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ORIGINAL PAPER

Do plant chloroplasts contain histidine kinases?

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Abstract Plastids are evolutionary descendants of cyanobacteria and retain many proteins of cyanobacterial origin including histidine kinases (HKs). Histidine kinases form a major group of protein kinases in cyanobacteria but a minor group in angiosperms; no HK has been detected in plant plastids so far. This raises the question: have higher plant plastids retained some cyanobacterial His/Asp regulatory systems or the latter have been replaced with Ser/Thr or Tyr protein kinases more typical for eukaryotes? To answer this question H1 antiserum against conservative phospho-acceptor motif of HKs was raised and applied for the analysis of different chloroplast fractions. In maize, three polypeptides with apparent molecular masses about 26, 27.5, and 28 kDa were revealed in thylakoid membranes. The results of in organello phosphorylation suggest that these proteins may be His-phosphorylated. Polypeptides with similar molecular masses were revealed in various mono- and dicotyledonous plants. Bioinformatic analysis demonstrated that in angiosperms these polypeptides might result from alternative transcription initiation and/or alternative processing of mRNAs of genes encoding well-known HKs. Besides, the genome of the moss *Physcomitrella patens* contains much more genes that could code for plastid HKs. The data obtained let us suppose that plastids of contemporary plants have HK(s) that could be involved in regulation of plastid gene expression.

Keywords Histidine kinase · Chloroplasts · Terrestrial plants · Evolution

Abbreviations

CDS	Coding DNA sequence
CSK	Chloroplast sensor kinase
HK	Histidine kinase
LHCII	Light-harvesting complex of PSII
PSI	Photosystem I
PSII	Photosystem II
RR	Response regulator
STK	Ser/Thr kinase

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Introduction

Plastids are evolutionary descendants of cyanobacteria (Mereschkowsky 1905; Dyall et al. 2004) and retain many proteins of cyanobacterial origin including those in photosynthetic complexes, gene expression machinery, and regulatory systems. Protein kinases are very important components of diverse regulatory pathways. Cyanobacteria and plants have two distinct groups of protein kinases: histidine kinases (HK) and serine/threonine kinases (STK). In cyanobacteria, HKs form a major group; e.g., the genome of *Synechocystis sp*. PCC 6803 contains 47 genes for His kinases (Ashby and Houmard 2006) and only 12 genes

for Ser/Thr kinases (Zhang et al. 2007; Zorina et al. 2011); some cyanobacteria have no genes for Ser/Thr kinases (Zhang et al. 2007). In contrast, higher plants have a large Ser/Thr kinase family and a much smaller HK family. The genome of *Arabidopsis thaliana* contains more than 800 genes for protein kinases and the genome of *Oryza sativa*—more than 1,300 (Krupa et al. 2006) but only eight of them encode genuine HKs (cytokinin receptors, ethylene receptors of subfamily 1, CKI-type kinases) (Hwang et al. 2002; Moussatche and Klee 2004; Pareek et al. 2006; Schaller et al. 2007).

A typical HK contains two major domains: catalytic (HATPase_c) and dimerization/phospho-acceptor (HisKA). Domain HATPase_c is characteristic of several types of ATP-binding proteins: DNA gyrase B, topoisomerase, heat shock protein 90, and others. Besides, HK may include signal sensor domain(s) and a receiver (REC) domain. In two-component systems, histidine kinase accepts a signal, autophosphorylates His in the dimerization/phospho-acceptor domain, transduces phoshoryl group to Asp of the receiver domain in a response regulator (RR), and the latter regulates transcription of target genes (Stock et al. 2000). Several plant proteins that had originated from HKs lost their His phospho-acceptor motif and now function as STKs (e.g., plant phytochromes, ethylene receptors of subfamily 2, CSK) (Yeh and Lagarias 1998; Moussatche and Klee 2004; Puthiyaveetil et al. 2008).

Plants accepted many genes from a cyanobacterial endosymbiont (Martin et al. 2002) and, perhaps, all plant HKs were acquired from cyanobacteria. At least, the best-studied plant HK-like proteins (phytochromes, ethylene and cytokinin receptors) have the closest homologs among cyanobacterial proteins (Yeh and Lagarias 1998; Mount and Chang 2002). However, it is more likely that all well-known HKs function outside contemporary plastids. All protein kinases identified in plastids so far are of Ser/Thr or Tyr type (Schliebner et al. 2008). This is not surprising as STKs are the prominent group of plant protein kinases. Still, the question remains: have higher plant plastids retained some cyanobacterial His/Asp regulatory systems or all of them have been replaced with Ser/Thr protein kinases more typical for eukaryotes?

Plastome is a part of plant genome that is compartmentalized into plastids and it is a relict of cyanobacterial genome. Plastome genes encode plastid proteins and non-coding RNAs. Genes encoding proteins homologous to HKs (*ycf26*, *dfr, tsg1*) and RRs (*ycf27*, *ycf29*) have been found in plastomes of many different algae; gene *ycf27* has been detected in plastome of *Chlorokybus atmophyticus* (Duplessis et al. 2007). *Chlorokybus atmophyticus* belongs to Streptophyta phylum that includes a number of green algae and all the land plants; therefore it is reasonable to suggest that a green algal progenitor of higher plants had a two-component regulatory system in its chloroplasts. However, such genes are absent in plastomes of terrestrial plants and their nearest relatives—chara algae (Lysenko, unpublished). In land plants these genes may have been lost or transferred into nucleus.

Several attempts have been made to investigate whether any nuclear genes code for plastid targeted histidine kinases and/or their response regulators. Applying the bioinformatic approach, plastid localization for some nuclear encoded HKs and RRs in arabidopsis and maize, including ZmRR1 (BAB20581), ZmRR2 (BAA85112), and ZmRR6 (BAA85113) was predicted (Forsberg et al. 2001). With the use of the GFP-fusion technique it was revealed that ZmRR1, ZmRR2, and ZmRR6 are targeted to other cell compartments (Asakura et al. 2003) but this experimental approach could be misleading too (Kodama and Sano 2006). In spinach chloroplasts, TCP34 protein was detected (Weber et al. 2006), and this protein contains RR domain with conserved Asp residue and DNA-binding motif. Genes homologous to spinach TCP34 were found in rice and arabidopsis, but not in cyanobacteria (Weber et al. 2006). Last year, a chloroplast sensor kinase (CSK) encoded by the nuclear locus *At1g67840* was revealed in *A. thaliana* (Puthiyaveetil et al. 2008). CSK is homologous to cyanobacterial HK but its phospho-acceptor motif is not conserved and lacks His; CSK is able to autophosphorylate but, probably, on Tyr residue (Puthiyaveetil et al. 2008). Thus, CSK is a descendant of histidine kinase but it changed its specificity and cannot transduce phosphate to Asp of RR.

