Heterologous expression of *HpBHY* and *CrBKT* increases heat tolerance in *Physcomitrella patens*

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1. Introduction

Plants experience various kinds of stress during their life cycles, such as drought, salt, and high temperature. With the effects of global warming, high temperature has become an increasingly severe problem for plants (Ding et al., 2018). High temperature adversely affects photosynthesis (Fan et al., 2017), hormone homeostasis (Maestri et al., 2002), and secondary metabolite contents (Wahid et al., 2007). Plants have evolved a number of responses to heat stress, evidence suggests that carotenoid accumulation and ABA and SA signaling appear to mediate plant defense (Wahid et al., 2007; Du et al., 2010). However, understanding how these processes interact to mediate plant defense against heat stress remains a major challenge in botany.

Previous studies have shown that plant stress defense is mediated by carotenoids and enzymes in the carotenoid biosynthetic pathway. Two key enzymes in the carotenoid biosynthetic pathway are \(\beta\)-carotene hydroxylase and \(\beta\)-carotene ketolase. \(\beta\)-Carotene hydroxylase, which is common in photosynthetic plants, catalyzes the conversion of \(\beta\)-carotene to zeaxanthin (Davison et al., 2002); \(\beta\)-carotene ketolase, which is present only in some bacteria and green algae, catalyzes the conversion of \(\beta\)-carotene to canthaxanthin (Mann et al., 2000; Huang et al., 2013). Overexpression of these genes has been shown to increase carotenoid levels in tobacco (Hasunuma et al., 2008; Mann et al., 2000), tomato (Huang et al., 2013), maize (Farré et al., 2016), and potato (Morris et al., 2006). Furthermore, previous studies that overexpressed \(\beta\)-carotene hydroxylase in *Arabidopsis* reported that the resulting plants were more tolerant of high temperature and high light (Davison et al., 2002).
Similarly, overexpressing a bacterial β-carotene ketolase in Arabidopsis increased plant tolerance of high light (Zhong et al., 2011). However, few studies have examined whether carotenoids respond to only high temperature stress, and if so, by what mechanism these molecules mediate plant response to high temperatures.

One promising model for examining the mechanisms of heat stress response in plants is the moss Physcomitrella patens. Mosses are small, as well as easy to cultivate and propagate (Schafer, 2001). In addition, moss growth is limited by high-temperature stress. P. patens is the only known plant that can be efficiently genetically modified by homologous recombination (Decker and Reski, 2004), and this high gene-targeting efficiency has made it a model plant for functional genomic studies (Schafer and Zryd, 2001). P. patens is tolerant of a number of abiotic stresses, including drought, salinity (Frank et al., 2005), and cold (Minami et al., 2005). However, few reports have examined its behavior under high-temperature stress.

In this study, we asked whether β-carotene hydroxylase and β-carotene ketolase increase heat stress tolerance in plants and, if so, what molecular mechanisms underlie this response. To answer these questions, we expressed a β-carotene ketolase gene from Chlamydomonas reinhardtii (CrBKT) and a β-carotene hydroxylase gene from Haematococcus pluvialis (HpBHY) in P. patens and subsequently examined plant response to heat stress.

2. Materials and methods

2.1. Culture conditions and treatment

Wild-type P. patens (Gransden 2004) plants (Li et al., 2017) were used for all experiments described in this study. All plants were grown on BCDAT medium, under a light cycle of 16 h light (60–80 µmol photons m⁻² s⁻¹) and 8 h dark at 25 °C; 40-day-old colonies were used for all experiments. For high-temperature treatments, 40-day-old plants were transferred to 45 °C under normal light intensity for 4 h, then allowed to recover under the normal conditions described above.

