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Identification of the *Ipomoea nil* cDNA clone encoding protein with one transmembrane domain by differential display PCR

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Abstract. A modified differential display procedure was used to compare *Ipomoea nil* gene expression under flower inductive and non-inductive light/dark conditions and a 170 bp cDNA fragment was displayed. Screening of the cDNA library from *I. nil* cotyledons led to the isolation of a 577 bp cDNA clone with high nucleotide and amino acids homology to One Helix Protein (OHP) genes from *Arabidopsis thaliana* and *Deschampsia antartica.* The membrane spanning helix of *In*OHP is 91% identical to the MSH of the *A. thaliana* OHP. MSH is located in the C-terminal part of *In*OHP protein and corresponds to third MSH of LHC proteins. *In*OHP transcripts are numerous in leaves of plants when grown under continuous light and are also present in grown under flower inductive condition but their quantity is lower. Southern analysis showed that *In*OHP is member of a gene family involved in photoprotection of photosystems against excessive light.

Keywords: chlorophyll a/b, binding protein (CAB), One Helix Protein (OHP), *Pharbitis nil,* flower induction, light stress, differential display PCR.

1. Introduction

Light is essential for normal plant growth and development not only as a source of energy but also as an environmental signal that regulates various developmental and metabolic processes. The light-regulated responses occur throughout the entire life cycle of the plant, including seed germination, seedling de-etiolation, leaf and chloroplast development, flowering and eventually senescence. It is well established that the leaf is the principal site of photoperiodic perception and that phytochrome is the primary receptor involved in photoperiodic flowering responses. The perception and transduction of the light signals are governed by at least three families of photoreceptors, including the phytochromes (red and far-red light receptors), blue-light receptors and UV receptors (Chamovitz & Deng, 1996; Pham et al., 2018). It has been reported that certain light-inducible genes, especially nuclear-encoded photosynthetic ones, are controlled also by a circadian rhythm (Piechulla, 1993; Ravichandran & Thangavelu, 2017).

The most abundant membrane proteins in plastids are the light-harvesting chlorophyll (Chl) *a/b* binding proteins (CABs), associated with photosystem (PS) I and II, those associated with PSII are designated as light-harvesting CABs of PSII (LHCII). The LHC proteins are a highly conserved family of nuclear-encoded proteins that contain

three membrane-spanning helices (MSHs) and bind at least 12 chlorophyll and two xanthophyll molecules. The two xanthophylls are thought to be essential for the prevention of photodamage resulting from the generation of singlet oxygen species in the PSII reaction center. The crystal structure determination of LHCII shows that the first and third MSHs are held together by ion pairs formed by charged amino acid residues that also bind Chl*a* (Kühlbrandt et al., 1994; Hey & Grimm, 2018). Residues involved in Chl*a* binding are strictly conserved in all members of the CAB family and in the less related fucoxanthin Chl*a/c* binding protein from the chromophytic algae (Bhaya & Grossman, 1993). The several distant relatives of the LHC protein family with conserved chlorophyll-binding residues include the photosystem II subunit S (PSBS) protein and the subfamily of proteins called early light-induced proteins (ELIPs) (Adamska, 1997; Montané & Kloppstech, 2000). The ELIPs subfamily includes three-helix ELIPs, two-helix stress-enhanced proteins (SEPs) and one-helix proteins (OHPs) also called high-light-induced proteins (HLIPs) or small chlorophyll a/b-binding-like proteins (SCPs) in prokaryotic organisms (Adamska & Kloppstech, 1991, Adamska et al., 1992; Staleva et al., 2015; Shukla et al., 2018). Two full-length cDNAs encoding OHP proteins from the leaves of *Arabidopsis* have been previously characterized (Hey & Grimm, 2018). Natomiast u *Pharbitis* poznano jedynie sekwencje genu *In*OHP2 (Stawski et al., 2008).

