Chloroplast-encoded serotonin N-acetyltransferase in the red alga Pyropia yezoensis: gene transition to the nucleus from chloroplasts

Yeong Byeon¹, Hyoung Yool Lee¹, Dong-Woog Choi² and Kyoungwhan Back¹,*

¹ Department of Biotechnology, Interdisciplinary Program of Bioenergy and Biomaterials, Bioenergy Research Center, College of Agriculture and Life Sciences, Chonnam National University, Gwangju, Republic of Korea
² Department of Biology Education, Chonnam National University, Gwangju 500-757, Republic of Korea

* To whom correspondence should be addressed. E-mail: kback@chonnam.ac.kr

Received 5 June 2014; Revised 31 July 2014; Accepted 1 August 2014

Abstract

Melatonin biosynthesis involves the N-acetylation of arylalkylamines such as serotonin, which is catalysed by serotonin N-acetyltransferase (SNAT), the penultimate enzyme of melatonin biosynthesis in both animals and plants. Here, we report the functional characterization of a putative N-acetyltransferase gene in the chloroplast genome of the alga laver (Pyropia yezoensis, formerly known as Porphyra yezoensis) with homology to the rice SNAT gene. To confirm that the putative Pyropia yezoensis SNAT (PySNAT) gene encodes an SNAT, we cloned the full-length chloroplastidic PySNAT gene by PCR and purified the recombinant PySNAT protein from Escherichia coli. PySNAT was 174 aa and had 50% amino acid identity with cyanobacteria SNAT. Purified recombinant PySNAT showed a peak activity at 55 °C with a $K_m$ of 467 µM and $V_{max}$ of 28 nmol min$^{-1}$ mg$^{-1}$ of protein. Unlike other plant SNATs, PySNAT localized to the cytoplasm due to a lack of N-terminal chloroplast transit peptides. Melatonin was present at 0.16 ng g$^{-1}$ of fresh mass but increased during heat stress. Phylogenetic analysis of the sequence suggested that PySNAT has evolved from the cyanobacteria SNAT gene via endosymbiotic gene transfer. Additionally, the chloroplast transit peptides of plant SNATs were acquired 1500 million years ago, concurrent with the appearance of green algae.

Key words: Endosymbiosis, laver, melatonin, Pyropia, red algae, serotonin N-acetyltransferase.

Introduction

Pyropia yezoensis, a marine alga laver known previously as Porphyra yezoensis (Sutherland et al., 2011), is used commercially in foods such as sushi. The products of laver are valued at around US$2 billion within East Asia (Sahoo et al., 2002). Due to its economic importance and nutritional properties, Pyropia cultivation is expanding to other countries (Noda, 1993). Around 75 species of Pyropia have been reported globally, and they can grow up to several metres in length with a linear or orbicular morphology. The thalli are in the gametophytic phase and consist of one or two thick cells containing one or two chloroplasts (Sahoo et al., 2002; Sutherland et al., 2011). In particular, Pyropia is known to be higher in vitamin C than oranges (Noda, 1993).

Melatonin was first isolated from beef pineal glands as an active ingredient for preventing the darkening of frog skin (Lerner et al., 1958). Since then, melatonin has been found to play pleiotropic roles in animals, including circadian rhythmicity (Reiter, 1991), seasonal reproduction (Barrett and...
Bolborea, 2012), the scavenging of free radicals (Garcia et al., 2014), enhancing innate immune responses (Calvo et al., 2013), and delaying the aging process (Hardeland, 2013). In contrast to animals, the presence of melatonin in plants was first uncovered in 1995 from various tissues and fruits (Dubbels et al., 1995; Hattori et al., 1995). Several reports over the past two decades have implicated plant melatonin in numerous biological activities, including root and leaf growth (Hernández-Ruiz et al., 2005; Chen et al., 2009; Park and Back, 2012; Pelagio-Flores et al., 2012; Sarropoulou et al., 2012), the promotion of germination (Tiryaki and Keles, 2012; Zhang et al., 2013), the delay of flowering (Kolář et al., 2003; Byeon and Back, 2014a), and increasing photosynthesis (Lázár et al., 2013). In addition, melatonin is generally thought to be involved in plant defense responses to various stresses such as cold (Posmyn et al., 2009; Kang et al., 2010; Bajwa et al., 2014), drought (Wang et al., 2013b; Zhang et al., 2013), herbicides (Park et al., 2013c), salt (Li et al., 2012), heavy metals (Tan et al., 2007a), senescence (Wang et al., 2012, 2013a), and pathogens (Yin et al., 2013). However, the precise role of melatonin in plant growth and development remains unclear. The role of melatonin has been explored using molecular genetic analysis of a melatonin-deficient mutant line and gain-of-function analysis with melatonin-rich transgenic plants (Kang et al., 2010; Okazaki et al., 2010; Byeon and Back, 2014a; Wang et al., 2014), which revealed similar functions to those mentioned above (Paredes et al., 2009).

