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RESEARCH REPORT

Characterization and expression analysis of *BcAMT1;4*, an ammonium transporter gene in flowering Chinese cabbage

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Abstract
Ammonium (NH_4^+) is generated during many endogenous metabolic processes in the leaves of plants, and there is increasing evidence that ammonium transporters (AMTs) play important roles in NH_4^+ transmembrane transport and distribution. However, the expression of different *AMT* genes is tissue-type specific and their functions differ. Information about *AMT* genes and their expression under different environmental conditions in flowering Chinese cabbage (*Brassica campestris* L.) is currently limited. Here, we isolated and characterized an *AMT* gene, *BcAMT1;4*, in flowering Chinese cabbage. BcAMT1;4 was localized to the plasma membrane and complemented NH_4^+ transport in NH_4^+ uptake-deficient yeast. The highest expression levels of *BcAMT1;4* were detected in the flowers and leaves of flowering Chinese cabbage. The expression of *BcAMT1;4* was induced by nitrogen deficiency and significantly inhibited by the reapplication of NH_4^+ (NH_4Cl or NH_4NO_3). In contrast, when plants pre-cultured in nitrate were transferred to an NH_4^+ nutrient solution, *BcAMT1;4* expression was significantly enhanced. *BcAMT1;4* exhibited a diurnal expression pattern, with higher expression levels during the light period than during the dark period, and a peak expression at the later stage of the light period. Knowledge of *AMT* genes in flowering Chinese cabbage will lay a foundation for enhancing our understanding of the functional roles of different *AMT* members in the regulation of its growth by NH_4^+, as *BcAMT1;4* seems to play an important role in leaf NH_4^+ transport.

Keywords Ammonium transporters · NH_4^+ · Leaf · Circadian rhythm · Flowering Chinese cabbage

1 Introduction

Ammonium (NH_4^+) is an important nitrogen (N) source that is absorbed through plant roots depending on its availability and the reduced state of N in the surrounding soil (Loqué and von Wirén 2004). Furthermore, plants will preferentially absorb NH_4^+ following exposure to N deficiency (Gazzarrini et al. 1999; Ruamrungsri et al. 2000) because the uptake and assimilation of NH_4^+ requires less energy than those of other forms of N (Bloom et al. 1992). However, excess NH_4^+ uptake can cause toxicity, and plants have evolved mechanisms to regulate NH_4^+ uptake and transport (Bittsánszky et al. 2015).

In addition to uptake from the environment, NH_4^+ is produced in healthy plant tissues during numerous endogenous metabolic processes including nitrate reduction, photorespiration, amino acid deamination, and phenylpropanoid metabolism (Masclaux-Daubresse et al. 2006; Bittsánszky et al. 2015). When coupled with the Calvin cycle, photorespiration is the most important process for generating NH_4^+ during vegetative growth, as the oxidative decarboxylation of glycine to serine releases NH_4^+ (Kumagai et al. 2011). Usually, most of the generated NH_4^+ is assimilated into glutamine (Guan et al. 2015) by the enzyme glutamine synthetase (GS; EC 6.3.1.2) while the remaining unassimilated NH_4^+ is transported through the membrane of the mesophyll cells into the leaf apoplast (Husted et al. 2002; Kumagai et al. 2011). Subsequently, NH_4^+ accumulates in the substomatal cavity in the form of ammonia (NH_3) and it is then

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released into the air (Husted et al. 2002; Rolny et al. 2016). Such emissions contribute to up to 5% of the N content loss from the shoots and can affect crop productivity (Schjoerring et al. 2000; Kumagai et al. 2011). However, the regulatory mechanism that controls the transfer of NH_4^+ into the leaf apoplast remains unclear.

