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Genes of carotenoid biosynthesis pathway in the moss *Hylocomium splendens*: identification and differential expression during abiotic stresses

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Abstract A promising approach to solve the problem of tolerance of plants in hostile environments is to focus of stress tolerance mechanisms of extremophilic plants, in particular mosses. Along with the universal stress mechanisms, bryophytes exhibit a unique spectrum of secondary metabolites such as carotenoids, a lipophilic metabolite derived from the isoprenoid pathway. The main representatives of carotenoids in mosses are α- and β-carotene, lutein, neo-, viola- and zeaxanthins. *Hylocomium splendens* is one of the most common and widespread mosses of Northern Hemisphere. The genome of this moss has not been sequenced, and the carotenoid biosynthesis pathway (CBP) genes of this species have not been reported to date. This is the first report to of an attempt to identify and characterize the CBP genes in *H. splendens*. As a result of cloning, sequencing, and *in silico* analysis, we identified and characterized ten CBP genes in *H. splendens* with a full ORF, and prediction of subcellular localization suggests chloroplast localization of CBP proteins. Using multiple alignments and phylogenetic and homology analyses, we demonstrated that the CBP genes of *H. splendens* share high similarity with the sequences in other bryophytes. Differential expression of CBP transcripts during abiotic stresses was more evident for genes in the middle and downstream steps of CBP. This work provides information on the molecular genetics of CBP in extremophilic bryophytes. Analysis of CBP genes can help to unravel the genetic evolution of carotenoid biosynthesis in plants.

Keywords *Hylocomium splendens* · Carotenoid biosynthesis · Abiotic stress · Gene expression · Mosses · Stress tolerance

1 Introduction

The ability of plants to adapt to unfavorable living conditions is a prerequisite for plant survival, evolution of the genotype, and maintenance of flora diversity. Detailed knowledge about the mechanisms of sensitivity and/or resistance of plant species to changing environmental conditions is required. A promising approach to solve the problem of plant resistance to stress is to study the mechanisms of stress resistance of "extremophiles", in particular mosses, higher non-vascular plants. The relative simplicity of the anatomical structure and the ability to survive in adverse environmental conditions make these

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evolutionary ancient plants ideal models for studying adaptation mechanisms.

Plants experience numerous stresses during their life cycles, such as drought, and low and high temperatures. Extreme temperature has an adverse impact on photosynthesis (Fan et al. 2017), hormone homeostasis (Maestri et al. 2002), and often results in changes in secondary metabolite concentration (Wahid et al. 2007). Among secondary metabolites, carotenoids are organic pigments, which are widespread in algae, mosses, ferns, gymnosperms and angiosperms (Park et al. 2021). In addition, they are found in the membranes of photosynthetic bacteria such as phototropic bacteria and cyanobacteria (Moeller et al. 2005; Sathasivam et al. 2021). Although classed as secondary metabolites, carotenoids have been implicated in a large number of biological functions of plant growth and development. In photosystem II (PS II), carotenoids contribute to the transfer of energy to the reaction center (Amarnath et al. 2016) and protect chlorophyll from excessive energy by sequestering single oxygen (Ramel et al. 2012; Ruban 2016; Leuenberger et al. 2017). Furthermore, carotenoids play roles in protecting plants from oxidative damages as effective antioxidants (Truscott 1990). They are also the precursors in the production of apocarotenoid hormones such as abscisic acid and strigolactones (Ruiz-Sola and Rodríguez-Concepción 2012).

Several studies have shown that carotenoids contribute to the increased survival of plants under abiotic stress (Davidson et al. 2002; Li et al. 2008). It is well-known that abiotic stresses, such as drought, waterlogging, elevated or freezing temperatures, are accompanied by an increased production of reactive oxygen species (ROS) (Choudhury et al. 2013). Carotenoids as antioxidants are able to detoxify various ROS and also directly quench triplet chlorophylls that are sources of $^1O_2$ in the leaves (Ramel et al. 2012).

High temperatures adversely impact photosynthesis (Fan et al. 2017), hormone homeostasis (Maestri et al. 2002), and metabolism of secondary compounds (Wahid et al. 2007). Plants display a variety of responses to heat stress, including accumulation of carotenoids; heat stress initiates abscisic acid (ABA) and salicylic acid mediated signaling (Wahid et al. 2007; Du et al. 2010). However, understanding how these processes are involved in plant defenses against heat stress remains unclear. Jackson (2015) reported tissue-specific changes in gene expression and carotenoid accumulation in strawberry at elevated temperature. Changes in carotenoid concentration under elevated temperature have also been observed in some green algae, e.g. in *Haematococcus pluvialis* astaxanthin formation increased three fold, while in a *Chlorococcum sp.* a two-fold increase in total carotenoid concentration occurred (Juneja et al. 2013). High temperatures increase lutein accumulation in microalgae such as *Muriellopsis* sp. and *Scenedesmus almeriensis* (Del Campo et al. 2000; Sanchez et al. 2008). In contrast, low temperature slows lutein accumulation in microalgal species (Bhosale 2004). In Angiosperms, water deficit and prolonged waterlogging cause changes in carotenoid concentration, which are highly depend on plant species, and the duration and intensity of the water restriction or flooding period. A decrease in the concentration of carotenoids during drought have been reported in cherry tomatoes, wheat, sorghum, sunflower, and some plants of the family *Asteraceae* (Hammad and Ali 2014; Manivannan et al. 2014; Cicevan et al. 2016). It is likely that carotenoid concentration tend to decrease under drought and moderate flooding but slightly increase in response to severe drought stress (Sudrajat et al. 2015). Thus, the concentration of carotenoids in plants can change following abiotic stresses such as temperature variations, drought, and high salinity.