2.2. Phylogenetic analyses of BHY and BKT

The amino acid sequences of HpBHY and CrBKT were obtained by translating the coding sequences (CDS) of HpBHY and CrBKT, which were downloaded from the National Center for Biotechnology Information (NCBI) (accession numbers BD250390 and AY860820, respectively), with the translate tool in the ExPaSy web server (Artimo et al., 2012) (https://web.expasy.org/translate/). To analyze the relationship of β-carotene hydroxylase and β-carotene ketolase, multiple sequence alignments of β-carotene hydroxylase and β-carotene ketolase amino acid sequences were conducted with the ClustalW program of MEGA6 software, the trees were constructed with MEGA6 software using the neighbor-joining method and the significance of the branching order was tested by bootstrapping (1000 replicates). Trees were visualized with iTOL (Ivica and Peer, 2016).

For β-carotene hydroxylase, we analyzed 30 amino acid sequences from 14 viridiplantae species, including 8 embryophyte and 6 chlorophyte species. For β-carotene ketolase, 16 species were analyzed, including 8 chlorophyte species and 7 marine bacterium species and one fungus.

2.3. Plasmid construction, in vitro translation, and protein import

The coding sequences of HpBHY and CrBKT were synthesized according to sequence information in GenBank (accession numbers BD250390 and AY860820, respectively). The gene sequence encoding a modified Pisum sativum RUBISCO chloroplast transit peptide (tp22) (Shi and Heg, 2013) was added before the sequences of HpBHY and CrBKT for targeting of the expressed proteins to chloroplasts. Then, these sequences were each inserted into the Nool-Sall sites of the vector pPOG1 under the control of the PpEF1-α promoter.

For the moss import assay, chloroplasts were isolated from 7-day-old moss protonema as previously described (Liu and Theg, 2014). In vitro translation was performed in a wheat germ system (Promega or tRNA Probes) in the presence of [35S]-leucine and other non-radioabeled amino acids as described (Lo and Theg, 2011). For in vitro protein import assays, moss chloroplasts were incubated with the resulting radiolabeled precursor proteins for 30 min in the presence of 3 mM ATP, and the reaction was stopped by adding 600 µl import buffer (330 mM sorbitol, 50 mM Tricine, and 3 mM MgCl₂, pH 8.0). The chloroplasts were pelleted by centrifugation at 3000 g for 5 min, resuspended in sample buffer (50 mM Tris–HCl at pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue), boiled for 10 min, separated by SDS-PAGE, and visualized by fluorography. Thermolysin treatments were performed as previously describe (Lo and Theg, 2011).

2.4. Moss transformation and genotyping

The generation of transgenic plants was performed using the polyethylene glycol (PEG)-mediated transformation method as described by Liu and Vidal (2011). For genotyping, moss genomic DNA was isolated using a CTAB method. HpBHY and CrBKT coding sequence amplification primers HpBHY-F/R and CrBKT-F/R (listed in Supplementary Table S1) were used for detection of HpBHY and CrBKT genes in P. patens, with 2 × MastTaq (Novoprotein, China) of 35 cycles of 94 °C for 20 s, 56 °C for 20 s, 72 °C for 30 s).

To detect the expression of HpBHY and CrBKT, total RNA was extracted using TRizol (Takara, Japan). For cDNA synthesis, 1 µg RNA was used following the manufacturer’s instructions for the PrimeScript™RT reagent Kit (Takara, Japan). The relative expression levels of HpBHY and CrBKT were measured using primers HpBHY-F/R (RT) and CrBKT-F/R (RT) (indicated in Supplementary Table S1) by semi-quantitative RT-PCR analysis, which was carried out with 2 × TaqMan (Novoprotein, China); the endogenous moss actin gene served as the reference gene (Vera et al., 2017). The quantification of gel bands was performed with the software ImageJ. The data were plotted in GraphPad Prism 6.