A major goal of our research on the photoperiodic control of flowering is to identify genes involved in these process. Flowering requires the developmental transition of the shoot meristem from vegetative to reproductive growth. In photoperiodic species of flowering plants, this developmental transition is regulated by the absolute duration of light and darkness in a 24-hour cycle (Vince-Prue, 1989). The experimental system we have used is *I. nil,* Choisy strain Violet, synonym: *Pharbitis nil* Choisy (Japanese morning glory), a short day (SD) species that has been well characterised with regard to the physiology and biochemistry of photoperiodic floral induction. *I. nil* is an ideal model for the study of the early events in the photoperiodic induction of flowering, because young, light-grown seedlings can be induced to flower by exposure to a single dark period of 16 hours (Mierek-Adamska et al., 2019). Draft *I. nil* genome has been published in 2016 year (Hoshino et al., 2016). Studies on *Arabidopsis thaliana* have demonstrated the importance of molecules that are present at low levels in the photoperiodic induction of flowering (Putterill et al., 1995). Ono and others (Ono et al., 1988, 1993, 1996; Sage-Ono et al., 1998) also predicted the existence of *P. nil* rare transcripts that are related to flowering. Hayama et al. (2019) found *P. nil* genes regulated by light to – dark transition.

Therefore, to examine this issue we decided to exploit a method for the isolation of preferentially expressed mRNAs, namely, differential display by PCR (DD PCR) (Liang & Pardee, 1992; Dąbrowska, 2001). The aim of this study was to identify a gene which is differentially expressed during photoperiodic induction of flowering. The subject we cover is important in the aspect of solving many ecological problems.

2. Material and methods

Plant material. The seeds of *I. nil* were mechanically scarified and then left for 12 hours in distilled water at 30°C. Imbibed seeds were sown into a mixture of humid sand and vermiculite (1:2). Plants were grown in a climatic chamber for 6 days under continuous light at 26°C. After that period cotyledons from non-induced plants were collected. Other plants were grown for another 16 hours under dark conditions and the cotyledons collected. The harvested material was frozen in liquid nitrogen and stored at -80°C. Some spare non-induced and induced plants were left in a climatic chamber as negative and positive controls of the inductive treatment.

Isolation of RNA. Total RNA from cotyledons was isolated according to the method of Chomczyński (1993) in accordance with a protocol of TRI-Reagent (MRC, Cincinnati) and was quantified spectrophotometrically (GeneQuant) and by electrophoresis in 1.5% agarose with ethidium bromide.

Differential display reverse transcription polymerase chain reaction. To prevent false signals arising from DNA contamination, the 2 µg of total RNA was treated with 2U of RNase – free DNase I (Promega, USA) for 15 min at 25°C. The final concentration of magnesium was 1 mM. After DNase digestion, the aliquot was incubated at 65°C for 10 min and used directly in the reverse transcription reaction.

1 µg of total RNA was used for reverse transcription in the presence of 20 mM dNTPs, 10 mM dithiothreitol, 1x RT-buffer, 200 U of reverse transcriptase (MMLV RT; Promega, USA) and 1 mM $(dT)_{12}$ VN-Primer (5'-T_{12}AG-3', 5'-T_{12}CA-3', 5'-T_{12}AC-3', 5'-T_{12}GT-3') in 20 µl reaction volume.

The PCR reaction mixture was prepared using 1/10 volume of the reverse transcription assay, 1 x PCR-buffer, 2 mM dNTPs, 1 U *Taq* DNA polymerase (Promega, USA), 1 mM $(dT)_{12}$VN-Primer and 1 mM arbitrary primer in a 25 ml reaction volume. Arbitrary primer sequences used in amplification were: TOR 1 – (5'-TACAACGAGG-3'), TOR 2 – (5'-TCGATACAGG-3'), TOR 3 – (5'-CTGCTTGATG-3'), TOR 4 – (5'-GATCCAGATC-3'), TOR 5 – (5'-T_{12}MG-3'; M-A,C,G degenerated mixture). Amplification was done for 40 cycles at 94°C for 30 s, 42°C for 2 min, 72°C for 30 s and an additional extension period at 72°C for 5 min. Afterwards,

10 ml of each sample was separated on a native 6% (w/v) polyacrylamide gel.

