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Article type : Original Manuscript

Rice histone deacetylase 10 and Arabidopsis histone deacetylase 14 genes encode *N*-acetylserotonin deacetylase, which catalyzes conversion of *N*-acetylserotonin into serotonin, a reverse reaction for melatonin biosynthesis in plants

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Running title: N-*acetylserotonin deacetylase genes*

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jpi.12460

Keywords:

Arabidopsis, melatonin deacetylase, *N*-acetyltryptamine deacetylase, rice

Abstract: In plants, melatonin production is strictly regulated, unlike the production of its precursor, serotonin, which is highly inducible in response to stimuli, such as senescence and pathogen exposure. Exogenous serotonin treatment does not greatly induce the production of *N*-acetylserotonin (NAS) and melatonin in plants, which suggests the possible existence of one or more regulatory genes in the pathway for the biosynthesis of melatonin from serotonin. In this report, we found that NAS was rapidly and abundantly converted into serotonin in rice seedlings, indicating the presence of an *N*-acetylserotonin deacetylase (ASDAC). To clone the putative *ASDAC* gene, we screened four genes that were known as histone deacetylase (*HDAC*) genes, but encoded proteins targeted into chloroplasts or mitochondria rather than nuclei. Of four recombinant *Escherichia coli* strains expressing these genes, one *E. coli* strain expressing the rice *HDAC10* gene was found to be capable of producing serotonin in response to treatment with NAS. The recombinant purified rice HDAC10 (OsHDAC10) protein exhibited ASDAC enzyme activity toward NAS, *N*-acetyltyramine (NAT), *N*-acetyltryptamine, and melatonin, with the highest ASDAC activity for NAT. In addition, its Arabidopsis ortholog, AtHDAC14, showed similar ASDAC activity to that of OsHDAC10. Both OsHDAC10 and AtHDAC14 were found to be expressed in chloroplasts. Phylogenetic analysis indicated that ASDAC homologs were present in archaea, but not in cyanobacteria,

which differs from the distribution of serotonin *N*-acetyltransferase (SNAT). This suggests that SNAT and ASDAC may have evolved differently from ancestral eukaryotic cells.

1 | INTRODUCTION

Melatonin is a universal molecule found in almost all organisms at levels that vary depending on tissue localization and diurnal changes.[1,2] Melatonin also plays a wide variety of physiological and ecological roles in animals and plants, including involvement in circadian rhythms, immune function, DNA repair, and defense against various stresses.[3,4]

Studies on the biosynthetic pathway and physiological roles of melatonin in plants have advanced rapidly, as melatonin was first identified only 20 years ago.[5] For example, melatonin functions in plants as a biostimulator, orchestrating an array of beneficial effects against biotic and abiotic stresses, including exposure to cold, senescence, cadmium, nitrate, drought, and pathogens,[6-15] via the well-known H_2O_2/NO signaling pathways.[16-19] However, the regulatory mechanisms of melatonin biosynthesis remain elusive.[20] In common with animals, melatonin biosynthesis in plants requires four enzyme-mediated steps. First, tryptophan is decarboxylated into tryptamine by tryptophan decarboxylase (TDC), which is followed by serotonin synthesis via the action of tryptamine 5-hydroxylase (T5H).[21,22] The penultimate enzyme is serotonin *N*-acetyltransferase (SNAT), which catalyzes the conversion of serotonin into *N*-acetylserotonin (NAS). SNAT enzymes are encoded by a small gene

family found in both chloroplasts and mitochondria.[23-26] The last enzyme in the pathway is *N*-acetylserotonin *O*-methyltransferase (ASMT), which converts NAS into melatonin via the action of multiple distantly-related *O*-methyltransferases, including caffeic acid *O*-methyltransferase (COMT).[27-30] These ASMT-encoding genes are expressed in either cytoplasm or chloroplasts, resulting in melatonin synthesis in different subcellular sites of the plant cells.[24,30]

Exogenous serotonin treatment was not found to significantly increase NAS and melatonin levels in transgenic rice overexpressing an animal *SNAT* gene or non-transgenic wild-type rice expressing an endogenous *SNAT* gene constitutively.[31] This result was consistent with subsequent reports that the increase in serotonin levels as a response to senescence or cadmium treatment was not proportionate to the increase in melatonin levels.[32,33] These results indicate that the conversion of serotonin into NAS is a bottleneck step in melatonin biosynthesis in plants, but the reason for the lack of NAS accumulation has not been elucidated.

In this report, we demonstrate for the first time that NAS is rapidly converted into serotonin upon exogenous NAS treatment in rice seedlings. We cloned the genes encoding NAS deacetylase (ASDAC) enzymes, characterized them in rice and Arabidopsis, and assessed their subcellular localization to chloroplasts. Based on enzyme functional analysis, it is clear that ASDAC plays a pivotal role in regulating melatonin biosynthesis in plants.

2 | METHODS

2.1 | Vector construction and expression of HDAC genes in *Escherichia coli*

Four full-length rice histone deacetylase (*HDAC*) cDNAs and one *Arabidopsis thaliana* *HDAC14* cDNA were provided by the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan).[34,35] Full-length cDNA fragments were amplified by PCR using primer sets with each forward primer containing 14-nucleotide (nt) *attB1* sequences (5'-AAA AAG CAG GCT CC-3') and each reverse primer containing a stop codon and 12-nt *attB2* sequences (5'-AGA AAG CTG GGT-3'), as described previously.[23] Because rice (*Oryza sativa*) HDAC10 and OsSIR2b were predicted to possess chloroplast transit sequences of 36 and 23 amino acids, vectors for the expression of these enzymes were designed to express the enzymes without the predicted transit sequences. The resulting *HDAC* cDNA fragments were further amplified by a second PCR using primers harboring the *attB* recombination sequence (forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CT-3'; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT-3'). The second PCR products were gel-purified and cloned into the pDONR221 Gateway vector (Invitrogen, Carlsbad, CA, USA) via BP recombination at 25°C for 1 hour, followed by protease treatment at 37°C for 5 min. The recombinant pDONR221 plasmids harboring various inserts of *HDAC* genes were recombined with the pET300 Gateway Destination Vector (Invitrogen) via the LR Recombination Reaction to form pET300-HDAC plasmid vectors, which were

transformed into BL21 (DE3) competent *E. coli* cells. *Escherichia coli* strains harboring various pET300-HDAC plasmids were incubated in the presence of possible substrates (1 mmol/L), such as NAS and isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mmol/L; Sigma) for 16 hours at 28°C. These cultures were spun down and supernatants were subjected to high performance liquid chromatography (HPLC) analyses.

2.2 | Chemicals

Melatonin (*N*-acetyl-5-methoxytryptamine), NAS, *N*-acetyltryptamine, *N*-acetyltyramine, 5-methoxytryptamine, serotonin, tryptamine, and tyramine were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were dissolved in ethanol and further diluted into water for analyses.