Reviews have summarized information about genes involved in the transcriptional regulation of the carotenoid biosynthesis pathway (CBP) in plants (Stanley and Yuan 2019; Sathasivam et al. 2021). The first important event in carotenogenesis is the production of phytoene from condensation of two geranylgeranyl diphosphate (GGPP) molecules, a reaction catalyzed by phytoene synthase (PSY). In several crop species, PSY is one of the most important and rate-limiting enzymes in the CBP (López Emparán et al. 2014). Four sequential dehydrogenation and two-isomerization increase the number of conjugated double bonds and transform 15-*cis*-phytoene into all-*trans*-lycopene (or simply lycopene), a pink colored carotenoid. In summary, during desaturation, phytoene is successively transformed to phytofluene, ζ-carotene, neurosporene and lycopene, with the enzymes phytoene desaturase (PDS), 15-*cis*-ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO) being involved in these reactions (Ruiz-Sola and Rodríguez-Concepción 2012).

The regulation of the CBP genes during transcription is critically important for the syntheses of photosynthetic pigments and plant hormones (Stanley and Yuan 2019). The CBP genes have been identified and characterized in several higher plants such as Arabidopsis, Chinese cabbage, citrus, *Ixeris dentate*, papaya, *Scutellaria baicalensis*, strawberry, and wolfberry (Tan et al. 2003; Kato et al. 2004; Devitt et al. 2010; Tuan et al. 2012; 2014; Zhu et al. 2015; Li et al. 2020; Reddy et al. 2017). The pathway for the biosynthesis of carotenoids in bryophytes has yet to be clarified. To date, there have only been few studies analysing CBP genes in the bryophytes, e.g. in *Physcomitrium patens* and *Marchantia polymorpha* (Takemura et al. 2014; He et al. 2019). Our preliminary analysis of the antioxidant activity and concentration of carotenoids in *Dicranum scoparium*, *Pleurozium schreberi*, *Hylocomium splendens*, and *Sphagnum magellanicum* indicated that the greatest antioxidant activity and highest concentration of carotenoids was observed in the moss *H. splendens* (unpublished data). This moss species was therefore chosen to study CBP genes.

Hylocomium splendens Hedw. is one of the most common and widespread mosses of the circumboreal forest and Arctic tundra, covering large areas of Canada, Alaska, northern Europe, and Siberia. To date, the full genome of *H. splendens* has not been sequenced yet. Moreover, CBP genes in *H. splendens* were not identified and characterized and their expression has not been studied. Therefore, here we aimed for identifying and characterizing the CBP genes (*HsPSY*_1, *HsPSY*_2, *HsPDS*, *HsZ-ISO*, *HsZDS*, *HsLCYb*, *HsCHYb*_1, *HsCHYb*_2, *HsLUT1*_1, *HsLUT1*_2) in the moss *H. splendens*. Gene expression following treatment of *H. splendens* with low negative (-20 °C) and elevated (+30 °C) temperatures, dehydration/rehydration was analyzed using quantitative reverse transcription-polymerase chain reaction (RT-qPCR). We hypothesized that differential expression of certain CBP genes in response to abiotic stresses can contribute to the targeted and timely defense of *H. splendens* against a particular unfavorable environmental factor.

2 Material and methods

2.1 Plant material

Hylocomium splendens Hedw. was collected in the Aisha Forest in Tatarstan, Russia (55°53 21.3 N 48°38 14.3 E). Plant material was placed between sheets of paper and left to dry slowly in the open air for 2 d before being stored in the refrigerator at +4 °C in the dark until use (Onele et al. 2022).

2.2 *In silico* identification of CBP genes

Metatranscriptome data of the moss *H. splendens* found in the Sequence Read Archive (SRA) NCBI GenBank database under the accession number SRR2518082 (Johnson et al. 2016) were used to *in silico* identify CBP genes. The *de novo* transcriptome assembly was constructed as reported in Onele et al. (2022). Sequences of CBP genes from *P. patens* were used as queries to search against *H. splendens* transcript/CDS databases using the BLAST + program (Camacho et al. 2009). To confirm the CBP genes members the predicted sequences were submitted against BLAST NCBI, BLAST Phytozome, NCBI Conserved Domains Da tabase (CDD), NCBI Conserved Protein Domain Family (Marchler-Bauer et al. 2017). The specific genes were identified and subjected to NCBI's open reading frame (ORF) finder program to identify whether the gene possesses the full ORF with a maximum nucleotide length.

2.3 Cloning of CBP genes

Total *H. splendens* RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). First strand and double strand cDNA were synthesized using Evrogen Mint 2 synthesis kit according to manufacturer's protocols (Evrogen, Moscow, Russia). cDNA was obtained in standard PCR with Taq polymerase (Evrogen) and specific primers listed in Table S1. The optimal annealing temperature was selected experimentally. The size of the PCR products was confirmed by agarose gel electrophoresis, cDNA of the expected sizes were eluted from gel and cloned into the pAL2-T vector (Evrogen).

2.4 Sequence analysis

Plasmid DNA containing cDNA insert (200 ng) of each gene was used for sequencing. The Sanger reaction was performed with the Big-Dye Terminator v3.1 Cycle Sequencing kit (Thermo Scinetific, Waltham, USA). The reaction was carried out using M13-For and M13-Rev primers. BLASTN software available online at (BLAST: Basic Local Alignment Search Tool (nih.gov)) was used to perform a homology search to compare the sequenced gene with other genes in the database. Files in Fasta format were downloaded from the NCBI database after BLAST search and then subjected to sequence alignments.