Silver staining. For visualization of PCR products by silver staining, the polyacrylamide gel was fixed in 10% glacial acetic acid followed by three washes in deionized water. The gel was stained for 30 min. in a solution containing: 1 g of silver nitrate and 1.5 mL of 37% formaldehyde in 1 L of water. Thereafter, the gel was rinsed for 30 s in deionized water and treated for 5 – 15 min in 250 mL of ice the cold developing solution (2 L of water containing 60 g of anhydrous sodium carbonate, to which 3 mL of 37 % formaldehyde and 400 µl of 1 % sodium thiosulfate are added just before use). The reaction was stopped by incubating the gel for 10 min in 250 mL of 10 % acetic acid solution, followed by rinsing in double-deionized water.

Elution of DD-PCR product. The pieces of polyacrylamide corresponding to the PCR bands of interest were cut from the gel using a sterile razor blade. DNA was recovered by dialysis against 100 mL of 10 mM Tris-HCl pH 8.9, 0.1 mM EDTA for 4-16 hrs at 37°C. The eluate was filtered through glass wool packed at the end of a 200 µL pipette tip. DNA was precipitated with ethanol in the presence of 0.3 M sodium acetate for 1h at -20°C in a 1.5 mL microcentrifuge tube, followed by a 15-min centrifugation at 12000 rpm at 4°C. The DNA pellet was then briefly washed with 75% ethanol, dried and resuspend in 40 µL of 0.1 mM EDTA pH 8.0 and stored at -20°C.

Reamplification of the PCR product. The PCR product was reamplified according to the conditions described by Liang and Pardee (Liang & Pardee, 1992) in a final reaction volume of 100 µL.

cDNA library screening. The cDNA library constructed from poly $(A)^+$ RNA isolated from cotyledons harvested from dark-induced *I. nil* was screened by hybridisation using a [a-^{32}P] dCTP – labelled differential display product of 170 bp. For primary screening approximately 4 x 10^5 colonies were screened (Sambrook et al., 1989).

Southern analysis of cDNA clones. Candidate colonies from secondary screening were cultivated in LB medium. Plasmid DNA was isolated by alkaline lysis (Sambrook et al., 1989) and digested with *Eco*RI and *Xho*I. Digested DNA was electrophoresed on 1% agarose gel and transferred to a nylon membrane. The DNA on the membrane was hybridised with the ^{32}P-labelled differential display product of 170 bp in hybridisation solution [1% bovine serum albumin (BSA, fraction V, Sigma), 1 mM EDTA, 0.5 M phosphate buffer (pH 7.2) and 7% sodium dodecyl sulfate (SDS)]. The nylon membrane was washed twice for 5 min at 37°C with buffer containing 0.5% BSA, 1 mM EDTA, 40 mM phosphate buffer pH 7.2 and 5% SDS and then four times for 5 min with buffer containing 1 mM EDTA, 40 mM phosphate buffer pH 7.2 and 1% SDS (Church & Gilbert, 1984). The washed membranes were exposed to X-ray film for 2 days at -80°C.

Sequencing and DNA sequence analysis. Sequencing was commissioned to DNA II-Gdańsk Laboratory. The nucleotide sequence of *In*OHP cDNA was determined with fluorescent primers and an automated DNA sequences. DNA sequences were analysed and compared with the GenEMBL databases. The derived amino acid sequence was compared with the SWISS-PROT databases, using the FASTA program or the NCBI BLAST network service.

Southern analysis of *I. nil* genomic DNA. Total DNA was isolated from *P. nil* cotyledons using the method described by Doyle and Doyle (1987) method. Genomic DNA was treated with *Eco*RI, *Hind*III or *Pst*I enzymes. Digested DNA was electrophoresed on 1 % agarose gel, transferred to a nylon membrane and hybridised with a ^{32}P-labelled *In*OHP cDNA as described above.

Dot-blot hybridization. Dot-blot experiments were performed on Hybond – N^+ membranes (Amersham, Germany). Using a standard protocol (Sambrook et al., 1989) 20 µg of total RNA per dot were applied. For positive control 10 ng of DNA differential display product was applied. Nucleic acids were immobilised by backing at 80°C for 2 hours. The hybridisation with the ^{32}P-labelled differential display product of 170 bp was performed as previously described.