2.3 | Affinity purification of OsHDAC10 and AtHDAC14 proteins

Aliquots (10 mL) of overnight cultures of *E. coli* strains containing pET300-OsHDAC10 and pET300-AtHDAC14 were inoculated into 100 mL of YEP medium (10 g/L Bacto peptone, 10 g/L Bacto yeast extract, 5 g/L NaCl) containing 50 mg/L ampicillin and incubated at 37°C until the optical density of the *E. coli* culture at 600 nm (OD_{600}) reached 1.0. After the addition of 1 mmol/L IPTG (Sigma), the cultures were shaken for 5 h at 28°C at 250 rpm. The cultures were centrifuged at 13 500 × *g* for 5 min at 4°C, and the cell pellets were used in

the protein purification steps. The purifications were carried out by affinity (Ni^{2+}) chromatography according to the manufacturer's instructions (Qiagen, Tokyo, Japan).

2.4 | HPLC quantification of amines

Supernatants from each of the *E. coli* cultures were mixed with equal volumes of methanol, and 20-μL aliquots of the solutions were subjected to HPLC using a fluorescence detector system (Waters, Milford, MA, USA). In brief, the samples were separated on a Sunfire C18 column (Waters; 4.6 × 150 mm) using isocratic elution with 6% methanol in 0.3% trifluoroacetic acid for 50 min at a flow rate of 1 mL/min. Serotonin, tyramine, tryptamine, and 5-metrhoxytryptamine were detected at 280 nm (excitation) and 348 nm (emission). All measurements were taken in triplicate.

2.5 | Subcellular localization of OsHDAC10 and AtHDAC14 proteins

The pER-mCherry vector, kindly donated by Dr. H.G. Kang (Texas State University, San Marcos, TX, USA), was used for subcellular localization of two HDAC proteins including OsHDAC10 (AK072557) and AtHDAC14 (At4g33470). Two full-length *HDAC* cDNAs were amplified by PCR using a primer set containing *Asc*I sites (GGCGCGCC). The resulting *HDAC* PCR products were cloned into the TA vector (RBC Bioscience, New Taipei City, Taiwan), and each *HDAC* insert was then digested using *Asc*I and cloned into the *Asc*I site of

the binary vector pER8-mCherry containing the estrogen-inducible XVE promoter (Pxve) to create pER8-HDAC-mCherry. The pER8-HDAC-mCherry plasmids were transferred into the *Agrobacterium tumefaciens* strain GV2260 using the freeze-thaw method, and confocal microscope (TCS-SP5; Leica, Wetzlar, Germany) transient expression analyses were performed as described previously.[36,37]

2.6 | Total RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR) analysis

Detached leaves of 4-week-old rice plants were treated with 0.2 mmol/L cadmium for various lengths of time. Pulverized powders from the leaves (100 mg) were subjected to total RNA extraction using the RNeasy Plant Mini Kit (Qiagen). The resulting total RNA (1 µg) was reverse-transcribed using RevertAid reverse transcriptase (Thermo Scientific Fermentas, St. Leon-Ro, Germany) and 500 ng of oligo(dT)18 primer at 42°C for 1 hour. The acquired cDNA (0.2 µL) was used as the template for PCR amplification. The rice ubiquitin-5 gene (UBQ5) served as a loading control. The primers (5'→ 3') used in the PCR reactions were as follows: *SNAT1* forward, 5'-GGG CTG CGG CAA CTT GGT CC-3' and *SNAT1* reverse, 5'-AGA AAG CTC GGT CTA AAA TCT GGG GTA-3'; *HDAC10* forward, 5'-ACT AGT ATG GAA CAG CTG TGG GTG-3' and *HDAC10* reverse, 5'-GAG CTC ACC ACG ATG CTT CGA AGT-3'; *UBQ5* forward, 5'-CCG ACT ACA ACA TCC AGA AGG AG-3' and *UBQ5* reverse, 5'-AAC AGG AGC CTA CGC CTA AGC-3'. RT-PCR-amplified fragments were

electrophoresed on ethidium bromide gels and photographed under ultraviolet (UV) light.

2.7 | Measurement of *in vitro* HDAC enzyme activity in rice seedlings

Seven-day-old rice seedlings were dissected into leaves and root parts. These samples (0.1 g) were ground in 50 mmol/L Tris-HCl buffer (pH 8.0) containing 10 mmol/L β-mercaptoethanol. The homogenates were centrifuged for 15 min at 13,000 × *g* and 4°C. The supernatants were used as a crude enzyme solution, 30 µL of which was used to assay ASDAC enzyme activity in a total volume of 100 µL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 1mmol/L NAS at 30°C for 90 min. The reaction was stopped by adding 20 µL of acetic acid. After brief centrifugation, a 20-µL aliquot was subjected to HPLC as described above. All measurements were conducted in triplicate. Protein concentrations were determined by the Bradford method using a protein assay dye (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.8 | Enzyme kinetics and phylogenetic analyses

The substrate affinity (K_m) and maximum reaction rate (V_{max}) were calculated from Lineweaver-Burk plots based on crude *E. coli* extracts containing the rice or Arabidopsis ASDAC proteins. The crude *E. coli extracts* (10 µL) or the purified recombinant proteins were used to assay ASDAC enzyme activity in a total volume of 100 µL of 50 mmol/L Tris-

HCl buffer (pH 8.0) containing 1 mmol/L NAS (or other substrates) at 30°C for 90 min. The reaction was stopped by adding 20 μL of acetic acid. After brief centrifugation, a 20-μL aliquot was subjected to HPLC as described above. The network-based method (TargetP) was used to identify chloroplast transit peptides and their cleavage sites from various N-terminal sequences of ASDAC homologs.[38] A phylogenetic tree was generated using BLAST-Explorer.[39]

3 | RESULTS

3.1 | Substrate feeding analyses in rice seedlings

To measure the *in vivo* substrate conversion rate of the melatonin biosynthetic pathway (Figure 1A), roots of 7-day-old rice seedlings were treated with various melatonin biosynthetic substrates for 24 hours. As shown in Figure 1B, NAS treatment (1 mmol/L) led to serotonin production of 1514 ng/g fresh weight (FW), whereas melatonin production was as low as 35 ng/g FW. These data suggested that the conversion rate of NAS into melatonin by ASMT was much slower than that of NAS into serotonin, which was strongly indicative of the presence of ASDAC. By comparison, the NAS conversion resulting from treatment with 1 mmol/L serotonin was 75 ng/g FW, whereas serotonin production resulting from treatment with 1 mmol/L tryptamine was as high as 8281 ng/g FW. Based on the substrate conversion rate, it was evident that rice plants express ASDAC enzyme, which catalyzes the conversion

of NAS into serotonin, and that the ASDAC enzyme has much higher *in vivo* catalytic activity than SNAT.