In accordance with the pleiotropic roles of melatonin in plants, melatonin levels vary significantly among plant species, ranging from a few picograms to micrograms per gram of mass (Chen et al., 2003; Rodriguez-Naranjo et al., 2011; Ramakrishna et al., 2012). On the other hand, melatonin has been found in all plant lineages including cyanobacteria, which are thought to be the origin of chloroplasts (Tilden et al., 1997; Tan et al., 2013), Chlorellaceae (Charophyceae (Chara australis), Chlorophyceae (Chlamydomonas spp., Dunaliella tertiolecta, and Acetabularia acutabulum), Phaeophyceae (Porphyra hirafuka, Laminaria digitata, and Petalonia fascia), Rhodophyceae (Graeciliria tenustipitata, Pteridium palmate, and Pyropia umbilicalis), alveolate (e.g. Gonyaulax polyedra, Alexandrium lusitanicum, and Ceratium horridum), and Pyrocystis lunula), and excavate (Euglena gracilis) (Balzer and Lüning, 1998; Hardeland, 1999; Tal et al., 2011). Like melatonin, which exists in all plant kingdom as described above, serotonin N-acetyltransferase (SNAT), the penultimate enzyme for melatonin biosynthesis in plants, is conserved throughout evolutionary plant lineages (Byeon et al., 2013; Kang et al., 2013). Homologues of SNAT genes are found in cyanobacteria, Chlorophyceae (Chlamydomonas reinhardtii and Volvox carteri), Rhodophyceae (Pyropia yezoensis and Cyanidioschyzon merolae strain 10D), Prasinophyceae (Ostreococcus tauri), bryophytes (Physcomitrella patens), Lycopodiophyta (Selaginella moellendorffii), and Euphyllophyta (e.g. Picea sitchensis, Arabidopsis thaliana, and Orzya sativa). Among these SNAT homologues, two SNAT genes from cyanobacteria (Byeon et al., 2013) and rice (Kang et al., 2013) were functionally characterized. Unexpectedly, a SNAT homologue from the alga laver was found in the plastid genome, whereas other SNAT genes from green algae to higher plants are present in the host nuclear genomes. This suggests that the plant SNAT gene originated from the incorporation of cyanobacteria into red algae via an endosymbiosis process.

In this study, we investigated whether the chloroplast-encoded SNAT homologue of the alga laver has SNAT activity and whether its activity is associated with melatonin production.

**Materials and methods**

**Laver material and growth conditions**

Laver (Pyropia yezoensis) was cultivated as follows. Leafy gametophytes of Pyropia yezoensis were cultured in modified Grun medium (McLachlan, 1973) at 10 °C with a photon flux density of 80 mol photon m⁻² s⁻¹ provided by cool-white fluorescent lamps with a photoperiod of 14/10 (light/dark) in a growth chamber. For high-temperature treatment, cultivation bottles growing Pyropia yezoensis were transferred to a 25 °C growth chamber under the same light conditions described above.

**Isolation of Pyropia yezoensis genomic DNA and PySNAT cloning**

For genomic DNA extraction, leafy gametophytes of Pyropia yezoensis (100 mg) were ground to a powder in liquid nitrogen using a mortar and pestle, and DNA was extracted with the buffer provided by a DNeasy Plant Mini kit (Qiagen, Tokyo, Japan). The full-length PySNAT sequence was cloned by PCR using the genomic DNA of Pyropia yezoensis with a primer set based on PySNAT sequence information (GenBank accession no. NC_007932). The forward and reverse primers were 5'-ATGATCCCTGTGAAAAAT-3' and 5'-TTATCTAGGATAAAA-3', respectively. The resulting PCR-amplified PySNAT product was ligated into the pTOP Blunt V2 vector to generate pTOP blunt V2:PySNAT, and the sequence of PySNAT was verified.

**Construction of Escherichia coli expression vectors for PySNAT protein purification**

The pET300 Gateway® vector (Invitrogen, Carlsbad, CA, USA) was used for N-terminal His-tagged expression of PySNAT. Full-length PySNAT and truncated Δ22PySNAT (lacking the N-terminal 22 aa) were amplified by PCR using appropriate primers harbouring the attB recombination sequences. For the full-length PySNAT, the primer set was as follows: forward primer 5’-AAAAAGCAGGCTCCATGATCTCTGAAA-3’, reverse primer 5’-AGAAAGCTGGTTTATCTTGGATACAAA-3’. The forward primer for the truncated Δ22PySNAT was 5’-AAAAAGCAGGGTCCATGAACATTGTGTTTAG-3’ and the reverse primer was the same as above. The resulting products were cloned into the pDONR221 Gateway vector (Invitrogen). The pDONR221-PySNAT and pDONR221-Δ22PySNAT entry vectors were then recombined with the pET300 Gateway destination vector via LR recombination to generate pET300-PySNAT and pET300-Δ22PySNAT. The pET28b vector (Novagen, Madison, WI, USA) was used for C-terminal His-tagged expression of PySNAT. The full-length PySNAT gene was amplified by PCR using 5’-AAAAAGCAGGCTCCATGATCTCTGAAAATT-3’ as the forward primer and NcoI restriction sites are underlined and the translation start codon is in bold), 5’-GTGCTCTTAGTTGGATACAAA-3’ (the XhoI site is underlined) as the reverse primer, and pTOP blunt V2:PySNAT as the

**Materials and methods**

**Laver material and growth conditions**

Laver (Pyropia yezoensis) was cultivated as follows. Leafy gametophytes of Pyropia yezoensis were cultured in modified Grun medium (McLachlan, 1973) at 10 °C with a photon flux density of 80 mol photon m⁻² s⁻¹ provided by cool-white fluorescent lamps with a photoperiod of 14/10 (light/dark) in a growth chamber. For high-temperature treatment, cultivation bottles growing Pyropia yezoensis were transferred to a 25 °C growth chamber under the same light conditions described above.

**Isolation of Pyropia yezoensis genomic DNA and PySNAT cloning**

For genomic DNA extraction, leafy gametophytes of Pyropia yezoensis (100 mg) were ground to a powder in liquid nitrogen using a mortar and pestle, and DNA was extracted with the buffer provided by a DNeasy Plant Mini kit (Qiagen, Tokyo, Japan). The full-length PySNAT sequence was cloned by PCR using the genomic DNA of Pyropia yezoensis with a primer set based on PySNAT sequence information (GenBank accession no. NC_007932). The forward and reverse primers were 5’-ATGATCCCTGTGAAAAAT-3’ and 5’-TTATCTAGGATAAAA-3’, respectively. The resulting PCR-amplified PySNAT product was ligated into the pTOP Blunt V2 vector to generate pTOP blunt V2:PySNAT, and the sequence of PySNAT was verified.