Northern analysis. Total RNA (20 mg) was electrophoresed on a formaldehyde-agarose gel and transferred to Hybond – N^+ membrane (Amersham, Germany). The RNA bound to the filter was allowed to hybridise with the ^{32}P-labelled *In*OHP cDNA in a hybridisation solution that contained 250 mM Na_2HPO_2, 1 mM EDTA, 7% SDS, 25 mM NaCl, 10% PEG 6000, 40% formamide, 3.75 mg/ml herring-sperm DNA at 42°C over night. The filter was washed with 2 x SSC and 0.5% SDS at 60°C for 15 min and then with 0.2 x SSC and 0.5% SDS at 60°C for 15 min. The washed membrane was exposed to X-ray film for 5 days at -80°C.

3. Results and discussion

Identification of a transcript whose level decreases during the inductive dark period

Differential display PCR reaction with TOR1 and anchored TOR5 primers led to the identification of a 170 bp differential product, which was present in plants non-induced and induced for flowering by 16 hrs of darkness. The difference was quantitative not qualitative. Dot blot northern analysis confirmed that the 170 bp product is a differential one (Fig. 1).

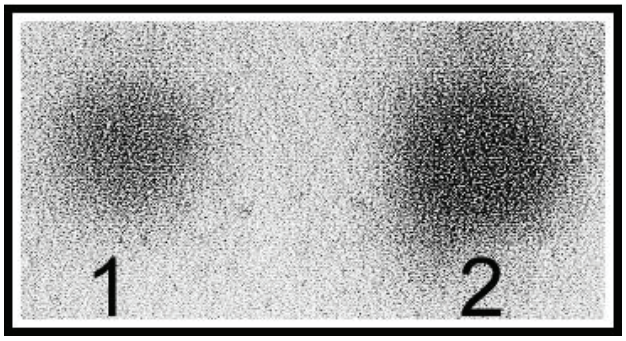

Figure 1. Dot blot northern analysis of *I. nil* seedlings induced (1) and non-induced (2) for flowering

Results indicate that the level of the hybridising transcript is lower in plants induced for flowering then in plants grown under continuous light.

Characteristics of the sequence of the cDNA clone

By screening a *P. nil* library constructed from poly $(A)^+$ RNA isolated from dark-induced cotyledons, we isolated few positive cDNA clones complementary to the 170 bp differential product. Southern analysis of cDNA clones led to the identification of only one clone with insert homologous to the DD PCR product.

The nucleotide sequence of this cDNA fragment shows high nucleotide (71% and 70%) and amino acid (81%) homology to OHP cDNAs from *A. thaliana* and *D. antarctica* respectively. The identified *I. nil* cDNA is henceforth referred to as *In*OHP (GenBank NCBI accession no. AY240874). The cDNA sequence of the *In*OHP consists of 577 bp, with an ORF of 116 amino acids (Fig. 2).

The amino acid sequence of *Arabidopsis thaliana* OHP has homology (51%) to *Synechocystis sp.* HLIP. The amino acid sequence of *In*OHP contains 10 from 13 amino acids, which form a HLIP characteristic motive (GFT--AE- -NGR-AMIGF), shown in blue (Fig. 3). The MSHs of shown peptides are underlined. Stars depict identical amino acids. The predicted cleavage site sequence of transit peptide is shown in red. Amino acids probably involved in pigment binding or helix-helix interaction are shown with green arrows.

There is no strict conservation of the transit peptide or the cleavage site sequence (VRAAK). The comparison amino acid sequences of the *In*OHP and the *A. thaliana* OHP suggests that the cleavage site could be positioned after the 47th residue and the mature *In*OHP protein is 69 amino acids long. The predicted molecular mass is approximately 7.48 kDa with a pI of 6.41. It seems that the protein contains one MSH (membrane-spanning helix) and that the N-terminus of the protein resides on the stromal side of the thylakoid membrane (Fig. 4).

The membrane spanning helix of *In*OHP is 91 % identical to the MSH of the *A. thaliana* OHP (Fig. 4). MSHs are located in the C-terminal part of OHP proteins and correspond to third MSH of LHC proteins.