2.5. Pigment analysis

Photosynthetic pigments were monitored by following the method described by Huang et al. (2011) with some modification. Pigments were isolated from the leafy gametophores of 40-day-old P. patens with the extract buffer which contain 50% acetone and 50% ethanol, and measured with an Infinite² 200 PRO (TECAN, Austria) through absorbance at 665, 642.5, 470, 485, and 474 nm. The contents of chlorophyll a, chlorophyll b, carotenoid and lutein were calculated as follows: C(Chlorophyll a) = 9.99 A₆₆₅ − 0.0872 A₆₄₂₅; C(Chlorophyll b) = 17.7 A₆₄₂₅ − 3.04 A₆₆₅; C(Lutein) = 10.2 A₄₇₀−11.5A₄₈₅−0.0036[A]−0.652[B]; C(Carotenoid) = 4.92A₄₇₄−0.0255[A]−0.225[B]. [A] in the formula indicates the concentration of chlorophyll a (mg/L), [B] indicates the concentration of chlorophyll b (mg/L).

2.6. Measurement of the maximal photochemical efficiency of PSII

The photochemical efficiency was estimated by measuring the maximal photochemical efficiency of PSII (Fₕ/Fₘ) of 40-day-old leafy gametophores with an IMAGING-PAM chlorophyll fluorometer and the Imaging Win software application (Walz, Germany).
After dark-adaptation of *P. patens* for 30 min, the $F_o/F_m$ was measured, where $F_o$ is the variable chlorophyll fluorescence yield and $F_m$ is the maximum chlorophyll fluorescence yield (Camejo et al., 2005).

2.7. Biochemical analysis

The amount of malonyldialdehyde (MDA) in the moss tissue, as an estimate of lipid peroxidation, was measured through a modification of the method of Cui and Wang (2006). About 0.3 g fresh tissue was ground in 3 mL 10% trichloroacetic acid (TCA) using a mortar and pestle. The homogenate was centrifuged at 12,000 $\times$ g for 10 min, 2 mL extract and 2 mL thiobarbituric acid (TBA) were mixed as the reaction mixture and was heated at 95 °C for 15 min, then cooled on ice quickly and centrifuged at 3000 $\times$ g for 10 min. The absorbance of the supernatant was determined at 532, 600, and 450 nm, respectively, with an Infinite® 200 PRO (TECAN, Austria). Equation $C_{MDA} = 6.45 (A_{532} - A_{600}) - 0.56 A_{450}$ was used to calculate the MDA content.

The activities of SOD and POD were measured by the methods of Meloni et al. (2003) and Fu and Huang (2001), respectively, with some modification. The enzymes were extracted with about 0.3 g fresh tissues and the samples were ground in 3 mL 50 mM sodium phosphate buffer containing 1% PVP (polyvinylpyrrolidone) using a mortar and pestle. The homogenate was centrifuged at 12,000 $\times$ g for 20 min to get the enzyme supernatant extract. For SOD, the reaction solution (3 mL) contained 0.3 mL of 0.75 mM NBT, 0.3 mL of 0.13 M methionine, 0.3 mL of 0.1 mM EDTA, 1.5 mL of 50 mM sodium phosphate buffer (pH 7.8), 0.05 mL distilled water, and 0.05 mL enzyme extract. Test tubes containing the reaction solution and enzyme extract were irradiated in a light incubator at 4000 lx for 10 min. The absorbances of the irradiated and non-irradiated solutions at 560 nm were determined with an Infinite® 200 PRO (TECAN, Austria). The amount of enzyme that would inhibit 50% of NBT photo-reduction was defined as one unit of SOD activity. For POD, the reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 1% guaiacol, 0.4% H$_2$O$_2$, and enzyme extract. Increase in the absorbance due to oxidation of guaiacol was measured at 470 nm for 1 min.

2.8. Measurement of plant phytohormones

Phytohormones (SA and ABA) were extracted from 0.3 g of frozen gametophores. Stable isotope-labeled CKs (0.01 ng) and phytohormones (SA 20 ng, ABA 0.6 ng) were added to the sample extraction buffer. Phytohormones were extracted and the content was determined by HPLC using the method described by Lee et al. (2015).

2.9. Statistical analysis

All experiments were performed with three or more biological replicates. Pigment contents and physiological indexes were analyzed by one-way analysis of variance; Tukey's multiple range test was used to identify the means of three or more groups; the significance thresholds were 0.05 (marked by *) and 0.01 (**). All data were analyzed with the software SPSS version 19.0 (International Business Machines Corporation, Armonk, NY, USA).

