terminus of the mature SiCHL P polypeptide.

Chl P sequences have been identified from limited plant species and a few photosynthetic bacterial species (Tanaka *et al*., 1999; Keller *et al*., 1988; Addlesee *et al*., 1996). Comparisons of the nucleotide and deduced amino acid sequences of SiCHL P with the *Chl P* sequences in the database revealed a high degree of similarity to the plant *Chl P*s. A phylogenetic tree based on the multiple comparisons of CHL P polypeptide sequences clusters SiCHL P with CHL P of tobacco, indicating that the closest evolutionary relationship to sesame CHL P is tobacco CHL P. The structural features revealed from the sequence analysis suggest putative identity of the clone as *SiChl P*. In contrast to the bacterial CHL Ps, the SiCHL P contains an amino-terminal extension that resembles a plastid transit peptide sequence, indicating the possible plastid localization of the enzyme.

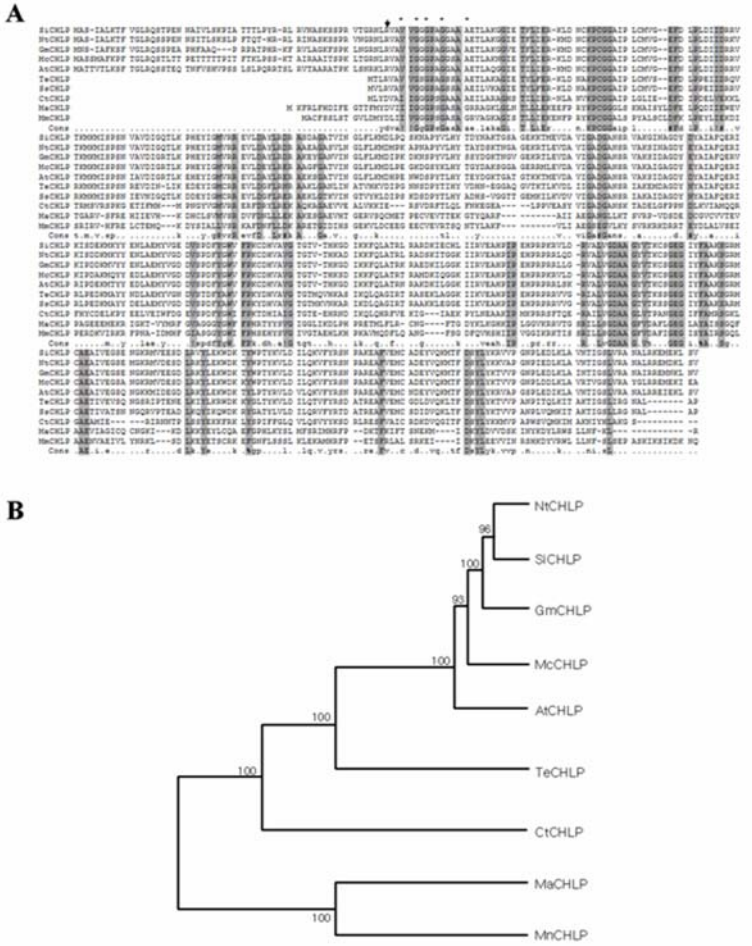

Fig. 2. A. Sequence alignment of geranylgeranyl reductases (CHL Ps). Alignment is shown for the deduced amino sequences of *Nicotiana tabacum* (NtCHLP, CAAO7683), *Glycine max* (GmCHLP, AAD28640), *Arabidopsis thaliana* (AtCHLP, CAA74372), *Mesembryanthemum crystallium* (McCHLP, T12299), *Thermosynechococcus elongates* BP-1 (TeCHLP, NP_680941), *Chlorobium tepidum* TLS (CtCHLP, NP_663129), *Methanosarcina acetivorans* C2A (MaCHLP, NP_616418), *Methanosarcina mazei Goe1* (MmCHLP, NP_634523), *Synechocystis sp* (SsCHLP, X97972). The arrow indicates the predicted protease cleavage site of the predicted signal peptide. Asterisks indicate the consensus sequence for the V/IXGX$_{1\text{-}2}$GXXGXXXG/A motif. Amino acid residues completely or strongly conserved among the sequences are indicated with uppercase letters or lowercase letters, respectively. The completely conserved residues are in gray. B. Phylogenetic relationship among geranylgeranyl reductases (CHL Ps). Amino acid sequences were aligned and the parsimonious tree was constructed using the Phylip program of GeneBee. Bootstrap support values are indicated above the branch lines. Abbreviations for the sequences are *Nicotiana tabacum* (NtCHLP, CAAO7683), *Glycine max* (GmCHLP, AAD28640), *Arabidopsis thaliana* (AtCHLP, CAA74372), *Mesembryanthemum crystallium* (McCHLP, T12299), *Thermosynechococcus elongates* BP-1 (TeCHLP, NP_680941), *Chlorobium tepidum* TLS (CtCHLP, NP_663129), *Methanosarcina acetivorans* C2A (MaCHLP, NP_616418), *Methanosarcina mazei Goe1* (MmCHLP, NP_634523), and *Synechocystis sp* (SsCHLP, X97972).