In this work a search for chloroplast histidine kinase(s) was also performed. For this purpose, antiserum to conserved phospho-acceptor motif of HKs was generated and different chloroplast fractions were examined with it. Two polypeptides with apparent molecular masses about 26 and 28 kDa that are thylakoid membrane proteins and, probably have His to be phosphorylated, were detected. Polypeptides with similar molecular masses were revealed in various mono- and dicotyledonous plants. Due to these results and the finding of TCP34 (Weber et al. 2006), a hypothesis that plastids of contemporary plants have a two-component system that could be involved in regulation of plastid gene expression may be advanced.

Materials and methods

Plant material

Maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), spinach (*Spinacia oleracea* L.), tobacco (*Nicotiana tabacum* L.), and rape (*Brassica napus* L.) plants were soil-grown at 21°C and 200 μmol photons m^{-2}

s^{-1} with 16 h light/8 h dark photoperiod. For experiments, plants of different age—from 7 days old up to 1–1.5 months old—were used. The age of seedlings was determined since their appearance from the soil surface. 4-month-old maize plants were grown in natural conditions and collected in early September.

Chloroplast isolation and sub-fractionation

Leaves (10–15 g) were homogenized in 60 ml of buffer A (0.33 M sorbitol, 50 mM Tris–HCl, pH 8.0, 2 mM EDTA, and 5 mM β-mercaptoethanol). The homogenate was filtered through one layer of cheesecloth and two layers of Miracloth (Calbiochem-Behring, USA) and centrifuged for 1 min at 1,600*g*. The pellet of organelles was resuspended gently in 15 ml of buffer A and fractionated in the discontinuous Percoll gradient (20/40/70%) by centrifugation for 10 min at 3,800*g*. Intact chloroplasts were collected at the interface of 20 and 40% Percoll (for cereals) or 40 and 70% Percoll (for dicots). For further fractionation, the chloroplasts were lysed by hypo-osmotic shock in deionized water containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM ε-amino-*n*-caproic acid, and 1 mM benzamidine) for 15 min with gentle stirring; NaCl up to 40 mM was added after lyses for better pellet precipitation, and thylakoids were separated from stroma and envelope membranes by centrifugation for 5 min at 5,000*g*. Thylakoid pellet was washed gently and thoroughly with 1 M NaCl solution containing the same protease inhibitor cocktail and then centrifuged for 5 min at 5,000*g*. To avoid contamination with fragments of thylakoid membranes, supernatant was centrifuged in new tubes one more time for 10 min at 5,000*g*. All operations were performed at 4°C.

Slightly different procedure was used for fractionation of the thylakoid membranes. Leaves were homogenized in buffer B (0.4 M sucrose, 20 mM Tris–HCl, pH 7.8, 5 mM $MgCl_2$, 15 mM NaCl, 2 mM EDTA, 1% BSA), the homogenate was filtered through two layers of nylon and centrifuged for 5 min at 4°C and 1,000*g*; the supernatant obtained was centrifuged for 15 min at 4°C and 3,000*g* and isolated chloroplasts were resuspended in buffer B without EDTA and BSA, and lysed in hypotonic buffer C (20 mM Hepes, pH 7.5, 35 mM NaCl, 5 mM $MgCl_2$). Thylakoids were collected by centrifugation for 20 min at 4°C and 5,000*g* and resuspended in buffer D (20 mM Mes-NaOH, pH 6.5, 35 mM NaCl, 0.33 M sorbitol). Thylakoid membranes were solubilized with 20% Triton X-100 solution with ratio of Triton X-100 to chlorophyll being 20:1 (w/w) for 20 min at 0°C as described in (Ford and Evans 1983). The PSII enriched fraction was separated by centrifugation for 40 min at 4°C and 35,000*g*. Supernatant was transferred into new tubes and PSI-enriched fraction was collected after centrifugation for 1 h at 4°C and 70,000*g*. Both fractions were washed two times with buffer D. Chlorophyll fluorescence spectra of isolated chloroplasts and fractionated thylakoid membranes were measured at liquid nitrogen temperature and excitation at 440 nm. Oxygen evolution rate was estimated in a closed 3-ml cell using a Clark-type electrode (Hansatech, UK) under continuous illumination with white light (600 μmol photons m^{-2} s^{-1}) at 20°C in the presence of 1 mM *p*-benzoquinone and 0.5 mM $K_3[Fe(CN)_6]$. Chlorophyll fluorescence was measured with a PAM 201 chlorophyll fluorometer at room temperature (Walz, Germany). Potential quantum yield of PSII photochemical reactions was calculated according to Krause and Weis (1991).

Electrophoresis and western blotting

In supernatant fractions, proteins were precipitated by adding four volumes of acetone (v/v) at 0°C and all pellets were resolved in the lyses buffer (50 mM Tris–HCl, pH 8.9, 5% SDS, 2% Triton X-100, 5 mM β-mercaptoethanol), boiled for 4 min and centrifuged for 3 min at 8,000*g* to remove insoluble particles. Solubilized proteins were precipitated with acetone and centrifuged as above. The protein pellets were washed with 80% acetone and resolved in double Laemmli sample buffer (125 mM Tris–HCl, pH 6.8, 4.6% SDS, 15% glycerol, 10% β-mercaptoethanol, 0.4‰ bromophenol blue), boiled for 4 min and separated in 12 or 15% SDS-PAGE according to Laemmli (1970). For western blot analysis, proteins were transferred onto either nitrocellulose or PVDF membranes according to Towbin et al. (1979). After blotting proteins were stained with chloramine T, KJ, and starch (Kumar et al. 1985). Membrane blocking and incubation with antisera were performed in buffer consisting of 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 1% ovalbumin at room temperature for 1 h. After each incubation with antiserum, membrane sheet was washed six times in PBS buffer with 0.05% Tween 20. Visualization was performed using peroxidase and 4-chloro-1-naphthol as a substrate. After in organello phosphorylation, the protein samples were not boiled and membrane sheets were not stained in acid KJ because of lability of phospho-histidine at acid condition and high temperature. Apparent molecular masses of polypeptides were calculated with Gel-Pro Analyzer 3.1 software (Media Cybernetix, USA).