3. Results

3.1. *HpBHY* and *CrBKT* have special evolutionary positions

To investigate the functions of *β*-carotene hydroxylases and *β*-carotene ketolases, we first analyzed the evolutionary presence of *β*-carotene hydroxylases and *β*-carotene ketolases. *β*-carotene hydroxylases are ubiquitous among the viridiplantae, we analyzed 8 embryophyte and 6 chlorophyte species, such as *Dunaliella salina* and *Haematococcus pluvialis* (Fig. 1A). In contrast, in the 17 viridiplantae species analyzed, *β*-carotene ketolases are present only in some chlorophytes, marine bacterium, and the fungus *Xanthophyllomyces dendrorhous* (Fig. 1B), which is widely used for biotechnological production of carotenoid (Ye et al., 2015). Further, *β*-carotene ketolases are present in single copies in all species.

To examine carotenoid biosynthesis in *P. patens*, we chose to transgenically express *β*-carotene hydroxylase from *H. pluvialis* (*HpBHY*) and *β*-carotene ketolase from *C. reinhardtii* (*CrBKT*). *HpBHY* is closely related to *PpBHY*. *CrBKT* is most similar to the *β*-carotene ketolase from the fungus *X. dendrorhous* (*XdBKT*). In addition, both *HpBHY* and *CrBKT* are widely used in the carotenoid biosynthetic industry (Guerin et al., 2003).

3.2. Expression profiling of *β*-carotene hydroxylase and *β*-carotene ketolase under stress

To investigate the functions of *HpBHY* and *CrBKT*, we first analyzed available gene expression for these genes or their homologs under different abiotic stresses using data from public databases BAR (http://bar.utoronto.ca/) and JGI (https://jgi.doe.gov/). Specifically, we examined the expression of *HpBHY* homologs in response to various stressors in *Oryza sativa* (rice; Fig. 2A), *Arabidopsis* (Fig. 2B), *Glycine max* (soybean; Fig. 2C), and *P. patens* (Fig. 2D). In addition, we examined *CrBKT* expression in response to nitrogen deficiency and hydrogen peroxide treatment (Fig. 2E). The expression profiles of *HpBHY* homologs in *O. sativa*, *Arabidopsis*, *G. max*, and *P. patens* changed under abiotic stresses such as drought, cold, and heat (Fig. 2A–D). For example, the expression of the *P. patens* *β*-carotene hydroxylase homologs (*Pp3c21_16590V3.1 and *Pp3c19_16780V3.2*) increased between 4.3 and 5.3 times, respectively, in response to heat stress. In response to nitrogen deficiency treatment, *CrBKT* expression increased almost 2.5 times after 30 min. These results show that *β*-carotene hydroxylases and *β*-carotene ketolase play important roles in abiotic stress response.

3.3. Confirmation of protein targeting to chloroplasts

Before transforming *P. patens* with *HpBHY* and *CrBKT*, we first verified that these genes would be successfully imported into moss chloroplasts. To do this, we synthesized fusion proteins in which the transit peptide of the chloroplast protein RUBISCO was fused to the *β*-carotene hydroxylase and *β*-carotene ketolase. The amount of enzyme that would inhibit 50% of NBT photo-reduction was defined as one unit of SOD activity. For POD, the reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 1% guaiacol, 0.4% H$_2$O$_2$, and enzyme extract. Increase in the absorbance due to oxidation of guaiacol was measured at 470 nm for 1 min.

3.4. Creation of transgenic *P. patens* expressing *HpBHY* and *CrBKT*

To detect the functions of *HpBHY* and *CrBKT* in *P. patens*, we transformed *P. patens* with expression vectors (Fig. 4A, B). Twenty to thirty independent transgenic lines for the two expression constructs were obtained after genotyping by direct PCR (Fig. 4C), and the presence and expression of *HpBHY* and *CrBKT* were confirmed by RT-PCR analysis (Fig. 4D). The transformants V36 (hereafter, *HpBHY*-expressing plants) and T25 (hereafter, *CrBKT*-expressing plants) were used for further analysis.