2.5 Alignment and phylogenetic analysis

EMBOSS web service (EMBOSS Transeq > Sequence Translation Sites > EMBL-EBI) was used to translates nucleic acid sequences of the *H. splendens* CBP cDNA to their corresponding peptide sequences. BLASTP software available online at (BLAST: Basic Local Alignment Search Tool (nih.gov) was used to perform a homology search in the database of predicted protein sequences with proteins from other Bryophyta species. The homologous sequences of CBP proteins obtained after BLASTX and other known CBP proteins from the NCBI database were aligned by ClustalW (Thompson et al. 1994) in MEGA X (Kumar et al. 2018). A phylogenetic tree was constructed in MEGA X using the neighbor joining method for 1500 bootstraps (Saitou and Nei 1987).

2.6 Stress treatments

Before the experiment, 2 cm apical stem segments of dry mosses were prehydrated at +4 °C for 24 h on wet filter paper in the dark. For stress treatments, we followed the protocol developed in our early studies (Onele et al. 2022). Hydrated apical stem segments were thermally stressed by their exposure to − 20 °C or +30 °C during 12 h in a dark temperature-controlled chamber (Thermostat LOIP, St. Petersburg, Russia). For gene expression analysis, moss samples were taken after 1, 3, 6, and 12 h of temperature treatments. For each time point of temperature treatments, hydrated moss, which was kept at room temperature (+22 °C), served as a control.

For desiccation stress, three biological replicates per treatment were used, each containing 0.1 g dry mass from 2 cm apical stem segments. Initially, air-dry mosses were fully hydrated by immersing them in a 20 mL volume of distilled water for 1 h while slowly shaking them on an orbital shaker. Then, the hydrated moss was gently blotted with filter paper and placed in the desiccator above silica gel. After 12 h of desiccation, moss samples were rehydrated for 1 h. For gene expression analysis, moss samples were taken after 12 h of hydration (control), 6 and 12 h of desiccation (D), and after rehydration (R). The change in relative water content (RWC) was monitored according to the protocol previously described in Onele et al. (2022).

2.7 RNA extraction, cDNA synthesis and RT-qPCR

Samples exposed to stresses were immersed in liquid nitrogen, then, each sample was ground into a fine powder. For RT-qPCR, 0.1 g of material from each replicate was immediately frozen in liquid nitrogen and stored at − 80 °C until use. Extraction of total RNA from *H. splendens* thalli was performed using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration and purity were measured with NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the integrity was further evaluated by gel electrophoresis in a 1% (w/v) agarose gel. First strand cDNA was synthesized using protocols from the Evrogen Mint 2 synthesis kit (Evrogen, Moscow, Russia).

For RT-qPCR, ribosomal RNA (18 S), glyceraldehyde-3-phosphate dehydrogenase (GAPDH2) and *a*-tubulin (*a*-TUB1 and *a*-TUB2) were used as internal controls. The vector NTI Suite 9 software was used to design RT-qPCR primers with the following parameters: amplicon length from 60 to 300 bp and a Tm range of 55 to 65 °C. Primers used in this study are shown in Table S2. RT-qPCR conditions used in our study followed the protocol described by Onele et al. (2022). Gene expression at each time point was calculated relative to the corresponding control using ΔCt method (Livak and Schmittgen 2001).

The visualization of expression analysis of CBP genes in the heatmap was conducted using an online heat mapper software ggplot R package.

2.8 Data analysis

Three biological and six analytical replicates were used to run all reactions. Gene expression differences were assessed using normalized expression (Cq) in the Bio-Rad CFX Maestro Software version 2.3 and were found to be significant for $p \leq 0.05$ (a), $p \leq 0.01$ (b), $p \leq 0.001$ (c) after ANOVA and Shapiro-Wilk Normality tests. The standard errors of the mean are shown as vertical bars ($n = 6$).

3 Results

3.1 *In silico* identification and sequence analysis of CBP genes

As the full genome of *H. splendens* is not sequenced, we found the coding sequence (CDS) of *H. splendens*, predicted based on transcriptome assemble (Johnson et al. 2016). ORFs corresponding to seven known CBP genes were determined using this cDNA library by the bioinformatic approach. One gene was identified for each of the transcripts encoding 15-*cis*-phytoene desaturase (PDS), zeta-carotene isomerase (Z-ISO), zeta-carotene desaturase (ZDS), lycopene beta-cyclase (LCYb). Two genes were identified for transcripts encoding phytoene synthase (PSY), *HsPSY*_1 and *HsPSY*_2, *β*-carotene-3-hydroxylase (CHYb), *HsCHYb*_1 and *HsCHYb*_2, as well as carotene epsilon monooxygenase (LUT1), *HsLUT1*_1 and *HsLUT1*_2.

3.2 Sequence analysis of CBP genes

For each ORFs found, cDNA was obtained, cloned, and sequenced. The sizes of the obtained cDNA products for *HsPSY_2*, *HsZ-ISO*, *HsPDS*, *HsCHYb*_1, *HsLCYb*, *HsLUT1*_1 and *HsLUT1*_2 corresponded to the sizes of transcripts predicted *in silico*, while the sizes of *HsPSY_1*, *HsCHYb*_2 and *HsZDS* cDNA differed from those of the predicted transcripts (Table 1). The *HsPSY1* cDNA sequence had a fragment corresponding to the *P. patens* PSY_2 mRNA (LOC112276781) that was missing from the bioinformatic transcript sequence, causing the cDNA to be larger than expected (Table 1).

Homology between the cDNA sequences of *HsPSY*_1 and *HsPSY*_2 was not found, however, their respective protein sequences obtained by bioinformatics coincided by 71%. Both *HsPSY*_1 and *HsPSY*_2 showed homology to *P. patens* phytoene synthase 2, chloroplastic-like mRNA, encoded by two different genes (LOC112276781 and LOC112276694, respectively) (Table 2). The sequences of *PpPSY2* (LOC112276781) and *PpPSY2* (LOC112276694) did not share any homology, but their respective protein sequences were 74% identical.