3.2 | *De novo* molecular cloning of rice ASDAC

Of the 16 *HDAC* genes in the rice genome,[40] three encoded proteins that were localized to sites other than the nucleus. OsHDAC6 was exclusively localized in chloroplasts, whereas OsHDAC10 was observed in both chloroplasts and mitochondria.[41] OsSIR2b, a rice ortholog of yeast sirtuin 2, was detected in mitochondria, as was its human ortholog hSIRT3.[42] Based on the subcellular localization of these rice HDAC proteins[41] and the fact that melatonin biosynthesis occurs in the chloroplasts and mitochondria of plants,[20,26] we predicted that these proteins might have activity to deacetylate NAS into serotonin. To analyze the function of these *HDAC* genes, we first expressed the genes in *E. coli* and incubated the recombinant strains in the presence of NAS to measure possible production of serotonin via ASDAC activity. Additionally, *OsSRT1*, a rice ortholog of yeast sirtuin 2, was included as an *E. coli* expression control because OsSRT1 protein is a histone deacetylase that is expressed in the nucleus[43] (Figure 2A). Among four transgenic *E. coli* strains, an *E. coli* strain harboring the Δ36:OsHDAC10 construct was found to produce serotonin in response to treatment with 1 mmol/L NAS at a concentration of 0.6 μg/mL culture, whereas other *E. coli* strains, including the strain expressing OsSRT1, were unable to produce serotonin upon treatment with NAS.

This suggested that *OsHDAC10* encoded ASDAC enzyme activity. To further verify the ASDAC activity of OsHDAC10, various substrates were incubated with the *E. coli* strains. As shown in Figure 2C–E, the *E. coli* strain expressing OsHDAC10 produced tyramine (42 μg/mL), tryptamine (1.8 μg/mL), and 5-methoxytryptamine (0.4 μg/mL) when incubated with *N*-acetyltyramine, *N*-acetyltryptamine, or melatonin, respectively. Our finding therefore demonstrated that OsHDAC10 had a potent ASDAC enzyme activity catalyzing the conversion of a number of *N*-acetylamines, such as *N*-acetyltyramine and NAS, as well as melatonin.

3.3 | *In vitro* ASDAC enzyme activity of purified recombinant OsHDAC10

TargetP analysis predicted a possible chloroplast transit sequence of 36 amino acids.[38] However, the results from the amino acid comparison between OsHDAC10 and its Arabidopsis ortholog AtHDAC14 suggested that the 66 N-terminal amino acids of OsHDAC10 might encode the chloroplast transit sequence. Therefore, we generated three constructs of OsHDAC10: full-length OsDHAC10 (FL:HDAC10), *N*-terminal 36-amino-acid-truncated OsHDAC10 (Δ36:OsHDAC10), and *N*-terminal 66-amino-acid-truncated OsHDAC10 (Δ66:OsHDAC10). The resulting recombinant HDAC10 enzymes were purified by nickel affinity chromatography (Figure 3A–C), with the highest soluble expression found in the Δ66:OsHDAC10-producing recombinant strain. Interestingly, both the purified

Δ36:OsHDAC10 and Δ66:OsHDAC10 proteins did not show any *in vitro* ASDAC enzyme activity, but the ASDAC enzyme activity was recovered by the addition of *E. coli* BL21(DE3) crude extract (Figure 3D). Upon the addition of boiled crude *E. coli* extracts, no ASDAC activity was observed, which suggests that some heat labile substances or proteins found in *E. coli* were necessary for *in vitro* ASDAC enzyme assay. The Δ66:OsHDAC10 protein exhibited threefold higher ASDAC activity than that of the Δ36:OsHDAC10 protein, which indicates that the chloroplast transit sequence is about 66 amino acids rather than 36 amino acids (Figure 3E). Given these findings, our further analyses employed the Δ66:OsHDAC10 enzyme. When the substrate specificity of the enzyme was assessed using a number of *N*-acetylamines, *N*-acetyltyramine showed the highest ASDAC activity (18 pkat/mg protein) followed by NAS (5 pkat/mg protein) and *N*-acetyltryptamine (1.6 pkat/mg protein). We observed that Δ66:OsHDAC10 was also able to deacetylate melatonin into 5-methoxytryptamine (Figure 3F), indicating that rice HDAC10 exhibits affinity for multiple substrates, as do the SNAT enzymes of plants.[20]

3.4 | Optimal ASDAC enzyme activity of OsHDAC10

Because NAS is a direct substrate for melatonin biosynthesis via the ASMT enzyme, we measured ASDAC activity in the presence of NAS as a substrate. As shown in Figure 4, ASDAC enzyme activity increased as the concentration of Δ66:OsHDAC10 increased. The

ASDAC enzyme demonstrated high activity at a broad range of pH values (from 6.8 to 8.8). The optimum temperature for the enzyme was 30°C, but moderate ASDAC enzyme activities were also detected at temperatures as high as 45°C and 55°C. In the presence of sodium butyrate, a potent histone deacetylase inhibitor, no ASDAC inhibition was observed. This indicates that the ASDAC enzyme activity of OsHDAC10 is resistant to the histone deacetylase inhibitor.

3.5 | Chloroplast localization of OsHDAC10

To examine the ASDAC enzyme activity in rice plants, we dissected seven-day-old rice seedlings into root and shoot, and assayed for *in vitro* ASDAC enzyme activity using NAS as a substrate. ASDAC enzyme activity was observed in both tissues, but higher activity levels were observed in the root tissue than in the shoot tissue (Figure 5A). Transcript levels of *OsHDAC10* were monitored in four-week-old detached rice leaves challenged with 0.2 mmol/L cadmium. As shown in Figure 5B, *HDAC10* mRNA was constitutively expressed at low levels in the control, but expression levels declined slightly when assessed at 12 and 24 hours after cadmium treatment, returning to the basal level at 72 hours after treatment. When assessing the subcellular localization of the enzyme in the *Nicotiana benthamiana* transient expression system, OsHDAC10 protein was found to be expressed in chloroplasts (Figure 5C). Our results partially contrast with previous reports that OsHDAC10 is expressed in

mitochondria in tobacco BY2 cells and in chloroplasts in transgenic Arabidopsis.[41] Since no mitochondrial expression of OsHDAC10 was detected in transgenic Arabidopsis,[41] this targeting discrepancy may be attributable to the use of different host systems. Overall, when our data are considered in view of the prior data, it is evident that OsHDAC10 is expressed in chloroplasts.

3.6 | Enzymatic characterization of the OsHDAC10 ortholog *Arabidopsis thaliana* HDAC14 (AtHDAC14),

The amino acid sequence of *Arabidopsis thaliana* HDAC14 (AtHDAC14) showed 70% sequence identity to the sequence of OsHDAC10, which suggests that it may be a functional ortholog of OsHDAC10. To examine whether AtHDAC14 possesses ASDAC activity, the full-length AtHDAC14 (FL:AtHDAC14) and *N*-terminal 45-amino-acid-truncated AtHDAC14 (Δ45:AtHDAC14) were expressed in *E. coli* and serotonin biosynthesis was measured in the presence of 1 mmol/L NAS. As shown in Figure 6A, the *E. coli* expressing Δ45:AtHDAC14 produced serotonin at a concentration of 22 μg/mL culture, whereas the FL:AtHDAC14 yielded only 0.2 μg/mL culture, which suggests that AtHDAC14 possesses ASDAC enzyme activity. The recombinant Δ45:AtHDAC14 protein was purified using a nickel affinity system and assessed using the ASDAC enzyme assay (Figure 6B). AtHDAC14 showed an ASDAC-specific activity of 7.3 pkat/mg protein by the addition of *E. coli* crude

extracts when NAS was used as the substrate (Figure 6C). Similar to the findings for OsHDAC10, AtHDAC14 had the highest ASDAC activity when NAT was used as the substrate (30 pkat/mg protein), followed by the activity values obtained with NAS and *N*-acetyltryptamine as substrates. Melatonin was also deacetylated into 5-methoxytryptamine by AtHDAC14, with an ASDAC activity of 1.6 pkat/mg protein (Figure 6D). Confocal microscopy also revealed that the AtHDAC14 protein was localized in chloroplasts, as the mCherry fluorescence was found to be completely superimposed with the autofluorescence signals of chlorophyll (Figure 7A–D).