Expression of *HpBHY* and *CrBKT* was confirmed in both the *HpBHY*-expressing moss and the *CrBKT*-expressing moss,
respectively, whereas wild-type plants were not found to express either gene (Fig. 4D).

3.5. Pigment concentrations in transgenic P. patens are altered

To detect whether HpBHY and CrBKT affect pigment synthesis of P. patens, we measured pigment concentrations in the transgenic plants. The relative abundances of pigments in the transformants were different from those in wild-type plants (Table 1). The transgenic HpBHY-expressing and CrBKT-expressing lines had lower chlorophyll a, chlorophyll b, and total chlorophyll content but higher total carotenoids content than the wild-type plants, indicating that the expression of HpBHY and CrBKT changed the pigment synthesis. Notably, compared to wild-type plants, the HpBHY-expressing and CrBKT-expressing lines accumulated higher amounts of lutein.

Lutein is a carotenoid that has been reported to play several important roles in photoprotection. We therefore hypothesized that the higher lutein content in HpBHY-expressing and CrBKT-expressing transformants might lead to higher stress tolerance.
expression data. 

Treatments (D), as well as that of the whole plants showed bleaching and leaf necrosis after a heat treatment of 4 h at 45°C under ammonia, nitrate, and urea treatments (C), and three HpBHY homologs in P. patens under ABA, strigolactone, heat, and continuous light treatments (D), as well as that of CrBKT under nitrogen deficiency and H2O2 treatment (E). Images were prepared with the HemI software (Deng et al., 2014) with the log2 of relative expression data.

**Fig. 2.** Heat map of the relative gene expression of various HpBHY homologs and CrBKT under different stress treatments. Heat maps of the relative gene expression values under dehydration and rehydration treatments of three HpBHY homologs in O. sativa (A), two HpBHY homologs in Arabidopsis under cold, osmotic, salt, drought, UV-B, and heat treatments (B), three HpBHY homologs in G. max under ammonia, nitrate, and urea treatments (C), and three HpBHY homologs in P. patens under ABA, strigolactone, heat, and continuous light treatments (D), as well as that of CrBKT under nitrogen deficiency and H2O2 treatment (E). Images were prepared with the HemI software (Deng et al., 2014) with the log2 of relative expression data.

**Fig. 3.** Import assay of HpBHY and CrBKT precursors into 7-day-old moss chloroplasts. L, import assay in the light; L + Th, import assay in the light followed by thermolysin treatments; Tr, 20% radioabeled precursor proteins; Pr, precursor protein; M, mature protein.

### 3.6. HpBHY and CrBKT transgenic P. patens are resistant to high temperature

To detect the stress tolerance of the transgenic P. patens, we treated the wild-type and transgenic lines with different stressors. The result suggested that the transgenic lines had greater tolerance of high-temperature stress. In both wild-type plants and the HpBHY-expressing and CrBKT-expressing transgenic lines, the whole plants showed bleaching and leaf necrosis after a heat treatment of 4 h at 45°C followed by 5 days of recovery at normal temperature. However, the HpBHY-expressing and CrBKT-expressing lines were clearly greener and healthier than wild-type plants, and showed less necrosis. After 30 days of recovery, the gametophytes of the transgenic plants remained healthier than those of the wild-type plants (Fig. 5A). To estimate the damage to PSII, we also analyzed the changes in the maximal efficiency of PSII photochemistry (Fv/Fm). The Fv/Fm values for all plants decreased sharply after high-temperature treatment, but were higher in the transgenic lines than in the wild-type plants (Fig. 5B). After 5 days of recovery, the Fv/Fm values for all plants were partially restored to previous levels, but the values for wild-type plants were still lower than those for HpBHY-expressing and CrBKT-expressing plants. After 30 days of recovery, the Fv/Fm values for all plants had increased considerably, Fv/Fm values for transgenic lines recovered to 0.5–0.6, whereas the Fv/Fm values for wild-type plants were only restored to about 0.4, which were much lower than those under optimal growth conditions (0.75–0.8). Although additional evidence is needed, we concluded that the expression of HpBHY and CrBKT may result in improved high-temperature stress tolerance in plants.