There is increasing evidence that NH_4^+ is primarily transported and distributed throughout the plant by ammonium transporters (AMTs), a family of integral membrane proteins belonging to the ammonium transporter/methylammonium permease/mammalian rhesus (AMT/MEP/Rh) protein family (Marini et al. 1997; Ludewig et al. 2007). Plant AMTs are encoded by the *AMT1* and *AMT2* gene subfamilies (Loqué and von Wirén 2004; Adetunji et al. 2015), both of which contain the AMT signature motif (Couturier et al. 2007; McDonald et al. 2012). Analysis of the expression characteristics of the AMT1 subfamily in rice (*Oryza sativa*) showed that *OsAMT1;1* is constitutively expressed in the shoots and roots, whereas *OsAMT1;2* and *OsAMT1;3* are specifically expressed in the roots (Sonoda et al. 2003). In *Arabidopsis thaliana, AtAMT1;4* is specifically expressed in pollen (Yuan et al. 2009). *BnAMT1;2* is highly expressed in the leaves of rapeseed (*Brassica napus*) and exhibits 97% sequence similarity to *AtAMT1;3*, although *AtAMT1;3* is only expressed in the roots (Gazzarrini et al. 1999; Pearson et al. 2002). The expression characteristics of the *AMT* genes are closely related to the N nutritional status and external availability of the different N forms (Li et al. 2017; Lupini et al. 2017), as shown by the upregulation of *AtAMT2;1* in Arabidopsis and *PbAMT1;5* and *PbAMT2* in *Pyrus betulaefolia* under N-deficient conditions (Sohlenkamp et al. 2000; Li et al. 2015, 2016). Consistent with the tissue-specific expression of *AMT* genes, no significant difference in the expression of *BnAMT1;2* was observed in rapeseed plants grown under N-deficient conditions (Pearson et al. 2002), and *LeAMT1;1* and *LeAMT1;2* showed different expression patterns under exogenous N treatment of tomato (*Lycopersicum esculentum*) plants (Lupini et al. 2017). In summary, different *AMTs* from various species may have their own expression or regulatory characteristics depending on their physiological roles during environmental and nutrient adaptation (Ludewig 2006; Li et al. 2016). Indeed, it is necessary to characterize homologous *AMTs* from species that are important crops or agriculturally valuable to improve growth and cultivation programs.

Flowering Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* (L.) Makino *utilis* Tsen et Lee) is a natural subspecies of Chinese cabbage, widely cultivated in China, especially in southern China (Song et al. 2012; Huang et al. 2017). We previously found that increasing the amount of NH_4^+ in nutrient solutions promoted the growth and improved the quality of flowering Chinese cabbage (Song et al. 2012). However, the expression and function of *AMT* genes in flowering Chinese cabbage have not been reported. In the present study, we identified and characterized the *AMT* gene *BcAMT1;4* in flowering Chinese cabbage and investigated its expression in different tissues and in response to different N nutritional status, different N forms, and circadian rhythms using reverse transcription quantitative polymerase chain reaction (RT-qPCR).

2 Materials and methods

2.1 Plant material

The experiments were conducted under 25–30 °C under natural sunlight at the Horticultural Science greenhouse, Guangdong Provincial Engineering Technology Research Centre for Protected Horticulture, South China Agricultural University, Guangzhou, China. Sterile seeds of flowering Chinese cabbage ('Youlv 501 caixin', Guangzhou Institute of Agricultural Sciences, Guangzhou, China) were sown in plug trays with perlite as the substrate. After 3 weeks, the seedlings were transferred to plastic pots for hydroponic growth. Each pot contained 16 plants and 24 L of normal nutrient solution (4.0 mM $NaNO_3$, 1.0 mM KH_2PO_4, 2.0 mM KCl, 1.0 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.1 mM Fe-EDTA, 50 μM H_3BO_3, 12 μM $MnSO_4$, 1 μM $ZnC1_2$, 1 μM $CuSO_4$, and 0.2 μM Na_2MoO_4; pH 6.0, adjusted with 1 M NaOH or 10% HCl) with 25 μM ampicillin to inhibit microbial activity (Zhong et al. 2016). The growth solution was changed every 4 days (Zhong et al. 2016).

2.2 Experimental treatments

For the organ-dependent expression analysis of *BcAMT1;4*, flowering Chinese cabbage plants were cultured in the normal nutrient solution until the flowering stage, and the root, leaf, petiole, stem, and flower tissues were subsequently sampled. To examine the effect of N deficiency on the expression of *BcAMT1;4*, flowering Chinese cabbage plants were grown in the normal nutrient solution for 12 days and then transferred to a fresh nutrient solution without N (normal nutrient solution without $NaNO_3$; N-free nutrient solution). The expression of *BcAMT1;4* in the leaves was measured 0, 1, 3, 9, 24, and 48 h after transfer. To elucidate the effect of the different N forms on *BcAMT1;4* expression, plants were either cultured in the normal nutrient medium for 14 days (N-sufficient plants) or cultured in the normal nutrient solution for 12 days and then transferred to the N-free nutrient solution and cultured for another 2 days (N-deficient plants). Both N-sufficient and N-deficient plants were then transferred to nutrient solutions without $NaNO_3$, but containing either 4 mM NH_4Cl or 2 mM NH_4NO_3, for 24 h. Leaf samples were