**Construction of Escherichia coli expression vectors for PySNAT protein purification**

The pET300 Gateway® vector (Invitrogen, Carlsbad, CA, USA) was used for N-terminal His-tagged expression of PySNAT. Full-length PySNAT and truncated Δ22PySNAT (lacking the N-terminal 22 aa) were amplified by PCR using appropriate primers harbouring the attB recombination sequences. For the full-length PySNAT, the primer set was as follows: forward primer 5’-AAAAAGCAGGCTCCATGATCTCTGAAA-3’, reverse primer 5’-AGAAAGCTGGTTTATCTTGGATACAAA-3’. The forward primer for the truncated Δ22PySNAT was 5’-AAAAAGCAGGGTCCATGAACATTGTGTTTAG-3’ and the reverse primer was the same as above. The resulting products were cloned into the pDONR221 Gateway vector (Invitrogen). The pDONR221-PySNAT and pDONR221-Δ22PySNAT entry vectors were then recombined with the pET300 Gateway destination vector via LR recombination to generate pET300-PySNAT and pET300-Δ22PySNAT. The pET28b vector (Novagen, Madison, WI, USA) was used for C-terminal His-tagged expression of PySNAT. The full-length PySNAT gene was amplified by PCR using 5’-AAAAAGCAGGCTCCATGATCTCTGAAAATT-3’ as the forward primer and NcoI restriction sites are underlined and the translation start codon is in bold), 5’-GTGCTCTTAGTTGGATACAAA-3’ (the XhoI site is underlined) as the reverse primer, and pTOP blunt V2:PySNAT as the
template. Truncated PySNAT (Δ22PySNAT) was amplified by PCR using 5′-ACCATGGC AAAACTTATTTTGA3′ as the forward primer (Neol restriction sites are underlined and the translation start codon is in bold) and 5′-GTTGCTCGAGTTAGGACTACAAAAT (the XhoI site is underlined) as the reverse primer. The PCR products were cloned into a T&A vector (RBC Bioscience, New Taipei City, Taiwan), digested with NeoI and XhoI, gel purified, and ligated into the same restriction sites in the pET28(b) reading frame. E. coli BL21 (DE3) was used as the host strain for both pET300 and pET28(b) containing the PySNAT genes. Cell culture and purification procedures using a Ni-NTA column were performed according to the manufacturer’s instructions (Qiagen). The purified recombinant PySNAT proteins were dissolved in 10 mM Tris/HCl (pH 8.0), and 50% glycerol solution and stored at −20°C until further analysis.

**Analyses for SNAT enzyme activity and enzyme kinetics**

Purified recombinant PySNAT proteins were incubated in a total volume of 100 μl containing 0.5 mM serotonin and 0.5 mM acetyl-CoA in 100 mM potassium phosphate (pH 8.5), as described previously (Byeon et al., 2013). Briefly, all SNAT assays were conducted at 55°C (or varying temperatures) for 1 h and stopped by the addition of 50 μl of methanol. A 10 μl aliquot was subjected to high-performance liquid chromatography (HPLC) using the fluorescence detector system, as described previously (Byeon et al., 2014). Non-enzymatic reaction products, which were generated in the absence of the PySNAT enzyme, were subtracted. The substrate affinity (Km) and maximum reaction rate (Vmax) were calculated from Lineweaver–Burk plots. The protein concentration was determined using the Bradford method and a protein assay dye (Bio-Rad, Hercules, CA, USA). The analysis was performed in triplicate.

**Subcellular localization of PySNAT**

Confocal microscopic analysis was performed to investigate the subcellular localization of PySNAT. The pER-mCherry vector was used for subcellular localization analysis (a kind gift from Dr H. G. Kang, Texas State University, San Marcos, TX, USA). Full-length PySNAT cDNA was amplified by PCR with the primer set harbouring an AscI site (forward 5′-GGGGGCGCGCCATGATCTTCTGGAAAA-3′; reverse 5′-GGGGGGCGCGCTCTTCTTAGGATACAAAAA-3′). The resulting PCR product was cloned into the TA vector (RBC Bioscience) and the PySNAT insert was digested using the AscI restriction endonuclease, purified, and ligated into the AscI site of the binary vector pER8-mCherry containing the oestrogen-inducible XVE promoter (Pxve) to create pER8-PySNAT-mCherry. The pER-PySNAT-mCherry plasmid was transformed into Agrobacterium tumefaciens strain GV2260 using the freeze-thaw method and transient expression analyses were performed as described by Voinnet et al. (2003). The pBiN61-GFP plasmid was used as a cytosolic expression marker (Byeon et al., 2014). Nicotiana benthamiana leaves were infiltrated with Agrobacterium strains and then induced with β-estradiol (10 μM) by infiltration as described previously (Byeon et al., 2014). Images were acquired using a Leica TCS-SP5 confocal microscope with the Leica LAS-AF software version 1.8.2. (Leica Microsystems, Exton, PA, USA). Green fluorescent protein (GFP) was excited with a blue argon ion laser (488 nm), and emitted light was collected between 494 and 546 nm. mCherry was excited with an orange He-Ne laser (594 nm), and emitted light was collected from 576 to 629 nm. Chloroplasts were excited with a blue argon laser (488 nm), and emitted light was collected from 660 to 731 nm. Signals were collected separately and later superimposed.