### 3.7. HpBHY and CrBKT increase high-temperature tolerance in P. patens through antioxidants

To further investigate the physiological changes that lead to high temperature stress tolerance in transgenic P. patens, we measured three physiological indexes of stress tolerance: malonylaldehyde (MDA) level, peroxidase (POD) activity and superoxide dismutase (SOD) activity. Under control conditions, the MDA levels of transgenic lines were lower than those of wild-type plants. After heat-stress treatment, the MDA levels of the transgenic lines increased less than those of the wild-type plants (HpBHY-expressing plants increased 7.56%, CrBKT-expressing plants increased 11.37%, and wild-type plants increased 18.84%) (Fig. 6A). As shown in Fig. 6B, the POD activities showed no significant difference among all plants under control conditions. After treating plants to high-temperature stress, however, POD activity increased more in the transgenic lines than in wild-type plants (HpBHY-expressing plants increase 149.33%, CrBKT-expressing plants increase 76.22%, and wild-type plants increase 57.77%). Similarly, after treating plants to heat-stress, SOD activity increased significantly more in transgenic lines than in wild-type plants (HpBHY-expressing plants increased 43.72%, CrBKT-expressing plants increased 71.04%, wild-type plants increased 7.67%). Taken together, these results indicate that HpBHY-expressing and CrBKT-expressing transgenic lines have increased antioxidant capacity.

### 3.8. HpBHY and CrBKT enhances P. patens high-temperature tolerance through damage repair pathways related to ABA and SA signaling

Endogenous hormone levels are also reported to be unstable under heat stress (Wahid et al., 2007). To investigate whether the

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**Fig. 4.** Import assay of HpBHY and CrBKT precursors into 7-day-old moss chloroplasts. L, import assay in the light; L + Th, import assay in the light followed by thermolysin treatments; Tr, 20% radioabeled precursor proteins; Pr, precursor protein; M, mature protein.
endogenous hormone levels of transgenic *P. patens* change under heat stress, we examined the endogenous levels of plant hormones such as abscisic acid (ABA) and salicylate (SA) in *HpBHY*-expressing plants and *CrBKT*-expressing plants and wild-type plants. After heat stress, the ABA levels of transgenic lines increased much more than wild-type plants. (*HpBHY*-expressing plants increased 162.30% and *CrBKT*-expressing plants increased 119.08%, whereas wild-type plants increased 28.51%) (Fig. 6D). In contrast, the SA levels in wild-type plants decreased markedly in response to heat stress, whereas SA levels in transgenic lines did not change after heat stress for 4 h. Under control conditions, ABA and SA levels were not significantly different in all plants (Fig. 6E).

Because the two transgenic lines had higher ABA and SA contents in response to high-temperature stress, we speculated that ABA and SA might mediate increased heat resistance by inducing damage-repair pathways.

### 4. Discussion

#### 4.1. Expression of *HpBHY* and *CrBKT* in *P. patens* increases carotenoids contents

β-carotene hydroxylase and β-carotene ketolase are key enzymes in the carotenoid biosynthetic pathway that are known to play significant regulatory roles in the biosynthesis of carotenoids (Giuliano, 2014). Overexpressing these two genes has been previously reported to increase carotenoid content in tobacco (Hasunuma et al., 2008; Mann et al., 2000), tomato (Huang et al., 2013), maize (Farré et al., 2016), rice (Du et al., 2010) and potato (Morris et al., 2006). In this study, we investigated the functions of *CrBKT* and *HpBHY* in *P. patens* plants.