Table 1 List of cloned CBP cDNAs of *H. splendens*

Gene name		cDNA, bp	
		Predicted	Experimental
1a	*HsPSY_1*	970	1370
1b	*HsPSY_2*	1263	1261
2	*HsPDS*	1756	1840
3	*HsZ-ISO*	1188	1183
4	*HsZDS*	2419	1989
5	*HsLCYb*	1940	1789
6a	*HsCHYb_1*	1164	1176
6b	*HsCHYb_2*	1655	1160
7a	*HsLUT1_1*	1852	1981
7b	*HsLUT1_2*	1911	1884

The *HsCHYb*_1 and *HsCHYb*_2 cDNA nucleotide sequences and their respective protein sequences were 75% and 67% identical, with both predicted proteins showing homology to the same *P. patens* beta-carotene-3-hydroxylase 2, chloroplast-like protein (XP_024403077.1). *HsCHYb*_1 was homologous to *PpCHY* mRNA (LOC112295550), while *HsCHYb*_2 was homologous to *PpCHY* mRNA (LOC112273933) (Table 2). Both *PpCHY* transcripts sequences were 80% identical and their respective protein sequences were 100% identical. Thus, these two *HsCHYb* genes may be duplicates encoding the same protein.

No homology was found between the cDNA sequences of *HsLUT1*_1 and *HsLUT1*_2, while their respective protein sequences showed low (42%) homology. *HsLUT1*_1 cDNA and predicted protein were homologous to *P. patens* carotene epsilon-monooxygenase (CYP97C1), chloroplastic-like, mRNA (LOC112283348) and protein (XP_024377702.1) by 83 and 87% respectively (Table 2). *HsLUT1*_2 cDNA and predicted protein were homologous to *P. patens* cytochrome P450 97B2, chloroplastic-like mRNA (LOC112281394) and protein (XM_024517858.1) by 85% and 87%,

Table 2 Homology of CBP cDNAs and predicted proteins of *H. splendens* with four moss species

	Gene name	Homology							
		Physcomitrium patens		*Ceratodon purpureus*		*Rhytidiadelphus loreus*		*Thuidium tamariscinum*	
		cDNA	Protein	cDNA	Protein	cDNA	Protein	cDNA	Protein
1	*HsPSY_1*	82%	82%	85%	92%	97%	–	90%	–
2	*HsPSY_2*	81%	79%	83%	82%	98%	–	94%	–
3	*HsPDS*	85%	88%	86%	90%	98%	–	96%	–
4	*HsZ-ISO*	82%	77%	84%	81%	98%	–	95%	–
5	*HsZDS*	85%	84%	84%	93%	95%	–	95%	–
6	*HsLCYb*	80%	–	84	–	95%	–	96%	–
7	*HsCHYb_1*	79%	71%	83%	74%	95%	–	91%	–
8	*HsCHYb_2*	81%	68%	81%	68%	96%	–	–	–
9	*HsLUT1_1*	83%	87%	85%	84%	95%	–	–	–
10	*HsLUT1_2*	85%	87%	87%	90%	97%	–	–	–

respectively. *P. patens* CYP97C1 enzyme and cytochrome P450 97B2 had 43% homology. Thus, *HsLUT1*_1 is a gene involved in CBP, while the *HsLUT1*_2 gene most likely performs a different function.

Blast analysis of the cloned CBP cDNA from *H. splendens* revealed the sequence homology of all these transcripts with the corresponding transcripts of two mosses whose genomes have been completely sequenced, *P. patens* and *Ceratodon purpureus*, ranging from 81 to 87% (Table 2). All cloned *H. splendens* cDNAs showed also strong homology ranging from 90 to 98% to the genomic DNA of two mosses, namely, *Rhytidiadelphus loreus* and *Thuidium tamariscinum*, although this was limited by the scores of matches identified.

BLASTp alignment of the predicted peptide sequences also confirmed the extended homology of the *H. splendens* CBP proteins with the corresponding proteins of *P. patens* ranging from 68% (HsCHYb_2) to 88% (HsPDS) and of *C. purpureus* ranging from 68% (HsCHYb_2) to 92% (HsPSY_1) (Table 2). Interestingly, one of the predicted proteins, HsLCYb, showed no homology with the lycopene beta-cyclase protein sequences of these two mosses. The protein sequences of *R. loreus* and *T. tamariscinum* protein sequences are not available in the database.

We performed analyses of the protein sequence using online services. Physico-chemical properties such as protein length (aa), molecular mass (M_W, kDa) and isoelectric point (pI) were predicted (Table 3). All CBP proteins of *H. splendens* were predicted to be targeted to the chloroplast (CP).

3.3 Phylogenetic and homology analyses

To investigate molecular evolutionary relationships between CBP proteins in *H. splendens* and those in other bryophytes, neighbor-joining phylogenetic trees were constructed using all ten CBP sequences and a set consisting of each specific CBP sequence. Fig. S1 and Table S3 describe the phylogenetic relationships of the retrieved homologous peptide sequences. Phylogenetic analysis showed that the CBP proteins are highly conserved within bryophytes. HsPSY_1/HsPSY_2, HsCHYb_1/HsCHYb_2 and HsLUT1_1/HsLUT1_2 isoforms form clades with *Ceratodon purpureus* hypothetical proteins and *P. patens* respective isoforms. HsPDS or HsZDS belong to a clade with two homologous *C. purpureus* hypothetical proteins, each of which has common ancestor with respective *P. patens* 15-cis-phytoene desaturase or zeta-carotene desaturases. HsZ-ISO belongs to *P. patens* 15-cis-zeta-carotene isomerase clade with common *C. purpureus* ancestor. *S. magellanicum* / *S. fallax* and *Marchantia paleacea* / *M. polymorpha* hypothetical proteins are descendants of more remote ancestors for these groups of the proteins. HsLCYb is in a clade with *S. magellanicum* and S. *fallax* hypothetical proteins but this clade has also common ancestors with *P. patens* lycopene beta cyclase and *C. purpureus* hypothetical proteins.