**HPLC analysis of melatonin**

The frozen Pyropia yezoensis tissue samples (100 mg) were ground to a powder in liquid nitrogen using TissueLyser II (Qiagen) and extracted with 1.5 ml of chloroform overnight at 4°C. The chloroform extracts were evaporated and dissolved in 1.5 ml of 35% methanol. Aliquots of 10 μl were subjected to HPLC with a fluorescence detector system (Waters, Milford, MA, USA). The samples were separated on a Sunfire C18 column (4.6×150 mm; Waters) with a gradient elution profile (from 42 to 50% methanol in 0.1% formic acid for 27 min, followed by isocratic elution with 50% methanol in 0.1% formic acid for 18 min at a flow rate of 0.15 ml min−1). Melatonin was detected at 280 nm excitation and 348 nm emission.

**Prediction of chloroplast transit peptides**

The network-based method (ChloroP) was used to identify chloroplast transit peptides and their cleavage sites from various N-terminal sequences of SNAT homologues (Emanuelsson et al., 1999). The ChloroP predictor is available as a web server at http://www.cbs.dtu.dk/services/ChloroP/. A phylogenetic tree was generated using BLAST-Explorer (Dereeper et al., 2010).

**Statistical analysis**

One-way analysis of variance was used for all statistical evaluations. *P*<0.05 was considered to indicate statistical significance.

**Results**

**Characterization of the SNAT gene in the Pyropia yezoensis chloroplast genome**

The rice SNAT gene exists as a single copy in the rice genome and is highly conserved in plant lineages, including cyanobacteria (Byeon et al., 2013). According to BLAST analysis, plant SNAT homologues have been found in the nuclear genome in all members of Chlorophyta that were examined, such as green algae and terrestrial plants, but the SNAT homologue was located in chloroplasts in rhodophyta, including red algae such as laver (Pyropia yezoensis). Therefore, we explored whether the chloroplast-encoded SNAT in red algae showed SNAT enzyme activity. We cloned the chloroplast-encoded SNAT homologue from Pyropia yezoensis (PySNAT). PySNAT was 174 aa with a calculated molecular weight of approximately 20 009 Da (Fig. 1). The PySNAT polypeptides shared 50% identity with the cyanobacteria SNAT and also showed high identity to rice and loblolly pine at 45 and 43%, respectively, when compared with the level of mature polypeptides of rice and loblolly pine. The theoretical isoelectric point (pI) was higher in PySNAT (9.36) than loblolly pine and rice SNAT polypeptides shared 50% identity with the cyanobacteria SNAT and also showed high identity to rice and loblolly pine. The theoretical isoelectric point (pI) was higher in PySNAT (9.36) than cyanobacteria SNAT (pI 7.78), but G+C content decreased to 29% in PoPySNAT relative to 51% in cyanobacteria SNAT. Phylogenetic analysis revealed that PySNAT is placed in the same clade as the cyanobacteria SNAT, suggesting that PySNAT originated from cyanobacteria via endosymbiosis. Unlike loblolly pine and rice SNAT polypeptides, no N-terminal extension peptides were observed as a chloroplast transit peptide.

**Bacterial expression and purification of PySNAT**

An E. coli heterologous expression system was used to purify the PySNAT protein. To facilitate the purification of PySNAT, we utilized a His-tagged affinity purification system with either pET300 or pET28b vectors. pET300 generated an N-terminal His-tagged PySNAT, whereas pET28b resulted in a
Byeon et al.

C-terminal His-tagged PySNAT (Fig. 2). Full-length PySNAT expression using the N-terminal His-tagged system yielded high expression after isopropyl β-d-thiogalactopyranoside (IPTG) induction, but the majority of the PySNAT protein was in an insoluble form (lane 3 in FL of Fig. 2A). In contrast, truncated PySNAT (∆22PySNAT) expression with an N-terminal His-tag enhanced soluble PySNAT expression (lane 3 in ∆22 of Fig. 2A). For the C-terminal His-tagged system, a truncated form of PySNAT showed much higher soluble expression than the full-length form (Fig. 2B). To verify that the purified recombinant PySNAT proteins exhibit SNAT enzyme activity, we measured SNAT enzyme activity at 30 °C by measuring N-acetylserotonin (NAS) in the presence of serotonin, acetyl-CoA, and various forms of PySNAT proteins. As shown in Fig. 3, all forms of purified recombinant PySNAT proteins contained SNAT catalytic activities, of which the full-length PySNAT with a C-terminal His-tag showed peak SNAT enzyme activity. This suggested that the chloroplast-encoded PySNAT gene was a SNAT homologue, as found in other plants.

Enzyme kinetics of the purified recombinant PySNAT protein

We first measured the thermostability of the PySNAT enzyme since plant SNAT proteins (including the cyanobacterial SNAT) show thermophilic characteristics (Byeon et al., 2013, 2014). Purified full-length PySNAT with a C-terminal His-tag was incubated at varying temperatures for the in vitro SNAT assay. As shown in Fig. 4A, SNAT enzyme activity increased with temperature. For example, NAS was produced at 0.3 ng per assay at 25 °C, whereas 35 ng was produced at 55 °C (116-fold increase compared with production at 25 °C). The temperature-dependent rapid increase in SNAT enzyme activity is common in plant SNAT enzyme from rice. SNAT enzyme activity peaked at 55 °C, but a rapid decrease in SNAT enzyme activity was observed at 70 °C and no activity occurred at 95 °C. In contrast, the SNAT proteins from rice and cyanobacterium had high activity, even at 70 °C (Byeon et al., 2013, 2014). Thus, we further investigated the enzyme kinetics at the optimal temperature of 55 °C based on Lineweaver–Burk plots. The $K_m$ and $V_{max}$ values were 467 µM and 28 nmol min$^{-1}$ mg$^{-1}$ of protein, respectively, which were 1.8-fold lower and 18-fold higher, respectively, than those for cyanobacterial SNAT. Thus, the catalytic efficiency ($V_{max}/K_m$) of PySNAT was 31-fold higher than that of the cyanobacterial SNAT.