Southern analysis of genome DNA

To determine whether *In*OHP represents a single locus in the *I. nil* genome or a multicopy gene, genomic DNA gel-blot hybridisation analysis was performed using the

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aattaaccctcactaaagggaacaaaagctggagctccaccgcggtggcggccgctctaga

actagtggatccccgggctgcaggaattcggcacgaggacagaaatggcatcaccactc
                                                M  A  S  P  L
tcatcgtctttcctctcactctcaacaacacatacaccccaacacaatcaactcttcttc
 S  S  S  F  L  S  L  S  T  T  H  T  P  Q  H  N  Q  L  F  F
ttcactaatcgcattcatacctccacaatttcccacaaaccaatctcctccttcaaactt
 F  T  N  R  I  H  T  S  T  I  S  H  K  P  I  S  S  F  K  L
caagctgcaaagcttcctgccggagtggaattgcccaaggagcagccaaaacttgagccc
 Q  A  A  K  L  P  A  G  V  E  L  P  K  E  Q  P  K  L  E  P
ccatttctgggtttcaccagaactgctgaaatatggaattccagagcttgcatgattggt
 P  F  L  G  F  T  R  T  A  E  I  W  N  S  R  A  C  M  I  G
ctcattggaaccttcattgttgaactgatcttgaataggggaattcttcagcttattgga
 L  I  G  T  F  I  V  E  L  I  L  N  R  G  I  L  Q  L  I  G
gtggatgttgggaaaggccttgatcttcccctatgagtattggggtgtttttgccttgta
 V  D  V  G  K  G  L  D  L  P  L  -
acaccactggattncattcatgccatcacatatgtaattctgaaaacaagctaatattat
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Figure 2. Nucleotide and deduced amino acid sequence of the *In*OHP. The cDNA sequence of *In*OHP is 577 bp long. The ORF is 116 aa long

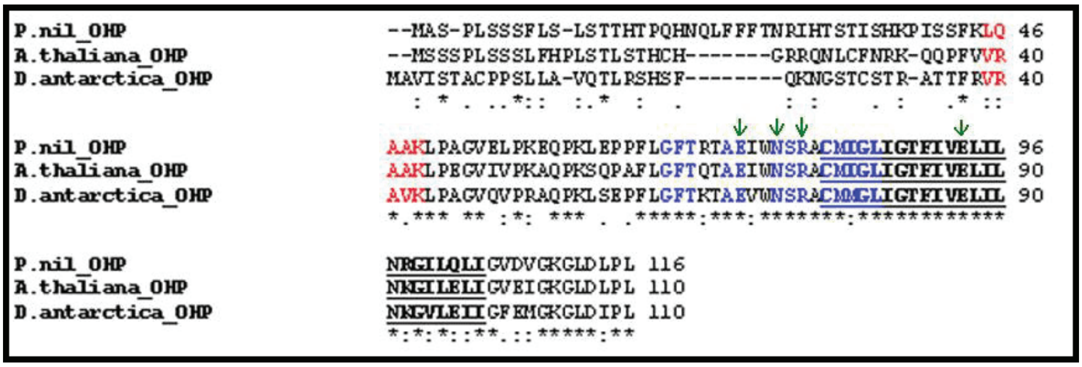

Figure 3. Comparison of amino acid sequences of the OHPs of *I. nil*, *A. thaliana* and *D. antartica*