The K_m and V_{max} values were measured using crude *E. coli* extracts obtained from recombinant strains expressing the enzymes. Rice HDAC10 had a lower K_m value (409 μmol/L) than that of Arabidopsis HDAC14 (701 μmol/L) (Figure 7B), whereas the V_{max} values of the two enzymes were similar. The K_m and ASDAC-specific activity of OsHDAC10 were similar to those for rice SNAT1.[23,24]

In summary, these data indicate that previously classified *HDAC* genes, such as *OsHDAC10* and *AtHDAC14*, encode enzymes with ASDAC activity. The functional significance of ASDAC *in vivo* requires further investigation with respect to melatonin synthesis in plants. The reversible acetylation and deacetylation of serotonin in plants may play a pivotal regulatory role in maintaining the steady-state level of melatonin biosynthesis in plants.

4 | Discussion

Deacetylase enzymes are classified into two types, depending on their substrates. One type deacetylates organic compounds and the other, proteins. The best protein substrate of deacetylase is histone proteins, which play a key role in modulating chromatin structure. The activity of enzymes known as histone deacetylases (HDACs) leads to the tightness of chromatin structure, which blocks the access of transcription factors and causes the repression of corresponding genes.[44] HDAC proteins have been identified in almost all eukaryotes, including animals, plants, fungi, and archaebacteria, and regulate many functions in transcription and the cell cycle.[45,46] HDAC proteins have also been shown to deacetylate non-histone substrates, such as α-tubulin, heat shock proteins, and β-catenin.[44] In contrast to HDAC, many deacetylases have been reported that are responsible for deacetylation of organic compounds. These include *N*-acetylglucosaminyl *N*-deacetylase,[47] acetylpolyamine amidohydrolase,[48] polyamine deacetylase,[49] and *N*-acetylornithine deacetylase.[50]

4.1 | Significance of plant *ASDAC* genes

Phylogenic tree analysis of 123 HDACs from eukaryotic and prokaryotic organisms identified five classes of the enzyme: class I is related to yeast RPD3, whereas classes II and III are associated with yeast HDA1 and yeast SIR2, respectively.[41] The annotated rice genome was found to possess 16 *HDAC* genes spanning all five classes of HDACs, seven of

which belong to class I and one which belongs to class IV. Among the 16 rice HDAC proteins, OsHDAC6 belongs to class IV, OsHDAC10 to class II, and OsSIR2b to class III. These three OsHDAC proteins are targeted to chloroplasts, mitochondria, or both, rather than the nucleus.[41] The localization data suggested that these three OsHDAC proteins may possess deacetylase activity toward organic compounds and/or other proteins, rather than histone. The functional roles of these three OsHDACs had not previously been assessed, but AtHDA2A and AtHDA14, the Arabidopsis orthologs of OsHDAC6 and OsHDAC10, were found to be involved in gene silencing and tubulin deacetylation, respectively.[51,52]

In this report, we uncovered for the first time that OsHDAC10 has a functional ASDAC activity which catalyzes the conversion of NAS into serotonin, a reverse pathway for melatonin biosynthesis (Figure 1). The ASDAC enzyme activity of 5 pkat/min/mg protein was almost identical to that reported for SNAT.[23] This reversible activity between the production of serotonin and NAS may play an important role in regulating a steady-state level of NAS in chloroplasts prior to the final catalysis by ASMT for the synthesis of melatonin. Additionally, the OsHDAC10 protein showed ASDAC activity toward other related *N*-acetylated amines, such as NAT, *N*-acetyltryptamine, and melatonin. The deacetylase activity of rice ASDAC (OsHDAC10) for multiple substrates is consistent with the acetylase activity of SNAT for multiple substrates.[25]

To further characterize the ASDAC activity of OsHDAC10, we assessed the function of AtHDAC14, an ortholog of OsHDAC10 (Figure 6). AtHDAC14 also exhibited ASDAC enzyme activity similar to that of OsHDAC10, suggesting the existence of conserved ASDAC proteins in plants. Genes orthologous to rice *ASDAC* have been widely distributed in plants, including green algae (GenBank accession no. XP_005851005) and even mycobacteria (GenBank accession no. EUA44530). Thus, the universal presence of melatonin in living organisms is further supported by the widespread existence of *ASDAC* genes. The functional significance and enzyme kinetics of ASDAC proteins from green algae and mycobacteria will shed light on the regulatory mechanism of melatonin in other organisms. Based on our results and previous reports, both OsHDAC10 and AtHDAC14 possess deacetylase activity for multiple substrates, such as *N*-acetylated amines and α-tubulin. However, it is more likely that the ASDAC activity of OsHDAC10 and AtHDAC14 proteins prevails over the α-tubulin deacetylase activity inside plant cells, because OsHDAC10 and AtHDAC14 reside in chloroplasts where α-tubulin is absent.

The cofactor requirements of rice and Arabidopsis ASDAC enzymes remain to be elucidated. Rice ASDAC enzyme activity was undetectable *in vitro* both in the absence of *E. coli* crude extracts and in the presence of boiled *E. coli* crude extracts, which suggests that heat labile cofactors may be required for enzyme activity. In contrast, Arabidopsis ASDAC had more than 40% activity in the absence of *E. coli* crude extracts, which indicates some

difference in the cofactor requirements of the two enzymes. The requirement of cofactors was also observed in glucosaminyl *N*-deacetylase, which was found to require *E. coli* crude extracts or a polycation polybrene for deacetylase activity.[52] However, the addition of polybrene was not effective to restore the activity of rice ASDAC, suggesting that other cofactors or proteins are required for rice ASDAC enzyme activity (data not shown).