After analyzing the phylogenetic position and expression profiles of β-carotene hydroxylase homologs and β-carotene ketolase, we found that abiotic stresses change the expression of these genes.
in various plant species. Thus, we hypothesized that \( \beta \)-carotene hydroxylase and \( \beta \)-carotene ketolase play significant roles in abiotic stresses response. To test this hypothesis, we constructed \( P. patens \) lines heterologously expressing each gene and analyzed the pigment concentration of the resulting transgenic plants.

We found that the total carotenoid level and lutein content increased (Table 1). Lutein is known to play a fundamental role in photoprotection (Jahns and Holzwarth, 2012; Matsubara et al., 2007; Dall’Osto et al., 2006). In our study, transgenic lines that expressed either \( \beta \)-carotene hydroxylase or \( \beta \)-carotene ketolase accumulated higher amounts of lutein than wild-type plants (Table 1). This increase in lutein content contrasts with lutein contents reported previously for various transgenic plants expressing carotenoid-pathway-related genes. For instance, in the transplastomic tobacco plants expressing both \( \beta \)-carotene hydroxylase (\( CrtZ \)) and \( \beta \)-carotene ketolase (\( CrtW \)) from \( Brevundimonas \) sp. SD212, the lutein content was decreased 12.4-fold (Fan et al., 2017), in addition, the lutein content in the transplastomic tomatoes leaves overexpressing \( CrBKT \) decreased 4.9-fold (Huang et al., 2013). This difference in how \( \beta \)-carotene hydroxylase and \( \beta \)-

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Fig. 5. Effects of high temperature on phenotypes and \( Fv/Fm \) in transgenic and wild-type \( P. patens \). (A) Phenotypic comparison of wild type and transformants grown under control conditions (Control), after heat stress treatment for 4 h followed by recovery for 5 days (Recovery 5 days), or after heat stress treatment for 4 h followed by recovery 30 days (Recovery 30 days). Scale bars, 20 mm. (B) \( Fv/Fm \) of transgenic and wild-type plants grown under control conditions (Control), after heat stress treatment for 4 h (Heat stress), after heat stress treatment for 4 h followed by recovery for 5 days (Recovery 5 d) and recovery 30 days (Recovery 30 d). Y36, \( HpBHY \)-expressing plants; T25, \( CrBKT \)-expressing plants; WT, wild type plants. Data are presented as means ± SEM of 5 replicates; t-test was used and asterisk indicates that the value of treatment is different from control (WT) *\( P < 0.05 \), **\( P < 0.01 \).

Fig. 6. Effects of high temperature on MDA contents, POD activity, SOD activity, ABA and SA content in transgenic and wild-type \( Physcomitrella patens \). Effect of high temperature on MDA content (A), POD activity (B), SOD activity (C), ABA content (D), and SA content (E). Measurements were taken under control condition (Control) and after direct heat treatment (Heat stress) at 45 °C for 4 h. WT, wild type plants; Y36, \( HpBHY \)-expressing plants; T25, \( CrBKT \)-expressing plants. Data are presented as means ± SEM of 5 replicates; t-test was used and asterisk indicates that the value of treatment is different from control (WT) *\( P < 0.05 \), **\( P < 0.01 \).
carotene ketolase affect lutein content suggests that these genes play distinct functions in the moss *P. patens*.

4.2. Antioxidant system and ABA and SA signaling are involved in tolerance of *P. patens* to heat stress

Plants have evolved various resistance mechanisms to stress, including adjusting photosynthesis; eliminating active oxygen species through increased activation of antioxidant enzymes and accumulation of some antioxidants; and accumulation of secondary metabolites, such as stress resistance hormones and carotenoids (Wahid and Ghazanfar, 2006). Carotenoids exhibit strong antioxidant properties and are important for plant photoprotection (Wahid et al., 2007); indeed, the levels of plant carotenoids may be related to stress tolerance (Wahid and Ghazanfar, 2006). For example, salt tolerance in mungbean has been tightly correlated with steady carotenoids levels (Wahid et al., 2004), and sugarcane improves its salinity tolerance through reduced chlorophyll and steady carotenoids levels (Wahid and Ghazanfar, 2006).