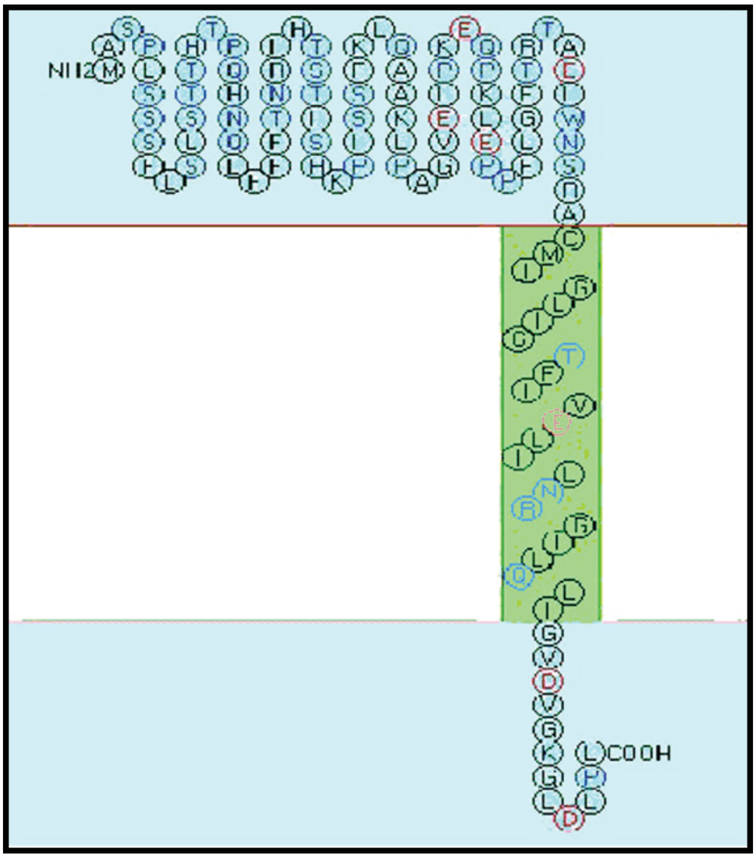

Figure 4. Schematic diagram of the *In*OHP peptide with a 23 amino acid long membrane helix (SOSUI program)

*In*OHP cDNA sequence as a probe. The results showed that the probe hybridised to at least three *Eco*RI restriction fragments (7400 bp, 5000 bp, 2400 bp), possibly meaning that *In*OHP is a member of a gene family (data not shown).

Regulation of the *In*OHP gene by light and darkness – northern analysis

The results of northern analysis showed that *In*OHP transcripts (600 bp) are numerous in leaves of plants when grown under continuous light. Transcripts are also present

in the leaves of plants when grown under flower inductive conditions but their quantity is lower (Fig. 5).

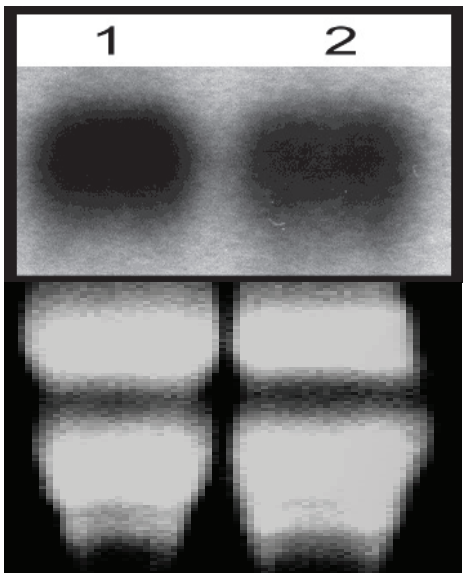

Figure 5. Northern analysis of mRNA levels of *In*OHP in continuous light (1) and darkness (2)

The light-regulated gene expression of plants is widely recognised (Piechulla, 1993). In most of the studies rather light then darkness is the external signal influencing the amplitude of cycling mRNA levels (Hayama et al., 2019). Indeed, darkness was observed to have a negative effect on the amplitude and transcriptional regulation of expression of *CAB* genes in tomato and maize (Taylor, 1989). The RNA population isolated from seedling of *I. nil* grown under continuous light and under flowering inductive conditions show qualitative and quantitative differences (Dąbrowska, 2001). The changes in expression of genes involved in control of *I. nil* flowering have quantitative character (Ono et al., 1996; O'Neil et al., 1994). Multiple genes are involved in the photoperiodic induction of flowering in *P. nil* (Ono et al., 1988; Sage-Ono et al., 1998).

Differential display PCR technique was used to compare *I. nil* RNA from cotyledons induced and non-induced for flowering. Exposure to a single dark period of 16 h induced flowering of *P. nil*. During inductive darkness a lot of genes are expressed and many of them are involved in other physiological processes.

The family one helix protein (OHP) in higher plants is light stress induced, nuclear-encoded, and related to light-harvesting chlorophyll a/b-binding (LHC) proteins. To learn more about possible function of *In*OHP we have isolated a cDNA fragment of 170 bp, and after screening of the cDNA library of *I. nil* cotyledons collected from plants induced to flowering by 16 h of darkness. We have found a *In*OHP cDNA clone of 577 bp. *In silico* analysis revealed that the predicted protein (116 amino acids) encoded by *In*OHP*1* shows homology to the LHC proteins. In previous studies Stawski et al. (2008) isolated cDNA encodes protein closely related to AtOHP2. *In*OHP2 has been synthesized as a 165-amino acid precursor protein containing an 76 amino acid long terminal transit peptide.