The anti-HK antiserum production

In order to raise the antiserum, the 19-aa oligopeptide (AKSQFLATVSHEIRTPMNG) was chosen as the antigen. The selected oligopeptide presents N-terminal part of HisKA domain that contains phosphorylation site and H

	H1 peptide	AKSQFLATVSHEIRTPMNG
	Ck receptors*	AKSQFLATVSHEIRTPMNG
	Et receptors*	ARNDFLAVMNHEMRTPMNA
	OsHK1	RAKQMLATMSHEIRSPLSG
	AtCKI2/AHK5	RAKQMLATMSHEIRSPLSG
	OsHK2	NKSNAFASASHDIRSALAA
	AtCKI1	NKSQAFANASHDIRGALAG
	AHK1	YKSQFLANMSHEIRTPMAA
cyano	MC7420_7660	AKSEFLATMSHEIRTPMNG
	PCC7002_A0148	AKSMFLATMSHEIRTPMNG
	alr2279	AKSNFLATMSHEIRTPMNA

Fig. 1 Conservation of N-terminal part of HisKA domain in plant and some cyanobacterial HKs *Ck* cytokinin, *Et* ethylene, *H1 peptide* the peptide selected for generation of H1 antiserum. Identical amino acid residues are against *dark-gray* background and similar ones are against *light-gray* background. *Asterisk* represents corresponding sequences are invariant among cytokinin receptors (AHK2-4, OsHk3-6, ZmHK1-3) and inside Ers branch of ethylene receptors (AtErs1, AtEtr1, OsErs1, OsErs2, ZmErs14, ZmErs25); sequences and gene names are given according to (Hwang et al. 2002; Yonekura-Sakakibara et al. 2003; Pareek et al. 2006; Schaller et al. 2007) except ZmErs14 (AY359577) and ZmErs25 (AY359578). Among 5 annotated sequences of cDNA of cytokinin receptor ZmHK1, one (AB042270) encodes A in the 9th position of the peptide, whereas other four (AB206390-AB206393) encode conservative V, therefore we didn't consider this deviation

motif—the characteristic feature of most HKs (Stock et al. 2000). The oligopeptide is very conservative in plant HKs and even in some HKs of cyanobacteria (Fig. 1).

The synthetic oligopeptide and its BSA-conjugated form were kindly provided by Dr. N.S. Egorova (Institute of Bioorganic Chemistry RAS, Moscow) and the latter was used for immunization. The complete Freund's adjuvant was used for the first immunization of a rabbit, incomplete for the second and the rest—in physiological NaCl solution. This scheme was employed to raise antibodies both against BSA-conjugated oligopeptide H1 and against pure BSA. The oligopeptide was applied to check the affinity of H1 antiserum. For specificity control, a mixture of the preimmune antiserum with the antiserum raised against pure BSA (1:1) was used.

In organello phosphorylation assay

Phosphorylation of chloroplast proteins was performed for 30 min at 26°C in a buffer containing 50 mM Tris–HCl, pH 7.5, 0.33 M sorbitol, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM NaF, 5.6 mM β-mercaptoethanol, 0.5 mM ATP, 0.1% Triton X-100; chloroplasts were added to final chlorophyll concentration 500 µg/ml; 1.3–1.5 MBq of [γ-^{32}P] ATP (148 PBq/mol) per 100 µl of reaction mixture was applied. Chlorophyll concentration was measured according to Lichtenthaler (1987). The reaction was stopped by adding the lyses buffer (see above). Samples for electrophoresis and western blotting were prepared without boiling. Immunodetected bands were cut from PVDF membrane and incubated for 16 h in 1 M HCl, 1 M NaOH, or 0.2 M Na-phosphate buffer, pH 7.2 at room temperature, or in 0.2 M Na-phosphate buffer, pH 7.2 at 45°C; then membrane slices were removed and radioactivity of solutions was determined in a scintillation cocktail appropriate for counting of water-containing solutions.

Bioinformatic analysis

Search for similar sequences was performed in GenBank databases with BLAST (Altschul et al. 1990). To compare the degree of similarity between different proteins, e-value parameter was used. ClustalW program was used for sequence alignment (Thompson et al. 1994).

Results

Search for potential HK in plant chloroplasts

In maize chloroplasts, H1 antiserum visualized two major bands with apparent molecular mass about 26 and 28 kDa and the third minor band—about 27.5 kDa (Fig. 2a, lane C). To investigate intra-chloroplast localization of these proteins, intact chloroplasts were lysed by hypo-osmotic shock and thylakoids were separated from stroma and envelope by centrifugation. Then thylakoid pellets were resuspended and washed with 1 M NaCl solution to remove loosely associated membrane proteins. After fractionation, no clear bands were observed in fractions that contained proteins of stroma and envelope (Fig. 2a, lane S) and proteins removed from thylakoid membranes with high-salt treatment (Fig. 2a, lane Na). However, all these bands were detected in washed thylakoid membrane fraction (Fig. 2a, lane T).

Patterns of bands recognized by H1 antiserum and preimmune antiserum were compared. Bands 28 kDa and 27.5 kDa were detected with H1 antiserum only but a band with apparent molecular mass about 26 kDa was revealed with both antisera (Fig. 2b). Consequently, bands 28 kDa and 27.5 kDa are recognized by antibodies to conservative phospho-acceptor motif; band 26 kDa may be recognized by preimmune antibodies but the possibility that both antisera recognize different proteins that co-migrated under our experimental conditions should not be ruled out. To clarify whether the detected proteins may have resulted from contamination with non-chloroplast membranes, thylakoid membrane fraction was solubilized in 2% Triton X-100. After centrifugation, green supernatant and white or light-greenish pellet were obtained. The pellet comprised