Subcellular localization of PySNAT

Based on the potential origin of PySNAT from cyanobacteria, PySNAT may target to chloroplasts in higher plants because some proteins (even in the absence of chloroplast transit peptides) can localize to chloroplasts (Ha et al., 2003; Jung et al., 2004). To determine whether PySNAT can target to chloroplasts in tobacco, we constructed a binary vector, pER8-PySNAT-mCherry, under the control of the
oestrogen-inducible XVE promoter. Agrobacterium cells harbouring the binary vector pER8-PySNAT-mCherry were infiltrated into tobacco leaves to examine subcellular localization using confocal microscopy. As shown in Fig. 5, PySNAT showed a strong red fluorescence in tobacco cytoplasm (Fig. 5A) that co-localized with the green fluorescence of GFP, a cytoplasmic marker protein (Fig. 5B). The red fluorescence of PySNAT merged with the green fluorescence of cytoplasmic GFP (Fig. 5D), which indicated that the chloroplast-encoded PySNAT is devoid of chloroplast transit peptides and lacks an intrinsic ability to translocate into chloroplasts of higher plants. Although the primary structure of PySNAT (including pI) differed from that of cyanobacteria SNAT, PySNAT itself cannot translocate into chloroplasts in higher plants and requires the acquisition of an N-terminal chloroplast transit peptide during evolution.

**Melatonin levels during the response to high-temperature stress**

During its natural life cycle, laver is exposed to various adverse environmental stresses such as drought and temperature changes (Ruangchuay and Notoya, 2003; Park et al., 2012a). Thus, we exposed laver culture to high temperature (25 °C) in vitro to characterize the melatonin response. The melatonin level in control laver was around 0.16 ng g⁻¹ of fresh weight (fw), and its levels were consistent within 3 h after heat stress (Fig. 6). However, melatonin increased to 0.23 ng g⁻¹ fw at 12 h after heat stress, suggestive of an induction of melatonin biosynthesis.
Subcellular localization of PySNAT. (A) Red fluorescence of PySNAT-mCherry. (B) Green fluorescence of cytoplasmic GFP. (C) Cyan fluorescence of chlorophyll. (D) The fluorescence images were merged. *Agrobacterium*-infiltrated tobacco (Nicotiana benthamiana) leaves with XVE-inducible PySNAT-mCherry were grown for 2 d in a growth room before XVE-induction and visualization by confocal microscopy. Bars, 20 μm. The GenBank accession number of PySNAT is NC_007932.

**Fig. 6.** Melatonin levels in response to heat stress in laver (*Pyropia yezoensis*). Leafy gametophytes of *Pyropia yezoensis* were challenged at a high temperature of 25 °C. Laver was collected at each indicated time point. Melatonin levels were quantified by HPLC. Data represent the means±standard deviation of three replicates. An asterisk indicates a significant difference from the wild type (P<0.05).

### Acquisition of chloroplast transit peptides during the evolution of plant SNAT proteins

All *SNAT* genes that have been examined in plant lineages possess an N-terminal chloroplast transit peptide according to ChloroP analysis (Emanuelsson et al., 1999). The lengths of chloroplast transit peptides vary among species and range from 14 to 83 aa (Fig. 7). The first acquisition of chloroplast transit peptides occurred in the unicellular green alga *Ostreococcus tauri*, which is thought to possess a 14 aa chloroplast transit peptide. The length of chloroplast transit peptides then increased to 30 aa in the multicellular green alga *Volvox carteri*, although *O. tauri* and *Volvox* belong to Prasinophyceae and Chlorophyceae, respectively, and are not closely related to the ancestors of the embryophytes. However, a moss (*Physcomitrella patens*) positioned between green algae and vascular plants during plant evolution contained an 83 aa chloroplast transit peptide. Other land plants such as maize and rice have various lengths of chloroplast transit peptides ranging from 45 to 83 aa. Thus, the chloroplast transit peptides were probably acquired 1500 million years ago during the evolution of unicellular green algae (Yoon et al., 2004), after which they progressively increased in length until the vascular plants emerged 450 million years ago. The average lengths of the chloroplast transit peptides among plant SNAT proteins was around 58 aa, which is the typical length of average plant chloroplast transit peptides (Zhang and Glaser, 2002). The chloroplast targeting functions of the 14 and 30 aa chloroplast transit peptides in *O. tauri* and *V. carteri*, respectively, requires further analysis.

### Discussion

Melatonin is found in almost all living organisms, including bacteria, animals, and plants, but the level of melatonin in Archaea has not yet been examined (Tan et al., 2012). Due to its ubiquity, melatonin is thought to be a basic component of living organisms (Pandi-Perumal et al., 2006). Melatonin appeared on earth 2.5 billion years ago during the transition from anaerobic to aerobic organisms (Tan et al., 2010). Although melatonin is derived from bacteria in both animals and plants, its bacterial origin differs between animals and plants based on a phylogenetic tree and the evolution of key melatonin biosynthetic genes (Coon and Klein, 2006; Byeon et al., 2013).