4.2 | Phylogenic significance of *ASDAC* genes in plants

The amino acid sequence of the OsHDAC10 protein possesses more than 60% identity to proteins of green algae, such as *Ostrecoccus tauri* (65%), *Micromonas pusilla* (65%), and *Chlorella variabilis* (64%). Also, the OsHDAC10 protein has 43% identity to that of the archeon *Methanosaeta thermophila* (Figure 8A). Although the functional analysis of the putative *M. thermophila ASDAC* gene is required, it is highly likely that the *M. thermophila ASDAC* gene possesses ASDAC enzyme activity, as the cyanobacteria SNAT homolog showed SNAT enzyme activity.[54] It is intriguing to note that rice *ASDAC* homolog genes were absent in cyanobacteria, but present in Archaea, while the rice *SNAT* homolog was present in cyanobacteria, not in Archaea. These data may suggest that plant melatonin synthesis originates from cyanobacteria via an endosymbiotic event, whereas the *ASDAC* gene evolved later via another endosymbiotic event, which supports the theory of multiple endosymbiotic events in plants.[55] Both *SNAT* and *ASDAC* genes are present in organisms from green algae to

higher plants, suggesting that both genes are necessary for plants to survive and thrive during normal plant growth and reproduction, as well as in response to various adverse environmental stresses, including cold, pathogens, and high temperature.[56]

Predicted transit sequences for subcellular localization, such as in chloroplasts, were found in *O. tauri* and *C. variabilis*, but not in *M. pusilla* (Figure 8B). These data suggest that the chloroplast targeting sequences of *ASDAC* genes were also acquired in the era of green algae, as were the *SNAT* genes.[57] The predicted transit sequence targeting mitochondria in *C. variabilis* is of interest and needs to be examined further.

Rice HDAC10 showed 35% identity with human HDAC10 (HsHDAC10). Whether HsHDAC10 has functional ASDAC activity or not remains to be investigated, but it is unlikely because it has a low sequence identity and a different gene structure. The gene for HsHDAC10 encodes 673 amino acids and consists of two catalytic domains: the N-terminal catalytic domain spanning amino acids 1–354, which is complete, and the C-terminal catalytic domain spanning amino acids 355–673, which has a partial catalytic domain.[58,59] Human HDAC10 is expressed in both the nucleus and cytoplasm with HDAC activity. Recently, HsHDAC10 was found to possess polyamine deacetylase (PADA) activity, which catalyzes the conversion of *N*-acetylspermidine into spermidine with a high K_{cat} value (0.28 S^{-1}).[60]

In summary, plants possess two genes encoding SNAT and ASDAC proteins, which act in the reversible biosynthesis of melatonin. SNAT favors melatonin synthesis, whereas ASDAC restricts melatonin synthesis. The simultaneous presence of two enzymes in chloroplasts suggests that melatonin synthesis is tightly controlled so that it is maintained at an optimal level that is essential for plant growth and development. These ideas are consistent with previous reports that the overexpression of melatonin biosynthetic genes, such as tryptophan decarboxylase,[61,62] *SNAT*,[9,29] and *N*-acetylserotonin methyltransferase,[31,63,64] did not abundantly lead to the accumulation of melatonin in plants and thus led to almost normal phenotypes. Further genetic alteration of plant *ASDAC* genes will shed light on the novel functions of melatonin that have not been previously identified in plants.

ACKNOWLEDGMENTS

This research was supported by grants (2010–0020141 and 2017R1A2A2A05069253) from the Basic Science Research Program of the National Research Foundation of Korea, funded by the Ministry of Education, Republic of Korea. We express sincere thanks to Dr. Ok Jin Hwang for technical assistance.

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FIGURE LEGENDS

FIGURE 1 Schematic diagram of the melatonin biosynthetic pathway and substrate feeding analysis. (A) Melatonin biosynthetic pathway and related enzymes. (B) *In vivo* conversion rates of various substrates. The 7-day-old rice seedlings grown in half-strength Murashige and Skoog medium were treated with 1 mmol/L substrates by root for 1 day. The upper leaves were collected and subjected to HPLC analysis. Values are means ± SD from three independent experiments. FW, fresh weight.

FIGURE 2 Production of amines in *Escherichia coli* expressing various rice *HDAC* genes. (A) List of *HDAC* genes used in this report. (B) Quantification of serotonin in recombinant *E. coli* strains upon treatment with NAS (1 mmol/L). (C) Quantification of tyramine in transgenic *E. coli* strains upon treatment with *N*-acetyltyramine (1 mmol/L). (D) Quantification of tryptamine in transgenic *E. coli* strains upon treatment with *N*-acetyltryptamine (NATM) (1 mmol/L). (E) Quantification of 5-methoxytryptamine (5-MT) in transgenic *E. coli* strains upon treatment with melatonin (1 mmol/L). Recombinant *E. coli* strains expressing various rice *HDAC* genes were treated with various substrates (1 mmol/L) and incubated at 28°C for 16 hours. Amines were quantified from *E. coli* cultures by HPLC. Values are means ± SD from three independent experiments. C, chloroplasts; M, mitochondria; N, nucleus.

FIGURE 3 Affinity purification of rice HDAC10 (OsHDAC10) proteins and their enzymatic activities. (A–C) Affinity purification of OsHDAC10 proteins from *E. coli*, and (D–E) their enzymatic characteristics. Various forms of the N-terminal truncated *OsHDAC10* genes were ligated in-frame with DNA sequences coding for amino-terminal hexahistidine sequences and transformed into *E. coli* BL21 (DE3) host cells. The recombinant proteins were induced with IPTG at 28°C for 5 hours. Protein samples were separated by SDS-PAGE and stained for proteins using Coomassie blue. MW, molecular mass standards; lane 1, total

proteins in 10-µL aliquots of bacterial culture without IPTG; lane 2, total proteins in 10-µL aliquots of bacterial culture with IPTG; lane 3, 20 µg of crude soluble protein; lane 4, 5 µg (A, B) or 10 µg (C) OsHDAC10 purified by affinity chromatography. ASDAC enzyme activities (D, E) were monitored by the conversion of serotonin from *N*-acetylserotonin. (F) The specific activities of ASDAC for the substrates *N*-acetyltyramine, *N*-acetyltryptamine, and melatonin were determined. Crude BL21 (DE3) extracts were prepared from the sonicated cells incubated for 24 hours at 37°C.

FIGURE 4 ASDAC activities as a function of (A) enzyme concentration, (B) pH, (C) temperature, and (D) histone deacetylase inhibitor concentration. *In vitro* serotonin production was measured using the purified Δ66:OsHDAC10 protein (2 µg) in the presence of 1 mmol/L *N*-acetylserotonin. Assay conditions are described in the Methods. The data represent the mean ± SD of triplicate experiments.