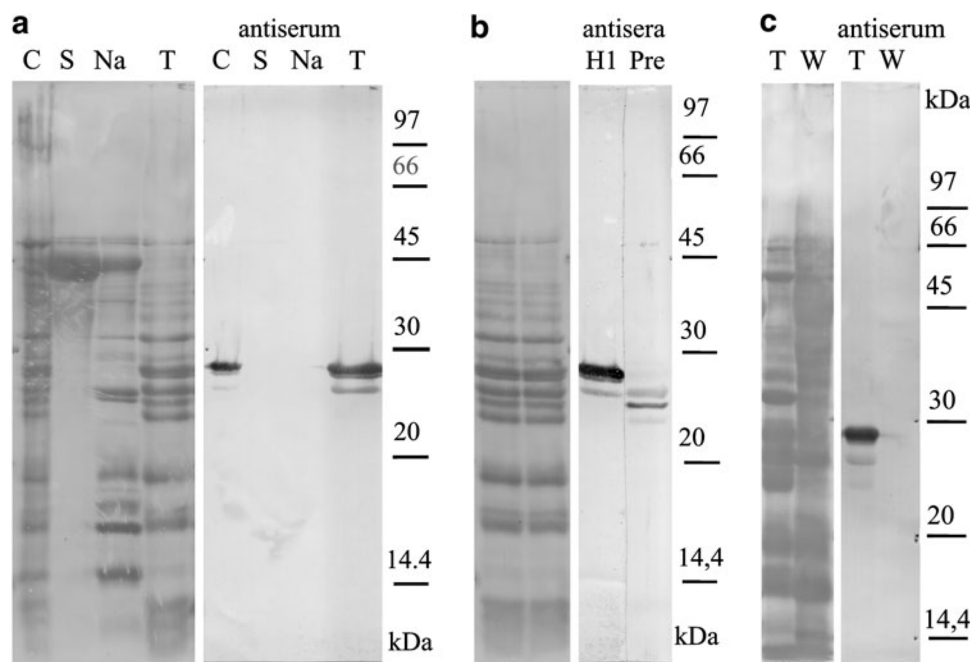

Fig. 2 Examination of different fractions of maize chloroplasts with the H1 antiserum After blotting, membranes were firstly stained for general proteins then washed and incubated with antibodies. **a** Chloroplast sub-fractionation. Chloroplasts (*C*), soluble proteins (*S*), proteins removed from thylakoid membranes by treatment with 1 M NaCl (*Na*), thylakoid membranes washed with 1 M NaCl (*T*). **b** Test for specificity of H1 antibody binding. Proteins of washed thylakoid membranes were separated and blotted. Lanes were incubated with H1 antiserum (*H1*) or with equal mix of preimmune antiserum and antiserum to BSA (*Pre*). **c** Search for possible contamination effect. Washed thylakoid membranes (T) were solubilized with 2% TritonX-100 and a very small amount of white or light-greenish pellet was obtained (*W*); probably, the last fraction (*W*) is greatly enriched in non-thylakoid membranes from the first fraction (*T*). 15 µg of protein was loaded for each lane

about 1% of solubilized thylakoid membrane fraction (data not shown) and, probably, was enriched with non-thylakoid membranes. The antibody binding was much lower in "white" fraction (Fig. 2c, lane W) than in initial thylakoid membrane fraction (Fig. 2c, lane T); the binding in "white" fraction may be due to the residues of thylakoid membranes in this fraction. Therefore, it may be concluded that the revealed bands represent thylakoid membrane proteins or several forms of the same protein and they do not result from contamination by other membranes.

Protein(s) with similarity to HK was detected but the question emerged whether it could function as a protein kinase? To answer the question, in organello phosphorylation followed by western blotting of total chloroplast proteins was performed. Bands revealed by H1 antiserum coincided with the zone of intensive phosphorylation (Fig. 3). Partially, the phosphorylation observed might be due to phosphorylation of protein(s) detected with H1 antiserum. Phospho-histidyl is known to be stable at alkaline and labile both at acid conditions and higher temperatures; vice versa, phospho-seryl and -threonyl are stable both in acid and at elevated temperatures but labile in alkaline solution (under all these conditions phospho-

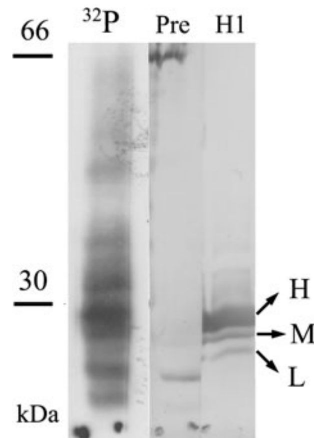

Fig. 3 In organello phosphorylation of chloroplast proteins After phosphorylation reaction, all chloroplast proteins were separated in 15% SDS-PAGE, blotted onto PVDF membrane and immunodetected with the equal mix of a preimmune antiserum and an antiserum to BSA (*Pre*) or with H1 antiserum (*H1*). First lanes were used for radioautography with X-ray film (^{32}P), second—for band excision; excised bands were denoted as *H*, *M*, and *L* and used for hydrolysis of phospho-amino acid bindings (see Table 1). 10 µg of protein was loaded for each lane

Table 1 Lability of phospho-amino acids in phosphorylated chloroplast polypeptides

	H		M		L	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
OH^-	1,384.0 ± 201.1	1,770.7 ± 310.3	297.7 ± 43.4	841.7 ± 61.2	169.0 ± 14.5	158.7 ± 31.6
H+	705.0 ± 45.2	728.0 ± 72.8	152.3 ± 16.7	162.3 ± 29.5	145.7 ± 36.0	144.7 ± 25.9
pH 7.2	257.3 ± 52.3	297.0 ± 77.2	45.7 ± 9.6	54.3 ± 10.7	30.7 ± 6.0	47.0 ± 4.0
45°C	998.7 ± 102.5	703.0 ± 11.6	172.0 ± 22.5	137.7 ± 21.4	146.0 ± 30.3	128.0 ± 3.2

After in organello phosphorylation and western blotting, bands (H, M, L, see Fig. 3) were excised carefully and incubated for 16 h in 1 N NaOH (OH^-), 1 N HCl (H^+), or 0.2 M Na-phosphate buffer, pH 7.2 at room temperature (about 22°C), or in 0.2 M Na-phosphate buffer, pH 7.2 at 45°C with gentle stirring. Then membrane slices were removed and radioactivity of liquids was counted. Mean values ± standard deviations are given as cpm