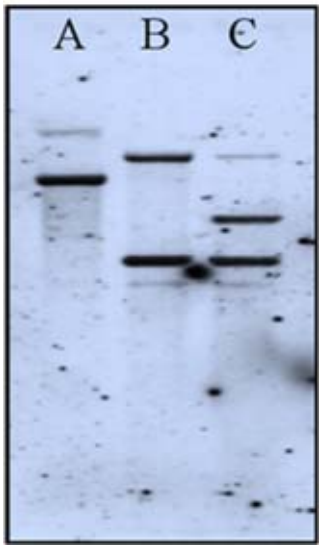

Fig. 3. Southern blot analysis of sesame genomic DNA digested with restriction enzyme *Bgl*II (A), *Nde*I (B), or *Bgl*II and *Nde*I (C). Ten micrograms of sesame genomic DNA digested with the restriction enzymes was separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane, then hybridized with alkaline phosphatase-labeled *SiChl P* insert DNA.

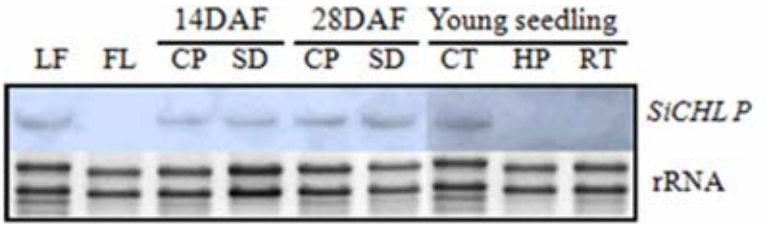

Fig. 4. Expression of *SiChl P* mRNA in the tissues of sesame plant. Leaf (LF), flower (FL), capsule (CP) and seed (SD) from 14 and 28 days after flowering (DAF). Cotyledon (CT), hypocotyl (HP), and root (RT) of seven-day-old seedlings. Twenty micrograms of total RNA was resolved in a 1.0% (w/v) agarose/formaldehyde gel, transferred to a nylon membrane, then hybridized with alkaline phosphatase-labeled *SiChl P* insert DNA. Ethidium bromide-stained rRNA bands as an indicator of equal loading (rRNA).

Because cultivated sesame is a cytogenetic diploid, the Southern blot result indicated that *SiChl P* is most probably present as a single gene with a remote sequence sharing low homology. Appearance of the one major band by *Bgl*II but two by *Nde*I digestion may indicate the presence of intron(s) containing *Nde*I-recognition sequences.

The expression patterns of *SiChl P* are in good agreement with the localization and roles of the CHL P enzyme in plants. The CHL P enzyme is localized in plastids and catalyzes the reduction of geranylgeranyl diphosphate to phytyl diphosphate, a molecule required for both tocopherol and chlorophyll biosyntheses (Tanaka *et al*., 1999; Grasses *et al*., 2001). Thus, higher expressions in developing seeds and green tissues supports similar

roles for CHL P in sesame. *Chl P* mRNA is expressed at higher levels during de-etiolation in *Arabidopsis* and during differentiation of chromoplasts in pepper (Keller *et al*., 1988).