SNAT is a key enzyme catalysing the penultimate step in melatonin biosynthesis in both animals and plants. In animals, SNAT is also termed arylalkylamine N-acetyltransferase. Given the common ancestor from aerobic bacteria, the SNAT gene is likely also to be well conserved in animals and plants. However, a study showed that animal SNAT genes have no homology with plant SNAT genes (Kang et al., 2013), suggesting that animal and plant SNAT genes have evolved independently. Animal SNAT homologues were identified in the genomes of Gram-positive bacteria, unicellular green algae, and fungi, suggesting the horizontal transfer of animal SNAT from Gram-positive bacteria (Coon and Klein, 2006; Tan et al., 2012). In contrast, plant SNAT homologues have been identified in the genomes of cyanobacteria, green algae, moss, gymnosperms, and angiosperms (Byeon et al., 2013; Kang et al., 2013). In addition, the plant SNAT homologue was found in the chloroplast genome of the red alga *Pyropia yezoensis*. The chloroplast-encoded red alga SNAT had higher SNAT catalytic activity than that of cyanobacterium (Fig. 4B). Collectively, these data suggest that plant SNAT was vertically transferred to descendants via endosymbiosis. Considering plant SNAT distribution, the cyanobacteria was
thought to have become the chloroplasts in red algae via endosymbiosis, and many plastid-encoded genes including SNAT were transferred into the nucleo in green algae since gene loss or transfer to the nucleus from plastids is a common phenomenon (Martin and Herrmann, 1998). During the evolution from red algae to green algae and moss, the acquisition of chloroplast transit peptides occurred progressively by extending the length of chloroplast transit peptides (Fig. 7). For example, the unicellular green alga O. tauri possessed a 14 aa chloroplast transit peptide, whereas the multicellular green alga V. carteri contained a 30 aa chloroplast transit peptide. The moss (Physcomitrella patens), phylogenetically close to the ancestors of vascular plants, harboured an 83 aa chloroplast transit peptide. Thus, it is intriguing to examine whether SNAT proteins from O. tauri and V. carteri target to the chloroplasts, which was observed for rice SNAT (Byeon et al., 2014). Analogous with the chloroplast origin of the plant SNAT gene, plant SNAT clearly localized to chloroplasts (Byeon et al., 2014).

The identification of melatonin in red algae has been reported previously (Hardeland, 1999), in which melatonin levels varied in a red alga (Pyropia unbullicus) grown in the laboratory and in the field (Lorenz and Lüning, 1998). In our report, melatonin levels in Pyropia yezoensis were induced in response to heat stress (Fig. 6). This heat-inducible melatonin increase may be associated with an increase in SNAT enzyme activity by high temperature, and not by an increase in SNAT mRNA and its translatable polypeptides. The high-temperature-induced melatonin increase was also observed in macroalga and rice (Tal et al., 2011; Byeon and Back, 2014b). In addition to high temperature, many abiotic factors are known to induce melatonin expression in plants, including agrochemicals (Vitalini et al., 2011; Park et al., 2013c), plant hormones such as ethylene and abscisic acid (Arnão and Hernández-Ruiz, 2013), high intensity light (Murch et al., 2000; Afreen et al., 2006; Tan et al., 2007b; Zhao et al., 2013), high temperature (Tal et al., 2011), senescence (Kang et al., 2010), and cold (Murch et al., 2009). Melatonin levels are also increased in specific tissues of plants such as flowers, fruits, seeds, and root tips under normal growth and development (Tan et al., 2007a; Hernández-Ruiz and Arnão, 2008; Okazaki and Ezura, 2009; Ramakrishna et al., 2012; Park et al., 2013b; Zhao et al., 2013). Based on a ubiquitous presence and well-conserved biosynthetic gene of melatonin in the plant kingdom, melatonin seems to play a critical role in plant growth and development. However, the role of melatonin in plants requires further study using a knockout mutant plant lacking a key melatonin biosynthetic gene such as SNAT. At this time, melatonin-rich transgenic plants through gain-of-function analysis have been generated, which showed similar results to plants challenged with exogenous melatonin treatment (Park and Back, 2012; Park et al., 2013c; Wang et al., 2014). A previous report applied the loss-of-function mutant approach to a melatonin biosynthetic gene, in which the tryptamine 5-hydroxylase gene was deficient or suppressed (Park et al., 2012b, 2013a). However, these mutant lines were melatonin-rich plants rather than melatonin-deficient plants due to the presence of an alternative melatonin biosynthetic pathway in plants. The SNAT genes that have been examined from all land plants and green algae possess chloroplast transit peptides, and their proteins are expected to localize to chloroplasts,
which are major sources of reactive oxygen species during photosynthesis. Melatonin or a melatonin precursor such as NAS may play a pivotal role in preventing oxidative damage of chloroplasts by scavenging reactive oxygen species or reactive nitrogen species, resulting in the increase of photosynthesis (Tan et al., 2012, 2013; Zhang et al., 2013). However, the exact role and benefit of chloroplast localization of SNAT in plants requires further study.

In summary, we cloned the chloroplast-encoded PySNAT gene and measured melatonin levels from the red alga Pyropia yezoensis. The purified recombinant PySNAT protein catalysed the conversion of serotonin into NAS at the optimal temperature of 55°C. According to the phylogenetic tree, the red alga PySNAT originated from cyanobacteria via endosymbiosis. Thereafter, the chloroplast-encoded SNAT in red algae was transferred into the nuclear genome in green algae through the acquisition of chloroplast transit peptides. Evolution of the plant SNAT gene may follow the direct gene transfer theory from bacteria via an endosymbiotic process.

Acknowledgements
This research was supported by grants from the Next-Generation BioGreen 21 Program (SSAC project no. PJ00949105) through the Rural Development Administration, and the Basic Research Program (2010-0020141) through the NRF of the Ministry of Education, Republic of Korea.

References
Afreen F, Zobayed SM, Kozai T. 2006. Melatonin in Glycyrrhiza uralsensis: response of plant roots to spectral quality of light and UV-B radiation. Journal of Pineal Research 41, 108–115.

Arnao MB, Hernández-Ruiz J. 2013. Growth conditions determine different melatonin levels in Lupinus albus L. Journal of Pineal Research 55, 149–155.

Bajwa VS, Shukla MR, Sherif SM, Murch SJ, Saxena PK. 2014. Role of melatonin in alleviating cold stress in Arabidopsis thaliana. Journal of Pineal Research 56, 238–245.

Balzer I, Hardeland R. 1991. Photoperiodism and effects of indoleamines in a unicellular alga, Gonyaulax polyedra. Science 253, 795–797.