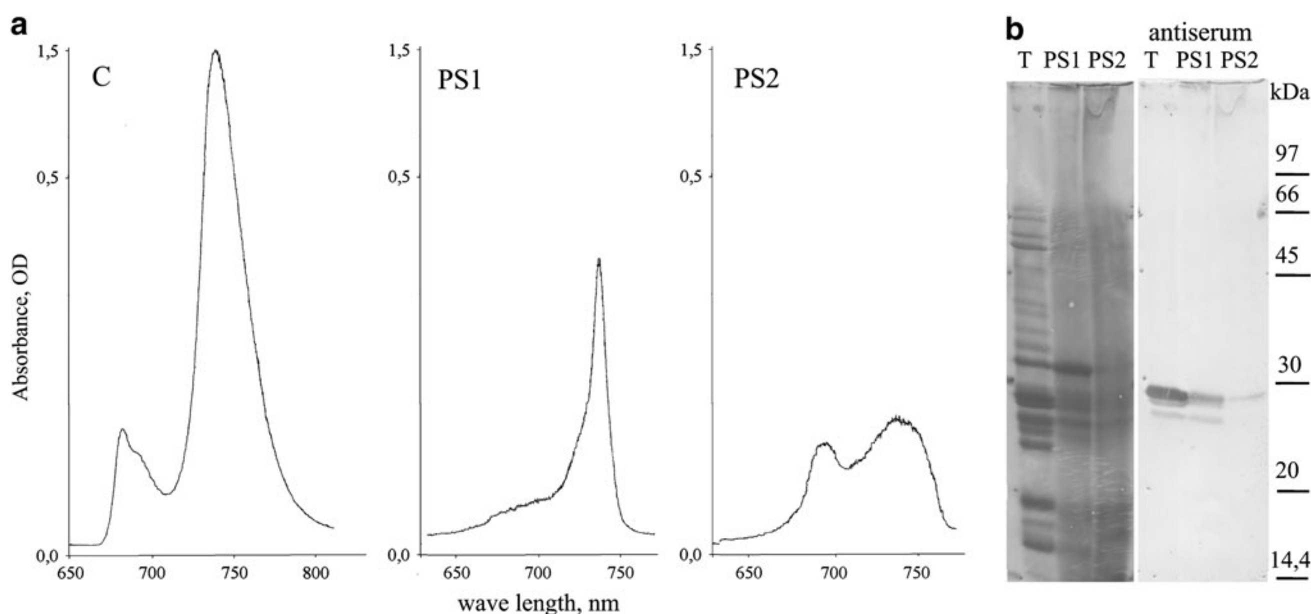

Fig. 4 Search for H1-binding protein(s) in stromal (PSI-enriched) and granal (PSII-enriched) parts of thylakoid membranes **a** Chlorophyll fluorescence emission spectra at liquid nitrogen temperature and excitation at 440 nm. The emission at 685 and 695 nm is attributed to PSII and LHCII respectively, whereas the emission at 735 nm originates from PSI. **b** Western blotting with H1 antiserum. *C* chloroplasts, *T* thylakoids, *PS1* (*PS2*) fraction of thylakoid membranes enriched with PSI (PSII). 15 µg of protein was loaded for each lane

aspartyl is labile and phospho-tyrosyl is stable) (Wei and Matthews 1991). Therefore, after in organello phosphorylation and western blotting, immunodetected bands were excised from PVDF membrane and incubated for 16 h in a neutral, alkaline, or acid solution at a room temperature or in a neutral solution at 45°C. Then membrane slices were removed and radioactivity of each solution was determined in a scintillation counter. The experiment was performed two times in triplicate for each treatment. The data obtained prove that in the regions of immunodetected bands phosphorylated Ser/Thr, His (Table 1), and even Tyr (data not shown) residues occur. Alkaline-labile phospho-Ser/Thr represents the major part of phosphorylated amino acids. Radioactivity removed by acid and elevated temperature was lower than that at alkaline conditions but higher than in control neutral solution. Therefore, it may be assumed that protein(s) detected with H1 antiserum was phosphorylated on His residues and may function as HK(s).

In plants and in bacteria, HKs often regulate transcription of target genes. In plastids, transcriptional machinery is localized in stroma. Unstacked (stromal) regions of thylakoid membranes contact with stroma and are enriched with photosystem I (PSI), whereas stacked (granal) regions of thylakoid membranes are enriched with photosystem II (PSII) and its functionally active light-harvesting complex (LHCII) (Allen and Forsberg 2001). Thylakoid membranes were solubilized with Triton X-100 and fractionated by sequential centrifugation at 35,000 and 70,000*g*. One of the obtained fractions was enriched in PSI (Fig. 4a) and contained few non-active PSII (Fv/Fm) with damaged transfer of electron excitation energy from antenna to reaction center (Fo) (Table 2); therefore this fraction was enriched

Table 2 Chlorophyll *a* fluorescence parameters and oxygen evolution rate of isolated thylakoid membranes of maize seedlings

Samples	Minimal fluorescence yield (Fo)	Maximum (potential) quantum yield of PS2 (Fv/Fm)	Oxygen evolution rate, µmol O_2/mg Chl per h
Thylakoids	0.504	0.628	195.2
PS1-enriched fraction	0.854	0.078	0.0
PS2-enriched fraction	0.266	0.654	250.3

in stromal regions of thylakoid membranes. The second fraction was depleted with PSI (Fig. 4a) and enriched in PSII with higher quantum yield (Fv/Fm), and oxygen evolution rate and lowered ratio of initial chlorophyll fluorescence (Fo) (Table 2); thus, the second fraction was enriched in granal regions of thylakoids. H1 antiserum clearly detected all three bands in PSI-enriched fraction and the major 28 kDa band was hardly detected in fraction enriched with PSII (Fig. 4b). Thus, it may be concluded that immunodetected protein(s) is mainly located in unstacked regions of thylakoid membranes and could be exposed into stroma.

All the experiments described above were performed on young maize seedlings and the question arose whether detected protein(s) is stage-specific maize protein(s)? To answer this question, thylakoid membranes from 7-day-old seedlings and from 1- and 4-month-old plants were isolated. The H1 antiserum revealed similar bands in thylakoids of young and mature maize plants (Fig. 5), which suggests that the protein(s) is expressed at different developmental stages.

Further chloroplasts of several mono- and dicotyledonous plants were analyzed (Fig. 6, Online Resource 1). In thylakoid membranes of tested plants, H1 antiserum recognized two bands with apparent molecular masses that usually varied from 26 to 28 kDa. However, in rape, the molecular mass of the second band was twice as large (approximately 56 kDa); in tobacco, one strong and several faint bands were revealed (Fig. 6); and in *A. thaliana*, only a strong band was visualized (data not shown). As with maize, no clear bands were immunodetected in fractions containing proteins from stroma and envelope, and proteins removed from thylakoid membranes with high-salt treatment in species studied (data not shown).

Search for genes that may code for potential HK in plant chloroplasts

Proteins with apparent molecular mass 26–28 kDa may consist of 250–300 aa. Besides, nuclear encoded

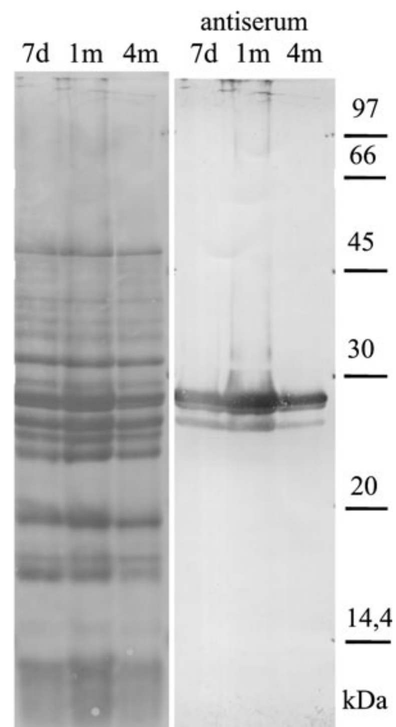

Fig. 5 Detection of H1-binding protein(s) in maize plants of different age Thylakoid membranes were isolated from 7 day- (*7d*), 1 month- (*1m*) and 4 month-old (*4m*) maize plants. Thylakoid membrane proteins were separated in 15% SDS-PAGE and probed with H1 antiserum; 15 µg of protein was loaded for each lane