Table 3 Physico-chemical properties and subcellular localization of CBP proteins in *H. splendens*[*]

Gene names	Full names	ORF (bp)	Protein (aa)	M_W (kDa)	Theoretical pI	Subcellular localization predictions
HsPSY_1	Phytoene synthase isoform 1	1255	477	53.5	8.02	CP
HsPSY_2	Phytoene synthase isoform 2	1263	488	54.67	7.55	CP
HsPDS	15-cis-phytoene desaturase	1677	558	62.07	5.62	CP
HsZ-ISO	Zeta-carotene isomerase	1188	396	44.1	8.37	CP
HsZDS	Zeta-carotene desaturase	1781	593	64.34	8.63	CP
HsLCYb	Lycopene beta-cyclase	1565	521	57.84	11.02	CP
HsCHYb_1	Beta-carotene 3-hydroxylase isoform 1	1008	336	36.89	6.8	CP
HsCHYb_2	Beta-carotene 3-hydroxylase isoform 2	1008	335	36.67	8.27	CP
HsLUT1_1	Carotene epsilon-monooxygenase isoform 1	1701	566	62.41	6.61	CP
HsLUT1_2	Carotene epsilon-monooxygenase isoform 2	1761	586	65.53	6.93	CP

[*]*ORF* open reading frame; *bp* base pair; *aa* amino acid; *MW* molecular mass; *pI* isoelectric point; *CP* chloroplast

3.4 Expression of CBP genes of *H. splendens* under different stress treatment

RT-qPCR analysis was used to analyze the expression patterns of CBP genes in response of *H. splendens* to different abiotic stress treatments such as exposure to elevated (+30 °C) and freezing (− 20 °C) temperatures and desiccation/rehydration cycles. PCR analysis revealed the presence of CBP transcripts in control plants. The results showed that all CBP genes were differentially expressed in *H. splendens* depending on the type of stress (Figs. 1, 2, 3 and 4). Expression of the CBP genes in moss at low negative temperature remained practically unchanged (Fig. 1), except for *HsLUT1_2*, which expression increased almost five times after 1 h treatment (Fig. 1).

Expression of CBP genes of *H. splendens* exposed to high (+30 °C) temperature differed from expression of these genes exposed to freezing temperature. Among these, the highest expression was observed for *HsPSY*_2, both isoforms of *HsCHYb* and *HsLUT1*_2 under high temperature treatment (Fig. 2), whereas the lowest expression was found for *HsZ-ISO*. Transcription of *HsPSY*_2 increased significantly by 3 h and gradually decreased during 6–12 h high temperature condition. Transcription of *HsZDS* also demonstrated the same trend as of *HsPSY*_2. Transcription of *HsCHYb_1* and *HsCHYb_2* gradually increased

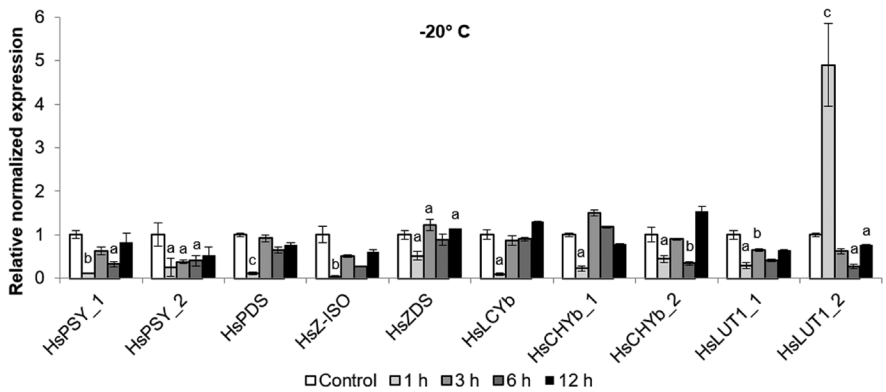

Fig. 1 qPCR analysis of the time-course of CBP gene expression in *H. splendens* exposed to − 20 °C. For each time point of temperature treatments, hydrated moss, which was kept at room temperature (+22 °C), served as a control. The transcripts concentration in the control was taken as one. $p \leq 0.05$ (**a**), $p \leq 0.01$ (**b**), $p \leq 0.001$ (**c**) denote significant differences between the control and treatments according to ANOVA ($n = 6$)

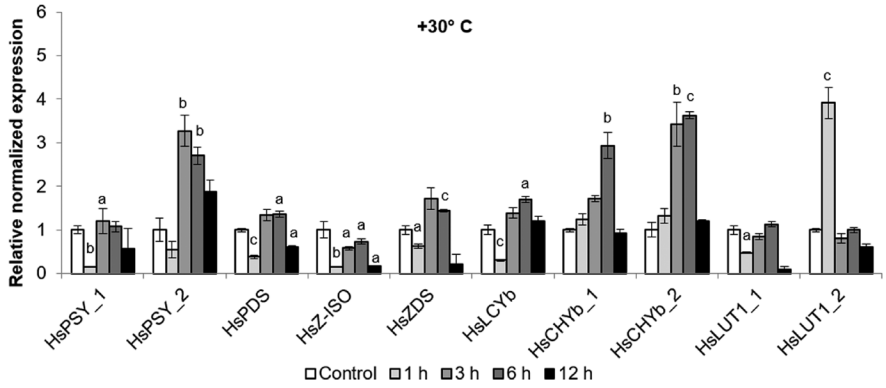

Fig. 2 qPCR analysis of the time-course of CBP gene expression in *H. splendens* exposed to +30 °C. For each time point of temperature treatments, hydrated moss, which was kept at room temperature (+22 °C), served as a control. The transcripts concentration in the control was taken as one. $p \leq 0.05$ (**a**), $p \leq 0.01$ (**b**), $p \leq 0.001$ (**c**) denote significant differences between the control and treatments according to ANOVA ($n = 6$)