Barrett P, Bolborea M. 2012. Molecular pathways involved in seasonal body weight and reproductive responses governed by melatonin. Journal of Pineal Research 52, 376–388.

Byeon Y, Back K. 2014a. An increase in melatonin in transgenic rice causes pleotropic phenotypes, including enhanced seedling growth, delayed flowering, and low grain yield. Journal of Pineal Research 56, 408–414.

Byeon Y, Back K. 2014b. Melatonin synthesis in rice seedlings in vivo is enhanced at high temperatures and under dark conditions due to increased serotonin N-acetyltransferase and N-acetylserotonin methyltransferase activities. Journal of Pineal Research 56, 189–195.

Byeon Y, Lee HY, Lee K, Park S, Back K. 2014. Cellular localization and kinetics of the rice melatonin biosynthetic enzymes SNAT and ASMT. Journal of Pineal Research 56, 107–114.

Byeon Y, Lee K, Park YI, Park S, Back K. 2013. Molecular cloning and functional analysis of serotonin N-acetyltransferase from the cyanobacterium Synechocystis sp. PCC 6803. Journal of Pineal Research 55, 371–376.

Calvo JR, González-Yanes C, Maldonado MD. 2013. The role of melatonin in the cells of the innate immunity: a review. Journal of Pineal Research 55, 103–120.

Chen GF, Huo YS, Tan DX, Liang Z, Zhang WB, Zhang YK. 2003. Melatonin in Chinese medicinal herbs. Life Sciences 73, 19–26.

Chen Q, Qi WB, Reiter RJ, Wei W, Wang BM. 2009. Exogenously applied melatonin stimulates root growth and raises endogenous indoleacetic acid in roots of elobated seedlings of Brassica junceae. Journal of Plant Physiology 166, 324–328.

Coon SL, Klein DC. 2006. Evolution of arylalkylamine N-acetyltransferase: emergence and divergence. Molecular and Cellular Endocrinology 252, 2–10.

Dereeper A, Audic S, Claverie JM, Blanc G. 2010. BLAST-EXPLORER helps you building datasets for phylogenetic analysis. BMC Evolutionary Biology 10, 8.

Dubrels R, Reiter RJ, Klenke E, Goebel A, Schnakenberg E, Ehlers C, Schiwa HW, Schloot W. 1995. Melatonin in edible plants identified by radioimmunoassay and by high performance liquid chromatography-mass spectrometry. Journal of Pineal Research 18, 29–31.

Emanuelsson O, Nielsen H, Von Hejne G. 1999. Chlorop, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Science 8, 978–984.

Fuhrberg B, Balzer I, Hardeland R, Werner A, Lühning K. 1996. The vertebrate pineal hormone melatonin is produced by the brown alga Pterygophora californica and mimics dark effects on growth rate in the light. Planta 200, 125–131.

García JJ, López-Pingarron L, Almeida-Souza P, Tres A, Escudero P, García-Gil FA, Tan DX, Reiter RJ, Ramirez JM, Bernal-Pérez M. 2014. Protective effects of melatonin in reducing oxidative stress and in preserving the fluidity of biological membranes: a review. Journal of Pineal Research 56, 225–237.

Ha SB, Lee SB, Lee Y, Yang K, Jang SM, Chung JS, Jung S, Kim YS, Wi SG, Back K. 2003. The plastidic Arabidopsis protoporphyrinogen IX oxidase gene, with or without the transit sequence, confers resistance to the diphenyl ether herbicide in rice. Plant, Cell and Environment 27, 79–88.

Hardeland R. 1999. Melatonin and 5-methoxytryptamine in non-metazoans. Reproduction, Nutrition, Development 39, 399–408.

Hardeland R. 2013. Melatonin and the theories of aging: a critical appraisal of melatonin’s role in antiaging mechanisms. Journal of Pineal Research 55, 325–356.

Hattori A, Migitaka H, Iigo M, Itoh M, Yamamoto K, Ohtani-Kaneko R, Hara M, Suzuki T, Reiter RJ. 1995. Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates. Biochemistry and Molecular Biology International 35, 627–634.

Hernández-Ruiz J, Arnao MB. 2008. Distribution of melatonin in different zones of lucen and barley plants at different ages in the presence and absence of light. Journal of Agricultural and Food Chemistry 56, 10567–10573.

Hernández-Ruiz J, Cano A, Arnao MB. 2005. Melatonin acts as a growth-stimulating compound in some monocot species. Journal of Pineal Research 39, 137–142.

Jung S, Lee Y, Yang K, Lee SB, Jang SM, Ha SB, Back K. 2004. Dual targeting of Myxococcus xanthus protoporphyrinogen IX oxidase into chloroplasts and mitochondria and high level oxyfluorfen resistance. Plant, Cell and Environment 27, 1436–1446.

Kang K, Lee K, Park S, Byeon Y, Back K. 2013. Molecular cloning of rice serotonin N-acetyltransferase, the penultimate gene in plant melatonin biosynthesis. Journal of Pineal Research 55, 7–13.

Kang K, Lee K, Park S, Kim YS, Back K. 2010. Enhanced production of melatonin by ectopic overexpression of human serotonin N-acetyltransferase plays a role in cold resistance in transgenic rice seedlings. Journal of Pineal Research 49, 176–182.

Kolář J, Johnson CH, Macháčková I. 2003. Exogenously applied melatonin (N-acetyl-5-methoxytryptamine) affects flowering of the short-day plant Chenopodium rubrum. Physiologia Plantarum 118, 605–612.

Lazar D, Murch SJ, Beilby MJ, Khazaalia SA. 2013. Exogenous melatonin affects photosynthesis in Characeae Chara australis. Plant Signaling and Behavior 8, e23279.