To investigate the role of two carotenoid biosynthetic enzymes — β-carotene hydroxylase and β-carotene ketolase — in stress resistance, we treated transgenic *P. patens* expressing each gene to heat stress (45°C for 4 h). We found that expression of β-carotene hydroxylase or β-carotene ketolase increased heat tolerance in transgenic *P. patens* plants. Specifically, PSI efficiency in transgenic plants expressing β-carotene hydroxylase or β-carotene ketolase recovered quicker from heat stress than in wild-type plants (Fig. 5B). Changes in PSI efficiency, measured by chlorophyll fluorescence, Fv/Fm ratio, are considered reliable diagnostic indicators of photoinhibition (Dew et al., 2015). Furthermore, following heat stress, transgenic lines expressing β-carotene hydroxylase and β-carotene ketolase were visibly greener and showed less leaf necrosis than wild-type plants (Fig. 5A). Similar results have been reported in *Arabidopsis*, for example, β-carotene hydroxylase overexpression in *Arabidopsis* increased resistance to high-light and high-temperature treatment (Davison et al., 2002) and overexpression *CrBKT* has been shown increase tolerance to high-light stress (Zhong et al., 2011). Similarly, in rice, overexpression of a putative β-carotene hydroxylase (DSMZ2) in rice increases the resistance to oxidative stresses and drought significantly (Du et al., 2010). These results suggest that overexpression of β-carotene hydroxylase and β-carotene ketolase can increase plant tolerance of stress and maintain relatively high PSI activity under stress. However, the mechanisms that underlie increased stress tolerance remain unclear. Peroxidative damage in membrane lipids, for which MDA content is a common indicator, is especially evident under stress (Liu and Huang, 2000). Higher activities of antioxidant enzymes, such as SOD and POD, are associated with less peroxidative damage and higher stress tolerance. In this study, the much lower MDA contents (Fig. 6A) and the higher POD (Fig. 6B) and SOD (Fig. 6C) activity indicated that the cell membranes of the transgenic lines were less damaged than those of the wild-type plants.

Carotenoids are precursors in ABA and SA biosynthesis (Du et al., 2010). Thus, we suspected that SA and ABA levels in β-carotene hydroxylase- and β-carotene ketolase-expressing transgenic plants may be higher under control condition. Interestingly, we found that before heat stress treatment, ABA and SA contents were no significant differences high. However, ABA contents increased and SA levels remained stable in β-carotene hydroxylase- and β-carotene ketolase-expressing transgenic plants under high-temperature treatment in our study (Fig. 6D, E). This finding suggested that ABA and SA play key roles in plants response to high temperature. Moreover, these results suggest that the increased heat-stress tolerance of the transgenic plants may be related to ABA and SA signaling. Taken together, we speculate that overexpression of β-carotene hydroxylase and β-carotene ketolase in *P. patens* may increase plants heat tolerance because of increased antioxidant capacity and improved damage repair related with ABA and SA signaling. This interpretation of our findings is consistent with previous reports which have shown that ABA and SA as important components of plant thermotolerance (Maestri et al., 2002).

Heterologous expression of *HpbHY* and *CrBKT* in *P. patens* not only increased carotenoid content but also improved heat tolerance in the transgenic plants, showing that these two genes could be valuable genetic resources for researchers seeking to develop plants with higher nutrition and stronger stress resistance. In this study, we confirmed carotenoids play important roles in plant heat stress response, through the antioxidant and damage repair metabolism, which is related to abscisic acid and salicylate signaling. It laid the foundation for further research on the mechanism of plant response to high temperature stress.

Author contributions

LL, HQH, JCH and JFH initiated and designed the research. JFH, PL, LNL and TT performed the experiments. JFH, MXH analyzed the data. LL contributed reagents/materials/analysis tools. JFH wrote the manuscript. LL, JFH and PL revised the manuscript. All authors read and approved the manuscript.

Conflict of interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2019.04.001.

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