FIGURE 5 Expression of rice HDAC10 and subcellular localization. (A) *In vitro* ASDAC enzyme activities in shoots and roots of 7-day-old rice seedlings. (B) *HDAC10* transcript levels in the 4-week-old detached leaves of rice upon cadmium treatment. (C) Localization of OsHDAC10 protein: left, the red fluorescence of HDAC10-mCherry; middle, chlorophyll autofluorescence; right, merged fluorescence images (left and middle). *Nicotiana*

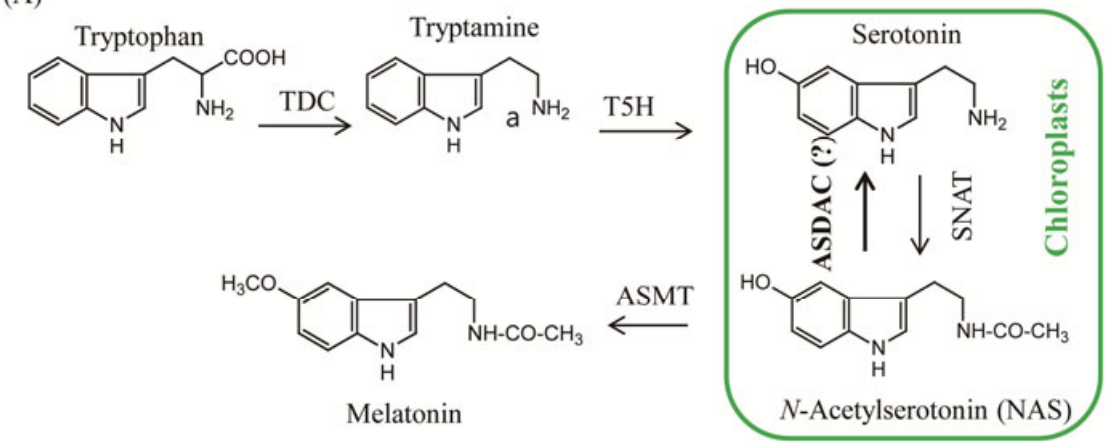
benthamiana leaves were infiltrated with *Agrobacterium* harboring the XVE-inducible HDAC10-mCherry construct and grown for 2 d in a growth room before visualization by confocal microscopy. Bars = 20 μm. Numbers in parentheses indicate the number of PCR cycles.

FIGURE 6 Identification of the Arabidopsis *HDAC14* gene encoding ASDAC activity. (A) Production of serotonin in *E. coli* expressing AtHDAC14 (At4g33470). (B) Affinity purification of an N-terminal 45-amino-acid-truncated Δ45:AtHDAC14. (C) Effects of *E. coli* crude extracts or Zn ion on ASDAC enzyme activity. (D) Substrate specificity measurement. The *E. coli* culture, purification steps, and ASDAC assay methods are described in Figure 3. $ZnCl_2$ and NAD were used at 1 mmol/L.

FIGURE 7 Localization of AtHDAC14 and enzyme kinetics. (A) Red fluorescence of AtHDAC14-mCherry, (B) chlorophyll autofluorescence, (C) green fluorescence of cytoplasmic GFP, and (D) merge of the three images (A+B+C). (E) Determination of K_m and V_{max} of OsHDAC10 and AtHDAC14 for NAS. *Escherichia coli* crude extracts were used for kinetic analyses. *Nicotiana benthamiana* leaves of 30-day-old seedlings were used as described in Figure 4. Bars = 20 μm.

FIGURE 8 Phylogenetic analysis of ASDAC homologs and *N*-terminal sequence comparisons. (A) Phylogenic analysis of ASDAC homologs from *Ostrecoccus tauri* (XP_003081007), *Micromonas pusilla* (XP_003057180), *Chlorella variabilis* (XP_005851005), *Oryza sativa* (AK072557), *Arabidopsis thaliana* (At4g33470), and *Methanosaeta thermophila* (WP_011695862). (B) Predicted transit peptides using TargetP analysis.[38] Numbers denote branch support values of each node. Bar, 0.4 substitutions per site. Shaded boxes indicate the predicted chloroplast transit sequences, whereas the open box denotes the predicted mitochondria transit sequences. Identical amino acids are denoted by stars (*) whereas similar amino acids are denoted by colons (:). Gaps are noted as dashes.

(A)



(B) *In vivo* conversion rates of various substrates in 7-day-old rice seedlings

Substrate (1 mmol/L))	Product	Product level (ng/g FW)	Related enzyme
Tryptamine	Serotonin	8281 ± 78	Tryptamine 5-hydroxylase (T5H)
Serotonin	NAS	75 ± 11	Serotonin *N*-acetyltransferase (SNAT)
NAS	Melatonin	35 ± 04	*N*-Acetylserotonin methyltransferase (ASMT)
NAS	Serotonin	1514 ± 51	*N*-Acetylserotonin deacetylase (ASDAC ?)

Fig. 1

(A)

No.	Name	Accession no.	aa length	Sub Loc	Chr. locus
1	OsHDAC6	AK100812	352	C	6
2	OsHDAC10	AK072557	443	C/M	12
3	OsSIR2b	AK067069	393	M	12
4	OsSRT1	AK069000	312	N	4

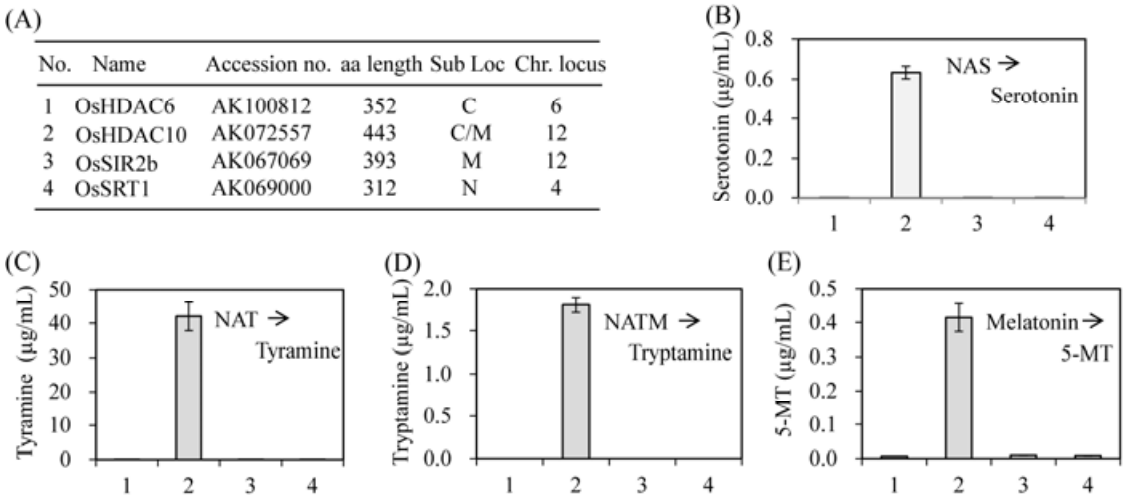
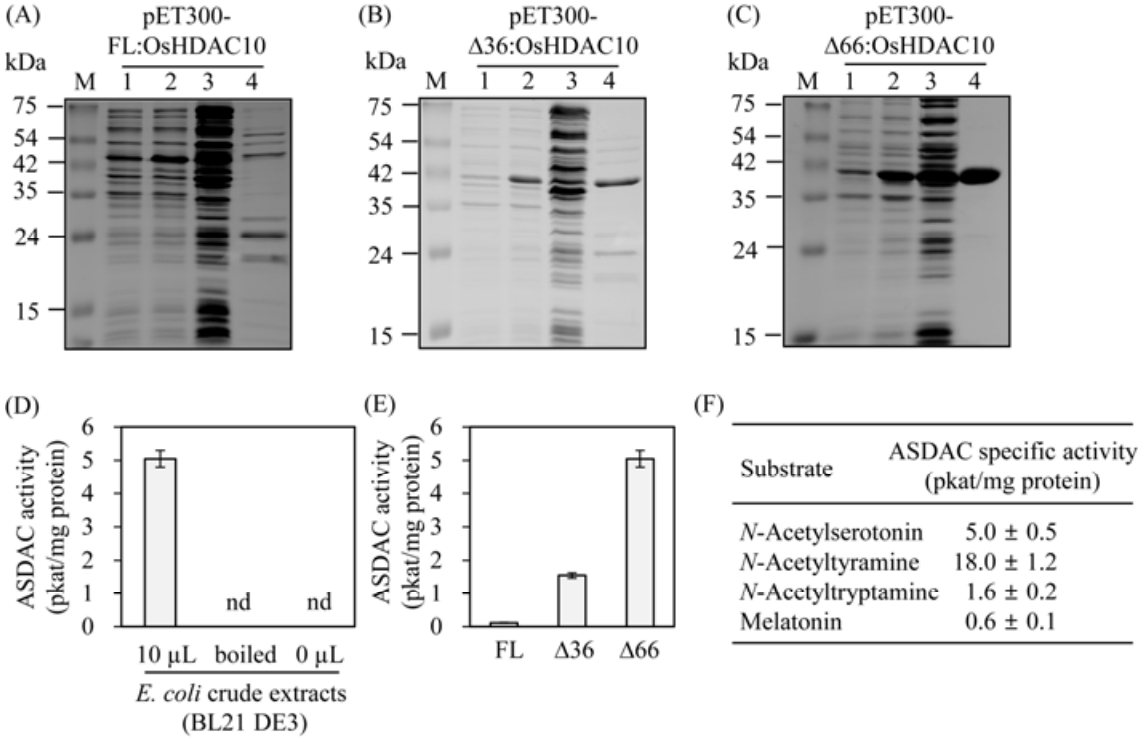