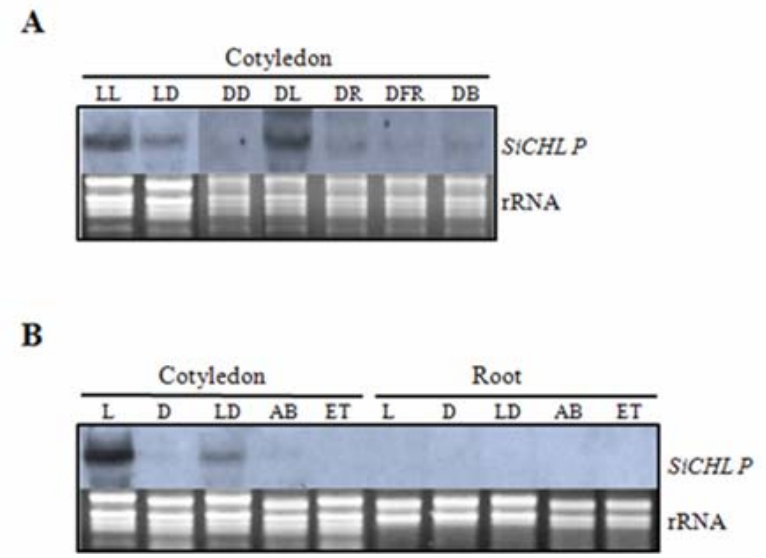

Fig. 5. Effects of light (A) and phytohormones (B) on *SiChl P* expression. A. Cotyledons dissected from one-week-old dark-germinated seedlings were immersed in sterile water and kept in the dark (DD) or treated with white light (DL) at 50 μmol/m^2/s, red light (DR) at 50 μmol/m^2/s, far-red light (DFR) at 50 μmol/m^2/s, or blue light (DB) at 50 μmol/m^2/s for 24 h at 25℃, respectively. Cotyledons from one-week-old light-germinated seedlings which were immersed in sterile water and kept in white light (LL) or transferred to the dark for 24 h (LD) are also presented for comparison. B. For phytohormone treatments, the cotyledons and roots of one-week-old light-germinated seedlings were used. The cotyledons and roots from light-geminated seedlings were immersed in sterile water (LD), 100 μM abscisic acid (AB) or 0.5 mM ethylene (ET) in the dark for 24 h at 25℃, respectively. The cotyledons and roots of one-week-old seedlings germinated in the light (L) or dark (D) before phytohormone treatments are also presented for comparison. Twenty micrograms of total RNA was resolved in a 1.0% (w/v) agarose/formaldehyde gel, transferred to a nylon membrane, then hybridized with alkaline phosphatase-labeled *SiChl P* insert DNA. Ethidium bromide-stained rRNA bands as an indicator of equal loading (rRNA).

Expression of *SiChl P* was stimulated by light in cotyledons, but the expression was repressed by dark. Although *SiChl P* was induced by red, far-red and blue light, red light was most effective in the induction of *SiChl P* expression. Light signals adjust plant growth and development in the prevailing environmental conditions (Casal *et al*., 2004). *Chl P* mRNA of peaches (Giannino *et al*., 1988) and tobacco (Tanaka *et al*., 1999) are induced by light. Plants tend to adapt the structure of their photosynthetic apparatus and pigment composition to light quality and quantity (Buschmann *et al*., 1978). Although regulation of development by light is mediated by photoreceptors in higher plants, different wavelengths of light activate different photoreceptors (Seo *et al*., 2004). *Arabidopsis* has five phytochromes (phyA through phyE), the photoreceptors that perceive red and far-red light (Quail *et al*., 1995). All members of the phytochrome family and chlorophylls are regulated by red light, which rapidly establishes a high

proportion of phytochrome active forms (Seo *et al*., 2004). Far-red light activates the high-irradiance response mode, which requires specific domains of the phyA molecule of *Arabidopsis* phytochromes (Yanovsky & Kay, 2002). Also, through studies of phytochrome A-deficient (*phyA*) mutants, it is known that *Arabidopsis Chl P* expression is up-regulated by phyA (Kuno *et al*., 2000). Blue light activates cryptochromes (Cashmore *et al*., 1999; Lin & Shalitin, 2003) and phototropins (Kasahara *et al*., 2002), which are specific blue light photoreceptors. Thus, it is probable that induction of *SiChl P* expression by light is mediated by the phytochrome family. However, convincing evidence for this assumption should be obtained from further investigation on the role of phytochromes in *SiChl P* expression in sesame.

Ethylene is required in the transduction pathway activated during injury. Ethylene plays an important role in mediating the wound responses of tomato plants (O'Donnell *et al*., 1996). In the ABA-dependent pathway, ABA is produced upon drought and cold stress with subsequent induction of expressions of various subsets of downstream genes (Uno *et al*., 2000; Yoshida *et al*., 2002). In peach leaves, *PpChl P* expression is diminished by cold stress and wounding (Giannino *et al*., 1988). The expression of *SiChl P* was not induced by ethylene or ABA treatment in cotyledons and roots. Therefore, this result indicates that *SiChl P* is negatively regulated by ethylene, which plays an important role in mediating the wound response, and a subset of genes regulated by ABA in an ABA-dependent pathway.

Taken together, the expression of *SiChl P*, which is most highly expressed in the leaves and developing seeds, is induced by light but repressed by dark, ABA and ethylene. This information can be used for further studies on the regulation of chlorophyll and tocopherol syntheses by light and phytohormones in sesame.

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