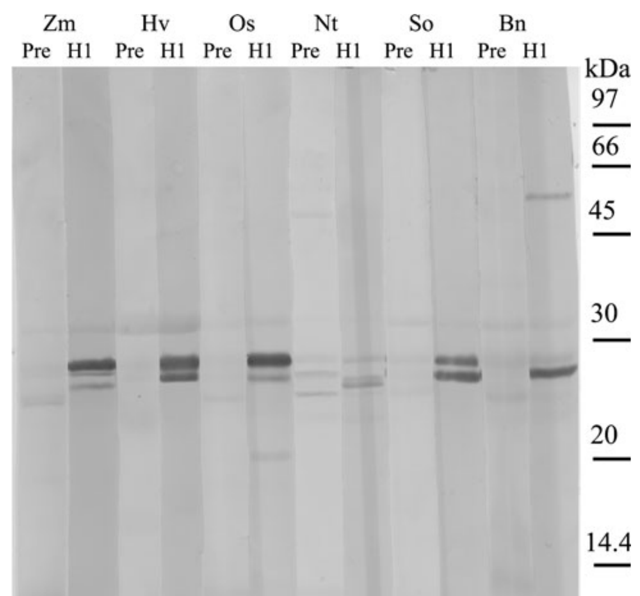

Fig. 6 Detection of H1-binding protein(s) in different mono- and dicotyledonous plants Thylakoid membranes were isolated from maize (*Zm*), barley (*Hv*), rice (*Os*), tobacco (*Nt*), spinach (*So*), and rape (*Bn*). Thylakoid membrane proteins were separated in 15% SDS-PAGE and immunodetected with equal mix of preimmune antiserum and antiserum to BSA (*Pre*) or with H1 antiserum (*H1*); 15 µg of protein was loaded for each lane. Staining of this membrane for total proteins is shown in Online Resource 1

chloroplast proteins usually contain transit peptide that is cleaved after delivery into organelle; transit peptides possess no extended blocks of sequence conservation and vary in length from 20 to more than 100 amino acid residues (Bruce 2000). Thus, a gene to be searched has to encode polypeptide with no more than 350–400 aa. However, in flowering plants (*A. thaliana*, *O. sativa*, and *Z. mays*), genes of well-known HKs encode much larger polypeptides. The size of cytokinin receptors, phytochromes, CKI1 orthologs and AHK1 vary from 1,000 to 1,200 aa, slightly smaller is the size of CKI2 orthologs (about 950 aa), and ethylene receptors consist of 600 to 800 aa (Hwang et al. 2002; Yonekura-Sakakibara et al. 2003; Pareek et al. 2006).

We searched through databases and found out that one gene from ethylene receptor family originates both full-length and substantially shortened mRNAs. For example, in *A. thaliana*, the set of mRNAs derived from gene *Ers1* includes the transcript (BX821649) that codes for a polypeptide of 321 aa. In *O. sativa*, gene *Ers2* originates mRNAs that encode polypeptides consisting of 635, 518, and 359 aa (Pareek et al. 2006). Its maize ortholog *Ers25* was found to give rise to mRNAs encoding polypeptides with 634 aa (AY359578) and 359 aa (BT038117). All these short transcripts, probably, resulted from alternative transcription initiation and/or alternative processing of mRNA 5′-end but not from alternative splicing. The encoded polypeptides contain domains essential for HK activity (HisKA and HATPase_c), small fragments of GAF domain and no receiver domain. The comparison of full-length and short mRNAs is presented for transcripts of maize *Ers25* gene (Online Resource 2).

After transit peptide cleavage (in chloroplasts) a polypeptide initially consisting of 359 aa may have apparent molecular mass of about 25–28 kDa. Neither TargetP nor ChloroP predicts chloroplast targeting for 359 aa polypeptides encoded by *Ers2* genes of rice and maize (data not shown), but such programmes are known to be unable to predict chloroplast localization for one-third of proteins experimentally detected in chloroplasts (Kleffmann et al. 2004). The search through DNA sequences of maize, complete genomes of rice and arabidopsis was performed, but we failed to find any other gene encoding protein of moderate size (from 200 to 350–400 aa) and containing appropriate epitope for antibodies raised against H1 peptide (Fig. 1).

Another approach was applied to reveal possible plastid HKs. We investigated which proteins of plants and green algae are the most similar to plastome encoded HKs of non-green algae. GenBank non-redundant protein database was searched through by BLAST (Altschul et al. 1990). The moss *Physcomitrella patens* was found to possess a number of predicted HKs of a relatively small size that are the most similar to the majority of plastid HKs of non-green algae (Tables 3, 4); HK with the highest similarity (XP_001786950) contains HAMP domain that is present in many plastome encoded HKs (Table 3). Proteins of other plant species demonstrated little similarity to plastome encoded HKs. Chlamyopsin-5 from *Chlamydomonas reinhardtii* (1,441 aa), putative ethylene receptor from rice (PK4, 653 aa), and predicted protein from *Ricinus communis* (EEF24099, 406 aa, incomplete coding DNA sequence—CDS) demonstrated relatively high similarity to the majority of plastome encoded HKs; besides, putative cytokinin receptor from *Vitis vinifera* (CAO41188, 936 aa) and predicted protein from *Populus trichocarpa* (XP_002336825, 693 aa) are very similar to some of them (Table 3, Online Resource 3). All these proteins contain predicted HK domains (HisKA, HATPase_c and REC, data not shown) and are represented by relatively big polypeptides except partial sequence of a predicted protein of *R. communis*.

Discussion

In the present work we raised antiserum H1 against conservative HK motif (Fig. 1) and applied it to search for potential HK(s) in chloroplasts of higher plants. The H1 antiserum detected a number of proteins in thylakoid membranes of various mono- and dicotyledonous plants (Figs. 2, 6). No clear bands were observed in fractions that contained proteins of stroma, envelope and loosely associated proteins of thylakoid membranes (Fig. 2 for *Z. mays*, for other plants data not shown). In maize, polypeptides recognized by the H1 antiserum were mainly located in unstacked (stromal) regions of thylakoid membranes (Fig. 4; Table 2) and, probably, were integral membrane proteins—they were not removed from the membranes with 1 M NaCl treatment (Fig. 2a) and after solubilization with Triton X-100 (Fig. 4). Similar results were obtained for barley (data not shown). The H1 antiserum visualized two or three bands in thylakoid proteins of all tested plants (only one band in the fraction of *A. thaliana* proteins, data not shown); bands lie close to each other and may represent different forms of the same protein or different proteins with similar apparent molecular masses. Only in *B. napus*, the H1 antiserum revealed proteins with quite different apparent molecular masses (Fig. 6), which suggests the presence of two potential HKs in thylakoid membrane of this plant species.