HLIP genes are located in the chloroplast genome but the *Arabidopsis thaliana* OHP is a nuclear-coded protein (Jansson et al., 2000). The *In*OHP sequence is not homologous to DNA from eukaryotic chloroplasts. It seems that *In*OHP is also a nuclear-coded protein, especially that the comparison of the amino acid sequences of *In*OHP and *A. thaliana* OHP showed that the N-terminal part of *In*OHP resembles a transit peptide directing the protein into the chloroplast.

Arabidopsis OHP proteins contain a single transmembrane domain, which encompasses an LHC motif (Hay & Grimm 2018). Jansson et al. (2000) shown that the third membrane-spanning helix of LHC proteins contains 31 amino acids and the *In*OHP*1* MSH is 23 amino acids long. C-terminus of *In*OHP2 also contains a single transmembrane α-helix (Stawski et al., 2008). Amino acids involved in chlorophyll binding are conserved and located in the first and third MSH of all LHC proteins and are also present in sequences of HLIP and *In*OHP. These amino acids in *In*OHP sequence are located at the same position like in LHC and HLIP sequences. The conserved amino acids are not located at the MSH of HLIP and OHP but near to a MSH at the N-terminus of the sequence of *In*OHP and the *A. thaliana* OHP (protein homologous to cyanobacterial high-light-inducible proteins).

A partially homologous motif to the motif present in the HLIP amino acid sequence involved in pigment binding is present in the *In*OHP. The last 4 amino acids from this motif (MIGF) are part of the *A. thaliana* OHP and *In*OHP MSH (Fig. 3). These all conclusions suggest that HLIPs and OHPs may be involved in pigment binding (Jansson et al., 2000). The first and third MSH in LHCb1 form an ion pairs between Glu and Arg residues (Montané & Kloppstech, 2000). These residues are conserved in proteins of HLIP and OHP families and are also present in HLIP characteristic motif (Fig. 3). It suggests that both OHP and HLIP may form dimers in thylakoid membranes (Jansson et al., 2000).

The shortest LHC members in *Arabidopsis* are two subclasses OHP1 and OHP2 (Engelken et al., 2012). Southern analysis shows that the *In*OHP*1* is a member of a gene family.

The genes encoding HLIPs, ELIPs and the *A. thaliana* OHP are known to be induced by strong light. The transcript of *A. thaliana OHP* was present in plants grown under low light conditions, but increased in abundance when plants were transferred to high light conditions (Jansson et al., 2000). Detailed expression analysis revealed that

OHP1 and OHP2 transcript levels are co-regulated during the diurnal light cycle, during de-etiolation and in response to excess light (Beck et al., 2017).

The results of northern analysis showed that *In*OHP transcripts were present in both plants, those grown under continuous light and those induced with 16 hours darkness. The difference was quantitative – *In*OHP expression is higher in plants cultivated under continuous light. Similarly, analysis of *In*OHP2 expression in *I. nil* cotyledons has been higher in light than in darkness (Stawski et al., 2008). CAB proteins seem to have two functions in photosystems. First of all they bind photosynthetic pigment like chlorophylls and carotenoids. They are also involved in photosystem protection against light stress especially ELIP family proteins. Latest research Hey & Grimm (2018) suggested that *Arabidopsis* OHPs play a substantial role in plastid development.

*In*OHP transcripts are present in plants grown under continuous light and under flower inductive darkness but the difference has quantitative character. The *In*OHP function is unknown. It seems that this protein may be involved in pigment binding processes or in responses related to stress. Nowadays there is no data showing relationship between light stress response and flower induction.

4. Conclusion

The family of One Helix Protein (OHP) in higher plants is nuclear-encoded, light stress induced, located in thylakoid membranes and related to light-harvesting chlorophyll a/b-binding (LHC) proteins. A photoprotective function was proposed for OHPs. Therefore, this subject is important from an ecological point of view and protection of plants against the negative effects of light.

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