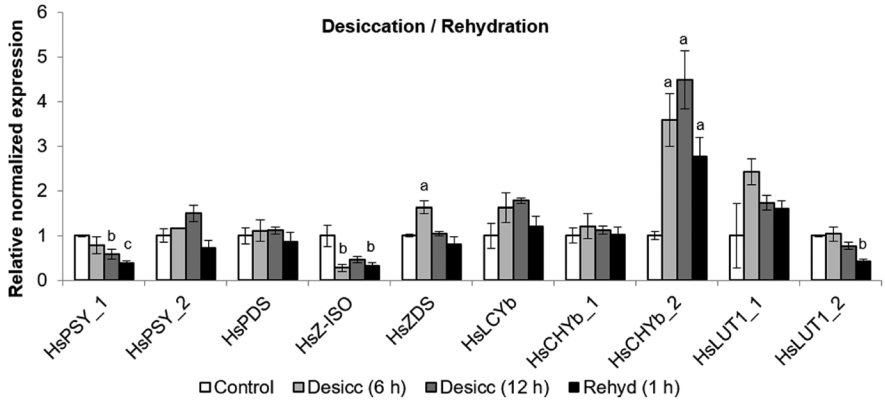

Fig. 3 qPCR analysis of the time-course of CBP gene expression in *H. splendens* during desiccation over silica gel and rehydration. For each time point of temperature treatments, hydrated moss, which was kept at room temperature (+22 °C), served as a control. The transcripts concentration in the control was taken as one. $p \leq 0.05$ (**a**), $p \leq 0.01$ (**b**), $p \leq 0.001$ (**c**) denote significant differences between the control and treatments according to ANOVA ($n = 6$)

during 1–3–6 h and declined sharply after 12 h high temperature treatment. Interestingly, *HsLUT1*_2 showed the highest expression after short-term (1 h) exposure to both low negative and high temperatures (Fig. 2).

Desiccation of the hydrated moss over silica gel resulted in a considerable drop in water content in the moss samples, down to 43.4% and 5.6% water content after 6 and 12 h of treatments, respectively. It was found that the downstream CBP genes are more sensitive to desiccation and rehydration cycles. Indeed, desiccation of the moss showed a gradual increase in *HsCHYb*_1 expression to 3.5 times after 6 h and 4.5 times after 12 h. Rehydration of the moss during 1 h after 12 h desiccation showed a slight decrease in *HsCHYb*_1 expression (Fig. 3). In addition, a slight increase in expressions was observed for *HsZDS*, *HsLCYb* and *HsLUT1*_1. Interestingly that *HsLUT1*_1 was only sensitive to this stress treatment.

4 Discussion

H. splendens is one of the most common and widespread mosses of Northern Hemisphere. The genome of this moss is not sequenced, and there have been no