After in organello phosphorylation, the bands visualized with H1 antiserum contained polypeptides phosphorylated on His residues (Fig. 3; Table 1). It is most improbable that the bands excised would contain a single protein because total chloroplast proteins were separated by SDS-PAGE. Therefore, the question is which polypeptides were His-

Table 3 Plastome encoded HKs of non-green algae and their closest homologues from Viridiplantae organisms

Taxon	Species	Gene	Predicted domains	aa	GenBank ID	Most similar Viridiplantae proteins		
						1st	2nd	3rd
Raphidophyta	*Heterosigma akashiwo*	*tsg1*	HisKA, HATPase_c	369	YP_001936324	*P. trichocarpa* XP_002336825 3e-18	***P. patens*** **XP_001786950** **5e-17**	*P. patens* XP_001755170 6e-16
Haptophyta	*Emiliania huxleyi*	*dfr*	HAMP, HisKA, HATPase_c	612	YP_277392	***P. patens*** **XP_001786950** **2e-23**	*P. patens* XP_001755170 1e-21	*O. sativa* PK4[c] 3e-21
Rhodophyta	*Cyanidium caldarium*	*ycf26*	HisKA (PAS)[a], HATPase_c	631	NP_045190	*P. patens* XP_001755170 3e-21	***P. patens*** **XP_001786950** **2e-20**	*R. communis* EEF24099 6e-19
	Porphyra purpurea	*ycf26*	HAMP, PAS, HisKA, HATPase_c	656	NP_054002	***P. patens*** **XP_001786950** **9e-27**	*V. vinifera* CAO41188 2e-26	*C. reinhardtii* chlamyopsin-5[b] 3e-22
	Porphyra yezoensis	*ycf26*	HAMP, PAS, HisKA, HATPase_c	653	YP_537073	*V. vinifera* CAO41188 6e-27	***P. patens*** **XP_001786950** **1e-26**	*C. reinhardtii* chlamyopsin-5[b] 3e-25
	Gracilaria tenuistipitata	*dfr*	HAMP, HisKA, HATPase_c	633	YP_063707	***P. patens*** **XP_001786950** **1e-32**	*C. reinhardtii* chlamyopsin-5[b] 1e-21	*O. sativa* PK4[c] 2e-18
Cryptophyta	*Rhodomonas salina*	*ycf26*	HisKA, HATPase_c	363	YP_001293515	*P. patens* XP_001755170 3e-18	*P. patens* XP_001778511 2e-14	*P. patens* XP_001769338 2e-14

P. patens XP_001786950 is in bold type. GenBank IDs of conservative domains: HisKA (cd00082), His_kinase (pfam06580), HATPase_c (cd00075), REC (cl09944), HAMP (cd06225), PAS (cl02459), PAC (smart00086). Degree of similarity between proteins is shown by means of e-value parameter

[a] Domain predicted in NCBI/Structure/cdd

[b] AAQ16277

[c] AAQ07255

phosphorylated: those recognized with H1 antibodies or others which co-migrated with the former? A simpler explanation is that the same polypeptides contained conservative phosphorylation motif recognized with H1 antiserum and were phosphorylated on His residues. Thus, we could suppose that protein(s) revealed with H1 antiserum could be functional HK(s) and autophosphorylate themselves on His residues.

Surprisingly, apparent molecular masses of the detected proteins vary from 26 to 28 kDa, except ~56 kDa protein in *B. napus* (Fig. 6), whereas all well-known HKs of *A. thaliana* and *O. sativa* are usually hybrid HKs and are represented by big or even immense polypeptides. The smallest of them (AtErs1) consists of 613 aa (Hwang et al. 2002; Pareek et al. 2006), and AtCSK—a chloroplast kinase that originated from HK but turned into STK—is of a similar size (611 aa) (Puthiyaveetil et al. 2008). However, non-hybrid HKs in other organisms may be small enough. Some of the smallest predicted HKs in cyanobacteria consist of 212 aa (glr1587), 277 aa (AM1_6067), 305 aa (alr0117); in algae, some plastome genes encode HKs consisting of approximately 360 aa (Table 3). Besides, a number of CDSs that share similarity with plastid HKs of non-green algae and encode enzymes of a moderate size (300–400 aa) were found in a land plant, the moss *P. patens* (Table 4). All these CDSs encode proteins with domains essential for HK activity (HisKA and HATPase_c) and have appropriate sites for binding with H1 antibodies (data not shown). It should be kept in mind that chloroplast proteins may be substantially shorter than it was predicted in silico (Kodama and Sano 2006).

It is interesting that some HKs of *P. patens* contain HAMP domain (Online Resource 4). HAMP is a signal sensor domain wide-spread among prokaryotic signaling proteins including HKs (Galperin et al. 2001) and the only sensor domain found in plastome encoded HKs (Table 3). One HAMP-containing HK of *P. patens* (XP_001786950) shared the highest similarity with plastid HKs, whereas others

Table 4 Similarity of plastome encoded HKs to predicted proteins of *P. patens*

Predicted proteins of *P. patens*			Similarity with plastome encoded HKs (e-value)						
Protein ID	aa	Predicted domains	Ha	Eh	Cc	Pp	Py	Gt	Rs
XP_001786950	364[a]	**HAMP**, HisKA, HATPase _c	3e−18	2e−24	1e−21	6e−28	6e−28	8e−34	
XP_001755170	336[a]	HisKA, HATPase_c, REC	4e−17	8e−23	2e−22	1e−22	4e−25	4e−18	2e−19
XP_001769338	369	HisKA, HATPase _c, REC[b]	6e−16	1e−14	1e−19	6e−18	3e−22	2e−15	2e−15
XP_001778511	328[a]	HisKA, HATPase _c, REC	3e−13	2e−15	3e−18	7e−21	5e−23	2e−15	1e−15
XP_001775981	436	HisKA, HATPase _c, REC		2e−17	5e−19	2e−18	1e−23	5e−15	3e−14
XP_001763917	379	HisKA, HATPase _c, REC[b]	4e−14	3e−13	3e−18	1e−20	6e−22	7e−15	2e−12
XP_001771969	1,040	MASE1, HisKA, HATPase _c, REC		4e−15	4e−18	9e−19	6e−22	2e−14	2e−15
XP_001767038	330[a]	HisKA, HATPase _c, REC[b]	2e−14	5e−18	4e−15	4e−16	4e−20	2e−17	1e−14
XP_001786575	315	HisKA, HATPase _c		4e−21	3e−12	2e−17	2e−16	5e−16	1e−11
XP_001770900	472	HisKA, HATPase _c, REC	5e−12	6e−16		1e−13	4e−15	5e−12	8e−11
XP_001757236	207[a]	HisKA, HATPase _c[b]		9e−13	1e−16	1e−16	1e−20	7e−11	5e−14
XP_001759657	927	HisKA, HATPase _c, REC	2e−14	3e−15		5e−19	4e−19	2e−13	1e−12
XP_001760203	1,191	HisKA, HATPase _c, REC	1e−11	4e−14	6e−11	9e−20	7e−22	1e−15	
XP_001754679	1,189	HisKA, HATPase _c, REC	2e−11	4e−14		2e−19	2e−21	3e−16	
XP_001762445	747	GAF, HisKA, HATPase _c, REC		6e−17	3e−11	2e−14	1e−17	8e−12	1e−12
XP_001760796	1,165	MASE1, HisKA, HATPase _c, REC		2e−16	1e−15	3e−19	8e−23	6e−15	4e−11[c]
XP_001764025	725[a]	HisKA, HATPase _c, REC		5e−14	7e−12	7e−21	9e−22	3e−16	
XP_001775670	298[a]	HisKA, HATPase _c	5e−11	6e−12	2e−14	2e−13	6e−15	3e−17	
XP_001752048	1,436	HisKA, HATPase _c, REC		2e−13	6e−12	5e−14	7e−18	5e−12	
XP_001753177	970	HisKA, HATPase _c, REC	7e−11	5e−12	1e−13	1e−14	7e−16		
XP_001774409	763	GAF, HisKA, HATPase _c, REC		3e−14	7e−12	2e−11	2e−15	2e−11	