Fig. 2



(F)

Substrate	ASDAC specific activity (pkat/mg protein)
N-Acetylserotonin	5.0 ± 0.5
N-Acetyltyramine	18.0 ± 1.2
N-Acetyltryptamine	1.6 ± 0.2
Melatonin	0.6 ± 0.1

Fig. 3

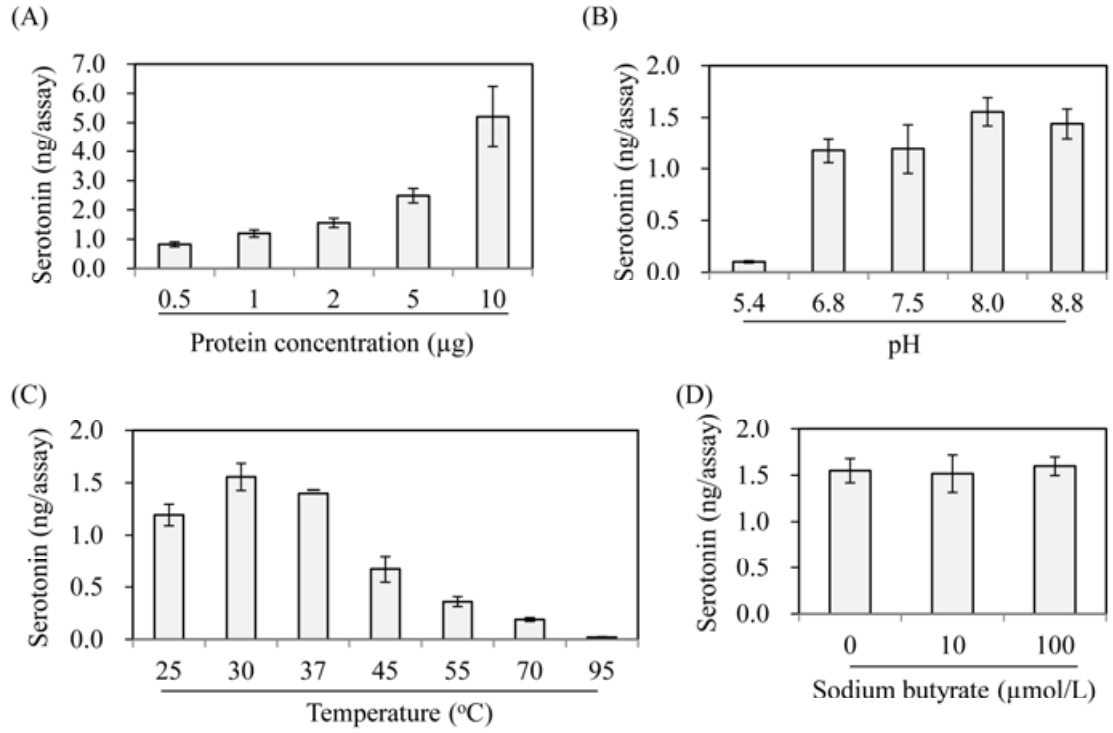


Fig. 4

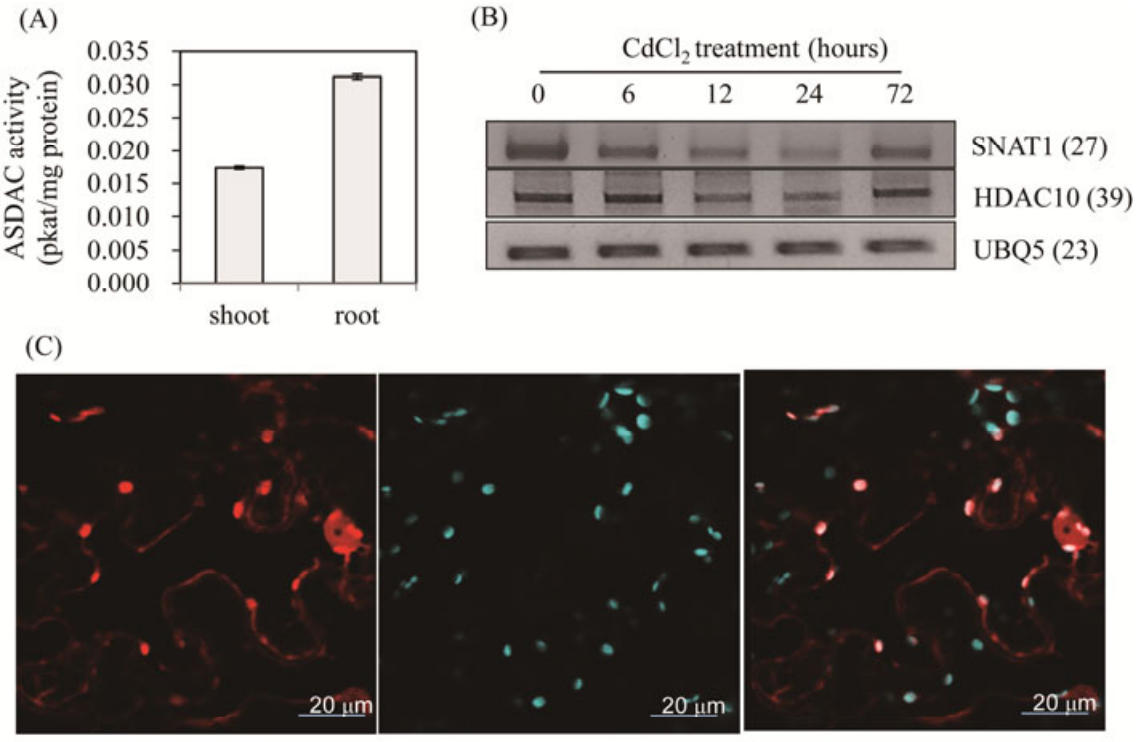
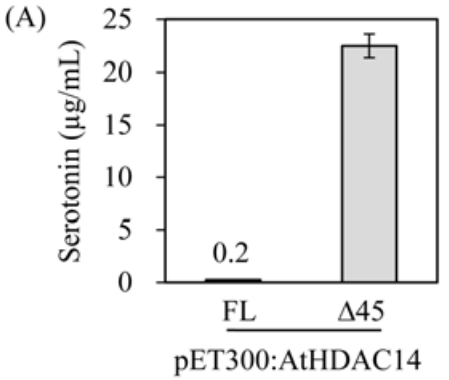
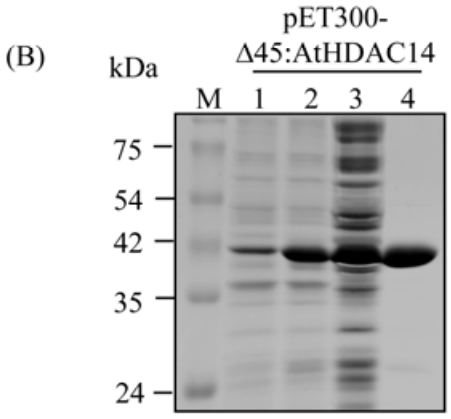
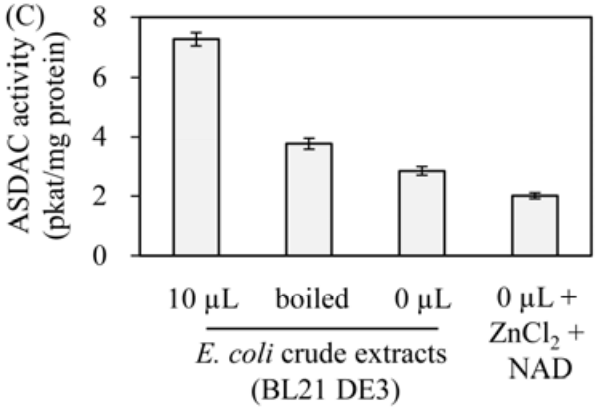


Fig. 5



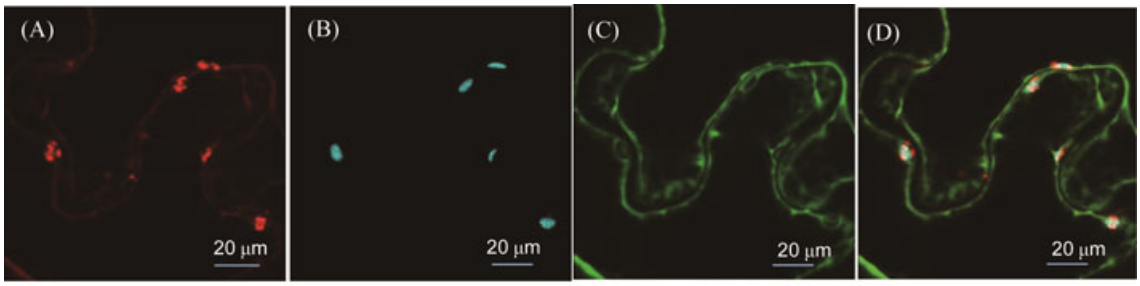




(D)

Substrate	ASDAC specific activity (pkat/mg protein)
N-Acetylserotonin	7.3 ± 1.1
N-Acetyltyramine	30.0 ± 2.3
N-Acetyltryptamine	2.4 ± 0.6
Melatonin	1.6 ± 0.3

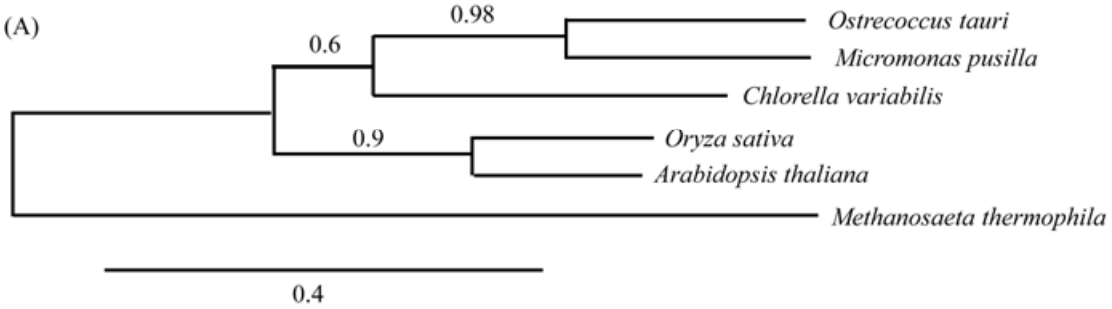
Fig. 6



(E)

Protein	K_m (μmol/L)	V_{max} (pkat/mg protein)	V_{max}/K_m
OsHDAC10	409	1.0	0.0024
AtHDAC14	701	1.2	0.0017

Fig. 7



(B)

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O. sativa          -MEQLWVPSLPILGGRILPMLRHYCGFGSHHPLTWRSLQITGRKQKHNGCWIAYCLPSHN
A. thaliana        MSMALIVRPFFVPGSAGISGSRNIC----------------KKNQ----WRKYLLKP-S
C. variabilis      ----------MIPHTASVQAARGRH-----------------------------------V
O. tauri           --MSALVRPSPIA-----------------------------------------------S
M. pusilla         ---------------------------------------------------------
M. thermophila     ---------------------------------------------------------M

O. sativa          GTSISDTNGVRKDLALPD-NLLRDAHILYCTSPAMGHNKEAHPETNKRVPAIVDALEKLE
A. thaliana        GSSINCSFSTEKNPLLPSIQQLADARLIYSVSAALGHNKESHPECSARVPAIVNALEMNE
C. variabilis      VGGAGPAARVVASALLLPPSTLSSARLLYATAGAPGHDYPGHPECAARVPAILEALDTHG
O. tauri           VCVVRKHISHVRSTTVPSASSNDAPKLIFVSSVASGHDLEGHPECAARVPAILKELEKGQ
M. pusilla         ----------------------------------MDHERVGHPECNARVPAILAALDAAR
M. thermophila     QIYNSCMTSIVYHPVY-----------------LMHETHEHPERKERLIAIMERIEAEG
                                                     *:   ***   *: **:  ::
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Fig. 8