Lerner AB, Case JD, Takahashi Y. 1985. Isolation of melatonin, a vertebrate pineal hormone, from the brown alga Pterygophora californica. Reptile Science 28–31.

Li C, Wang P, Wei Z, Liang D, Liu C, Yin L, Jia D, Fu M, Ma F. 2012. The mitigation effects of exogenous melatonin on salinity-induced stress in Malus hupenhensis. Journal of Pineal Research 53, 298–306.
Lorenz M, Lüning K. 1998. Detection of endogenous melatonin in the marine red macroalgae Porphyra umbilicalis and Palmaria palmata by enzyme-linked immunosassay (ELISA) and effects of melatonin administration on algal growth. In: Proceedings of the Conference News from the Plant Chronobiology Research, Markgrafeneide, Germany, 42–43.

Martin W, Herrmann RG. 1998. Gene transfer from organelles to the nucleus: how much, what happens, and why? Plant Physiology 118, 9–17.

Macklachlan J. 1973. Growth media—minute. In: Stein JR, ed. Handbook of phycoligical methods: culture methods and growth measurements . New York: Cambridge University Press, 25–51.

Murch SJ, Alan AR, Cao J, Saxena PK. 2009. Melatonin and serotonin in flowers and fruits of Datura metel L. Journal of Pineal Research 47, 277–283.

Murch SJ, KrishnaRaj S, Saxena PK. 2000. Tryptophan is a precursor for melatonin and serotonin biosynthesis in in vitro regenerated St John's wart (Hypericum perforatum L. cv. Anths) plants. Plant Cell Reports 19, 688–704.

Noda H. 1993. Health benefits and nutritional properties of nori. Journal of Applied Phycology 5, 255–258.

Okazaki M, Ezura H. 2009. Profiling of melatonin in the model tomato (Solanum lycopersicum L.) cultivar Micro-Tom. Journal of Pineal Research 46, 336–343.

Okazaki M, Higuchi K, Aouini A, Ezura H. 2010. Lowering intercellular melatonin levels by transgenic analysis of indoleamine 2,3-dioxigenase from rice in tomato plants. Journal of Pineal Research 49, 239–247.

Pandi-Perumal SR, Srinivasan V, Maestroni GJM, Cardinali DP, Tal O, Haim A, Harel O, Gerchman Y. 2011. Melatonin as an antioxidant and its semi-lunar rhythm in green macroalgae Ulva sp. Journal of Experimental Botany 62, 1903–1910.

Park S, Back K. 2010. Lowering intercellular melatonin levels by transgenic analysis of indoleamine 2,3-dioxigenase from rice in tomato plants. Journal of Pineal Research 49, 239–247.

Park S, Lee K, Kim YS, Back K. 2007. Transcriptional suppression of tryptamine 5-hydroxylase, a terminal serotonin biosynthetic gene, induces melatonin biosynthesis in rice (Oryza sativa L.). Journal of Pineal Research 45, 131–137.

Park S, Lee K, Kim YS, Back K. 2012. Melatonin regulates Arabidopsis root system architecture likely acting independently of auxin signaling. Journal of Pineal Research 53, 279–288.

Posmyk MM, Balabusta M, Wieczorek M, Sliwinska E, Janas KM. 2004. Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science. Journal of Experimental Botany 55, 577–597.

Posmyk MM, Balabusta M, Wieczorek M, Sliwinska E, Janas KM. 2003. Physiologicical responses of blade growth during chilling stress. Applied Phycology 15, 1131–1151.

Pandhi Perumal SR, Srinivasan V, Maestroni GJM, Cardinali DP, Tal O, Haim A, Harel O, Gerchman Y. 2011. Melatonin as an antioxidant and its semi-lunar rhythm in green macroalgae Ulva sp. Journal of Experimental Botany 62, 1903–1910.

Tiryaki I, Keles H. 2012. Reversal of the inhibitory effect of light and high temperature on germination of Phacelia tanacetifolia seeds by melatonin. Journal of Pineal Research 52, 332–339.

Vitalini S, Gardana C, Zanzotto A, Fico G, Faoro F, Simonetti P, Iriti M. 2011. From vineyard to glass: agrochemicals enhance the melatonin and total polyphenol contents and antiradical activity of red wines. Journal of Pineal Research 51, 278–285.

Wang L, Zhao Y, Reiter R, He C, Liu G, Lei Q, Zuo B, Zheng XD, Li Q, Kong J. 2014. Changes in melatonin levels in transgenic 'Micro-Tom' tomato over-expressing ovine ANAT and ovine HOMT genes. Journal of Pineal Research 56, 126–133.

Wang P, Sun X, Chang C, Feng F, Liang D, Cheng L, Ma F. 2013a. Delay in leaf senescence of Malus hupehensis by long-term melatonin application is associated with its regulation of metabolic status and protein degradation. Journal of Pineal Research 55, 424–434.

Wang P, Sun X, Li C, Wei Z, Liang D, Ma F. 2013b. Long-term exogenous application of melatonin delays drought-induced leaf senescence in apple. Journal of Pineal Research 54, 292–302.

Yoon HS, Hackett J, Ciniglia C, Pinto G, Bhattacharya D. 2004. A molecular timeline for the origin of photosynthetic eukaryotes. Molecular Biology and Evolution 21, 809–818.

Zhang N, Zhao B, Zhang HJ, Weeda S, Yang C, Yang ZC, Ren S, Guo YD. 2013. Melatonin promotes water-stress tolerance, lateral root formation, and seed germination in cucumber (Cucumis sativus L.). Journal of Pineal Research 54, 15–23.

Zhang XP, Glaser E. 2002. Interaction of plant mitochondrial and chloroplast signal peptides with the Hsp70 molecular chaperone. Trends in Plant Science 7, 14–21.