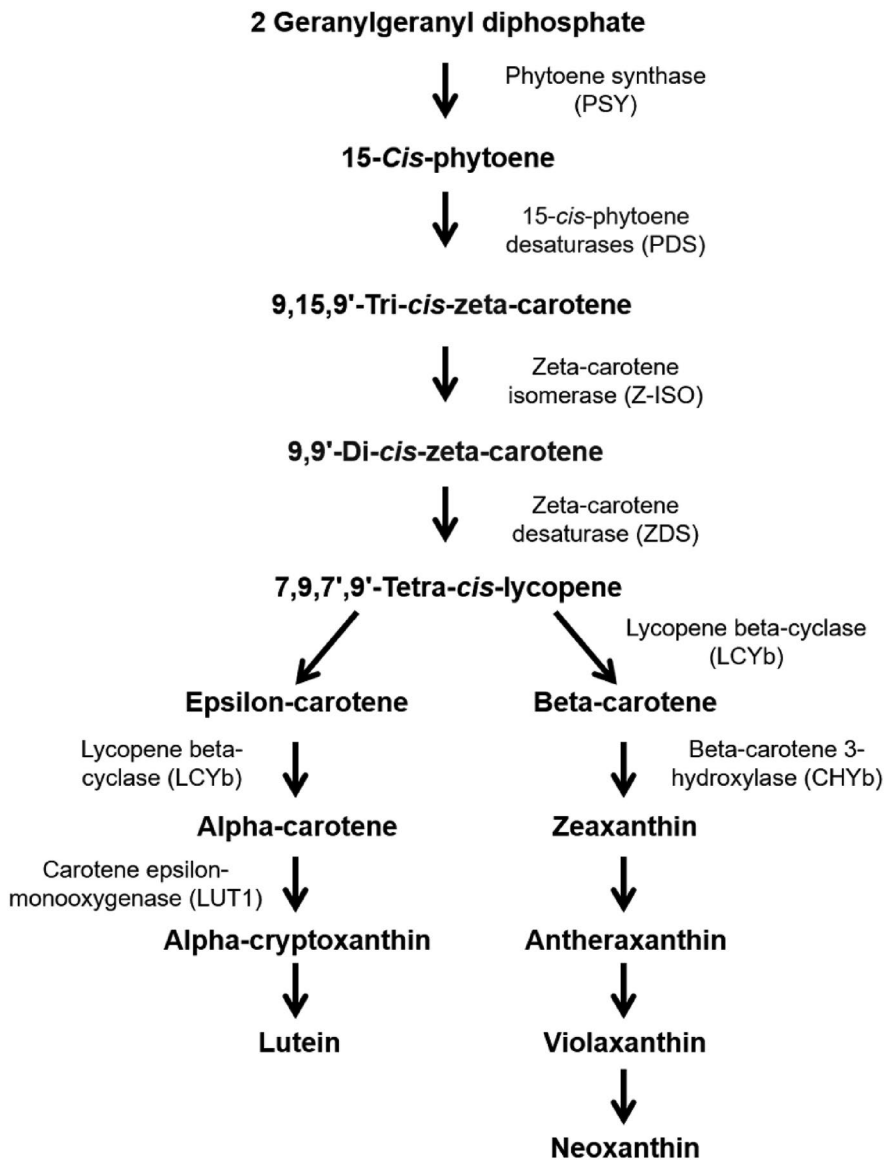

Fig. 4 Scheme of the CBP in *H. splendens*. In this scheme, enzymes whose genes are identified and characterized in this study are presented

reports on the CBP genes of this species. The present study is the first report to identify and characterize the CBP genes in the moss *H. splendens*. As a result of cloning, sequencing and *in silico* analysis, we identified and characterized ten CBP genes in *H. splendens* with a full ORF. Scheme illustrates CBP enzymes whose genes were identified in present work in *H. splendens* (Fig. 4).

Using multiple alignments and phylogenetic and homology analyses, we demonstrated that CBP gene sequences in *H. splendens* share high similarity with such sequences in other bryophytes. Prediction of subcellular localization suggests chloroplast localization of most CBP proteins of *H. splendens*. Differential expression of CBP genes demonstrates a stress-responsive patterns at the transcriptional with most CBP genes being highly upregulated during early stress response. A heatmap provides graphical representations of the expression profiles of CBP genes of *H. splendens* across the samples (Fig. 5).

The ten genes encoding seven enzymes of CBP were characterized, because three genes occur with two isoforms (*HsPSY*_1 and *HsPSY*_2; *HsCHYb*_1 and *HsCHYb*_2; *HsLUT1*_1 and *HsLUT1*_2). Most

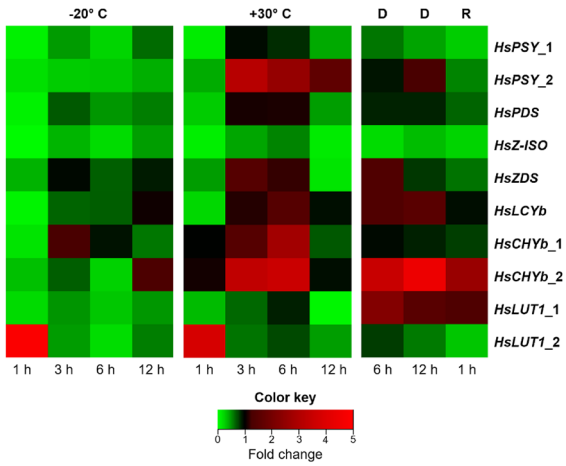

Fig. 5 Heatmap of the expression profiles of CBP genes of *H. splendens* exposed to low (− 20 °C) and high temperature (+30 °C), desiccation (D) and rehydration (R). The heatmap was constructed using fold-change values gained from RT-qPCR. Heatmap was plotted using ggplot R package. Down-regulated gene expression is indicated by green, while upregulated gene expression is indicated by red

genes generate multiple mRNA isoforms as a result of alternative splicing, intron retention, and alternative transcription start/stop sites. The result is a diversity of mRNA sequences, yielding isoforms that often differ in their protein-coding capacity. Isoforms with very similar sequences can have substantially different morphological outcomes that will reflect a host of gene expression changes in different organs of the plant (Syed et al. 2012). Analysis of the sequences of isoforms of CBP genes in *H. splendens* indicates similar sequences of ORFs and differ in 5′ and 3′ untranslated regions, which is characteristic of isoforms; in addition, they have differential expression patterns under abiotic stresses (Fig. 5).

Sequence homology analysis of the CBP genes of *H. splendens* exhibits high similarity with those of other bryophytes, more than 80% with sequences of *P. patens* and *C. purpureus*, as well as significant homology with the DNA genomes of *R. loreus* and *T. tamariscinum* (Table 2). The genera *Hylocomium* and *Rhytidiadelphus* are the parts of the family *Hylocomiacea*, and together with the genus *Thuidium* they belong to the order *Hypnales*, a group of feather mosses that is abundant and young compared to others (Merget and Wolf 2010). We demonstrate that the CBP genes of bryophytes are highly conserved and share higher sequence identities with vascular plants. *T. tamariscinum* exhibits an almost cosmopolitan distribution, as does *C. purpureus*, which grows in a wide range of habitats, mainly in urban areas and next to roads on dry sand soils. In contrast, *R. loreus* grows on decaying logs, the forest floor and as an epiphyte on living trees, and is considered to be a key component of a healthy, thriving forest ecosystem, serving as a water-retaining and thermal insulator. Genera *Ceratodon*, *Marchantia* and *Sphagnum* all belong to the other than *H. splendens* orders (respectively, *Dicranales*, *Marchantiales* and *Sphagnales*), and inhabit very different ecosystems. *C. purpureus* grows in a very wide variety of habitats, mainly in urban areas and next to roads on dry sand soils, Sphagnum mosses occur mainly in the Northern Hemisphere in peat bogs, conifer forests, and moist tundra areas, *M. polymorpha* is a widespread plant often found beside rivers. Thus, despite the wide distribution of these bryophytes in very different ecosystems, homology analysis of CBP gene suggests that these genes are universal and conserved.