The analysis was restricted to e-values less than 1e-10; *P. patens* proteins similar to the majority (at least five out of seven) of plastome encoded HKs were included in the table only. MASE1 prokaryotic sensor domain (GenBank ID: cl01367); GAF cGMP/cAMP binding domain (cl00853), IDs of other domains are given in the legend to Table 3. In plants, GAF + HisKA + HATPase_c domain structure accomplished with transmembrane domain is characteristic of ethylene receptors from Ers branch (Hwang et al. 2002; Pareek et al. 2006)

Ha H. akashiwo, *Eh E. huxleyi*, *Cc C. caldarium*, *Pp P. purpurea*, *Py P. yezoensis*, *Gt G. tenuistipitata*, *Rs R.* salina; names of corresponding genes and IDs of their proteins are given in Table 3

[a] Partial CDS

[b] Incomplete domain

[c] Protein has another similar stretch of amino acids with e-value 0.001

demonstrated no clear similarity to the latter (Tables 3, 4, Online Resource 4). Therefore, XP_001786950 is the best candidate for plastid HK in the moss *P. paten*. Some other small HKs presented in Tables 3 and 4 and HKs with HAMP domain (Online Resource 4) should also be considered as appropriate candidates for this role. We failed to find this domain in all flowering plant proteins available at GenBank except two sequences of *R. communis* that encoded putative phosphoserine phosphatase rsbU (EEF24369) and diguanylate-cyclase (EEF27419). Probably, flowering plants had lost HAMP domain. Thus, a lower terrestrial plant—the moss *P. patens*—demonstrated much more candidate genes for encoding chloroplast HKs than flowering plants.

The data presented in Fig. 6 suggest that detected potential chloroplast HK(s) is conservative in many flowering plant species. Nevertheless, no predicted gene(s) that may code for such a protein (CDS encoding 200–400 aa with HisKA and HATPase_c domains and with an appropriate site for binding with H1 antibodies) was found in GenBank. Perhaps, such genes have not been predicted yet for a variety of reasons. For example, in plants some nuclear (Kawasaki et al. 1999), plastid (Bonen 1993), and mitochondrial (Bonen 1993, 2008) genes are split and located in quite different parts of a genome; these parts are transcribed independently and their transcripts are merged due to *trans*-splicing.

Another possible explanation is that this protein(s) may be encoded by alternative transcript of a well-known plant HK gene. We searched through databases and found out that one gene from subfamily 1 of ethylene receptors (*AtErs1*, *OsErs2*, *ZmErs25*) gave rise to both full-length and substantially shortened transcripts. Probably, short mRNAs resulted from alternative transcription initiation and/or alternative processing of 5′-end of these mRNAs (Online Resource 2). The application of such a mechanism for encoding chloroplast protein was demonstrated

previously (Kodama and Sano 2007). Polypeptides encoded by short mRNAs of *AtErs1*, *OsErs2*, and *ZmErs25* contain domains essential for HK activity (HisKA and HATPase_c) but no receiver domain (Online Resource 2). The absence of REC domain is characteristic of non-hybrid HKs that transduce phosphoryl group directly to RR. After transit peptide cleavage (in chloroplasts) such proteins may be shortened to 250–300 aa and, probably, may be represented in SDS-PAGE by bands with apparent molecular mass 26–28 kDa. It is interesting that putative rice ethylene receptor PK4 is the closest flowering plant homolog of algal plastome encoded HKs (Table 3, Online Resource 3). Thus, it is not improbable that in flowering plants, chloroplast HK(s) may be encoded by alternative expression form of ethylene receptor gene(s).

Relatively small size of potential HKs revealed with the H1 antiserum (26–28 kDa) suggests that the protein(s) must be non-hybrid HK and may transfer a signal (phosphoryl group) directly to an acceptor protein in stroma. Candidate acceptor protein(s) may be similar to TCP34 (Weber et al. 2006) or RRs encoded by plastome genes *ycf27* and *ycf29*. These proteins were found in stroma and are supposed to regulate transcription. TCP34 specifically binds plastid DNA, especially in the region of *psaA* gene (Weber et al. 2006); the RR encoded by *trgI* (*ycf27*) gene of the alga *Heterosigma akashiwo* was predicted to bind a sigma factor—the general transcription factor of plastid multisubunit RNA polymerase (Duplessis et al. 2007). Besides, chloroplast RRs may be expressed as an alternative form of well-known nuclear genes (Pareek et al. 2006).

Conclusion

The data obtained in this work support the idea that plants retained HKs in their chloroplasts. We suppose that chloroplasts of flowering plants contain a small set of non-hybrid HKs that are located in unstacked regions of thylakoid membranes and may autophosphorylate themselves and transduce a phosphoryl group to RR-like proteins in stroma, thus influencing chloroplast transcription. The genome of the moss *P. patens* contains much more genes that may code for such chloroplast HKs than the genomes of flowering plants. Probably, in the process of evolution of terrestrial plants, diversity of chloroplast HKs was reduced. However, to prove strictly that polypeptides immunodetected with H1 antibodies are functional HKs we need to identify the polypeptides and investigate their molecular function in vitro and their biological role in vivo.

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