Analyses of the physico-chemical properties and the subcellular localization indicate that all CBP proteins of *H. splendens* share a high similarity with those of vascular plants. In *A. thaliana*, sweet potato, and in some other vascular plants, most CBP enzymes are localized within the chloroplasts (Han et al. 2015; Kang et al. 2018). Prediction of subcellular location of all CBP proteins of *H. splendens* also suggests a chloroplast localization (Table 3).

To understand the evolutionary relationships between CBP proteins of *H. splendens* and well-known CBP proteins from bryophytes, neighbor-joining trees were constructed using the deduced amino acid residues of these CBP proteins (Fig. S1 and Table S3). Analyses of these trees demonstrate that the CBP proteins of *H. splendens* are highly homologous to other CBP proteins in bryophytes.

Recently, some studies indicated that overexpression of the CBP genes results in an increased concentration of carotenoid and improved the tolerance of plants to abiotic stresses. For example, overexpression of IbZDS (Li et al. 2017) and *IbLCYb* (Kim et al. 2014) genes enhanced salt stress tolerance of transgenic sweet potato plants, while *Lycium chinenses* plants overexpressing *LcLCYe* displayed enhanced tolerance to chilling stress (Chen et al., 2015). In transgenic tobacco, overexpression *LcPDS*, *LcZDS*, or *LcCRTISO* led to an increased carotenoid

concentration in leaves and changed the ratio between various carotenoids (Li et al. 2020). This study indicated that *LcPDS*, *LcZDS*, and *LcCRTISO* are able to improve carotenoid concentration and salt tolerance in higher plant breeding.

It is known that isoforms of some genes can adopt new functions, with differential expression patterns. For example, in rice, both *OsPSY1* and *OsPSY2* containing light responsive *cis*-acting elements play predominant roles in carotenogenesis in green tissues, whereas *OsPSY3* is induced in the roots by high salt and/or drought stresses (Welsch et al. 2008). In the moss *H. splendens*, the *HsPSY*_1 and *HsPSY*_2 transcripts differ when moss experiences abiotic stresses (Fig. 5). Stresses do not change the expression of *HsPSY*_1, while the *HsPSY*_2 transcripts change in response to high temperature and desiccation (Fig. 5). The highest expression is observed for *HsPSY*_2 in response to high temperature treatment (Fig. 2). According to Busch et al. (2002), the overexpression of *NtPSY* significantly accelerates the biosynthesis of carotenoids and promotes accumulation of extra carotenoids in tobacco leaves. Interestingly, *HsPDS* and *HsZDS* transcripts involved in desaturation of phytoene do not change significantly under abiotic stresses (Figs. 1, 2 and 3). It is known that the dysfunction of these genes results in the deficiency of carotenoid accumulation and can lead to the death of the plants (Wang et al. 2009). Thus, among upstream CBP genes, *HsPSY* is a stress inducible gene, while *HsPDS*, *HsZ-ISO* and *HsZDS* are more stable in stress conditions.

Differential expression of CBP transcripts during abiotic stresses was more evident for genes in the middle and downstream steps of CBP. These genes encode the enzymes responsible for carotenoid diversity, such as β-carotene, violaxanthin, zeaxanthin, lutein, and neoxanthin, as a result of the activities of lycopene β-cyclase (LCYb), ε-cyclase (LCYe), and β-carotene hydroxylase (CHYb) (Bouvier et al. 2000; Kim and DellaPenna 2006; Zhu et al. 2008). As known, zeaxanthin, all-trans-violaxanthin, and neoxanthin are carotenoid precursors of ABA (Du et al. 2010). Another pattern of expression is observed for other pair of isoforms of CBP genes in *H. splendens*. Indeed, *HsCHYb*_1 and *HsCHYb*_2 respond similarly to temperature stress, in particular both are activated by elevated temperature but do not respond to freezing temperature. In contrast, these genes display differential response to desiccation and rehydration, e.g. *HsCHYb*_1 is not activated, while *HsCHYb*_2 is activated by this treatment. Overexpression of *CHYb* gene was shown to increase carotenoid concentration in tobacco (Hasunuma et al. 2008), tomato (Huang et al. 2013), maize (Farre et al. 2016), and potato (Morris et al. 2006). Furthermore, previous studies in *Arabidopsis* with overexpressed *CHYb* reported that these plants were more tolerant of high temperature and high light (Davison et al. 2002).

In the next pair of isoforms *HsLUT1*_1 and *HsLUT1*_2, the first is activated only during dehydration, while the second responds only to the short-term temperature stress (Fig. 5). It is possible that these gene isoforms contain different stress-sensitive *cis*-elements in promoter regions that can explain their differential stress induced expression and suggest the performance of various functions during carotenogenesis. Moreover, considering that moss *H. splendens* was collected in the woods, the potential contribution of endophytic bacteria to the gene expression can not be excluded, although such contribution would be minimal for the expression of genes in the apical stem segments.

Thus, an increase in CBP transcripts of xanthophyll cycle during elevated temperature, desiccation and rehydration may be related to the roles of xanthophylls in plant stress tolerance and involvement in ABA synthesis. Stresses will increase xanthophyll cycle activity because extra energy dissipation is needed when carbon fixation is reduced but light interception continues. High conservation of CBP genes during evolution indicates their essential role in carotenogenesis in photosynthetic organisms.

5 Conclusion

In the present study, ten genes involved in the carotenoid biosynthesis pathway in *H. splendens* were identified and found to share high similarity with those in other bryophytes. Adverse temperatures and desiccation/rehydration treatment induce the expression of some of these CPB genes, providing insights into the roles of CBP genes in response to abiotic stresses and expanding our understanding of the molecular mechanisms regulating carotenoid biosynthesis in mosses. Future research is needed to enable us to evaluate how differential expression of CBP genes can influence

the total carotenoid content and the concentrations of individual carotenoids in *H. splendens* in response to stress treatment.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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