collected from five plants at the end of the treatments. To examine the effects of the circadian rhythm on *BcAMT1;4* transcription, plants were cultured in the greenhouse for 12 days and then moved to a phytotron and grown under a 12 h (07:00–19:00) photoperiod with a luminal intensity of 150 μmol m^{-2} s^{-1}, at a constant temperature of ~ 25 °C, and 70% relative humidity. On day 3 after their transfer to the phytotron, leaf tissues were sampled every 3 h starting at 03:00. All samples were frozen in liquid N and then stored at − 80 °C until analysis.

2.3 Isolation of *BcAMT1;4*

The National Center for Biotechnology Information (NCBI) GenBank expressed sequence tag (EST) databases were used to identify several highly homologous *AMT1;4* ESTs from other plant species that were then used to design a pair of primers (5′-ATGGCGTCGTCGACAATC-3′ and 5′-TCAAAGAACACCTACATGTC-3′) for the isolation of *BcAMT1;4*.

Total RNA from flowering Chinese cabbage leaves was isolated via RNAiso Reagent (TaKaRa Bio Inc., Kusatsu, Japan) and then used to synthesize first strand cDNA with the PrimeScript 1st strand cDNA synthesis kit (TaKaRa Bio Inc.). The PCR amplification was performed using the high-fidelity PrimeSTAR Max DNA polymerase kit (TaKaRa Bio Inc.), and the resulting products were sub-cloned into a pMD19-T vector (TaKaRa Bio Inc.) and transformed into *Escherichia coli* Trans5α (Transgen Biotech, Beijing, China). Positive clones were screened using blue-white selection, verified by PCR and agarose gel electrophoresis, and amplicons from the positive clones that yielded a unique ~ 1500 bp band were sequenced (Invitrogen, Shanghai, China).

2.4 Yeast complementation assay

The yeast complementation assay was conducted using the ammonium uptake-deficient yeast strain 31019b (*Δmep1*, *Δmep2*, *Δmep3*, and *ura3*) (Marini et al. 1997; Yuan et al. 2007). For expression of *BcAMT1.4* in yeast, the pYES2 vector was used to construct the yeast expression vector pYES2-BcAMT1.4, and the recombinant (pYES2-BcAMT1.4) or empty (pYES2) plasmids were transformed into 31019b yeast cells using lithium acetate (Yuan et al. 2007). The transformed yeast lines were cultured in synthetic-defined selective medium without uracil (SD/-Ura) for screening positive transformants. Positive clones were pre-cultured in liquid yeast nitrogen-base medium (YNB) without amino acids and ammonium sulphate for yeast complementation experiments, as described by Zhu et al. (2018).

2.5 Subcellular localization of BcAMT1s

The open reading frame (ORF) of *BcAMT1;4* was amplified from the cDNA of flowering Chinese cabbage leaves, and, together with the green fluorescence protein (GFP), it was used to construct the fusion expression vector pBI121-BcAMT1;4:GFP, which was verified by sequencing. The construct and the pBI121-GFP control were then individually transformed into *Agrobacterium tumefaciens* strain GV3101, and transiently expressed in onion (*Allium cepa*) epidermal cells using the *A. tumefaciens*-mediated transient expression method (Shah et al. 2001). After 2 days of incubation in 1/2 Murashige and Skoog medium (pH 5.8, with 100 μM acetosyringone), the fluorescence of the GFP fusion protein was examined under an Axio Imager D2 positive fluorescence microscope (Zeiss, Oberkochen, Germany).

2.6 Bioinformatics analysis of BcAMT1;4

The molecular weight of three predicted BcAMT1;4 proteins was calculated using DNAStar 7.0 (DNAStar Inc., Madison, WI, USA), and transmembrane region prediction and signature motif analysis were performed using InterProScan (http://www.ebi.ac.uk/interpro/). The full-length amino acid sequences were aligned using DNAMAN 6.0 (Lynnon Biosoft, San Ramon, CA, USA), and the neighbour-joining method was used for phylogenetic analyses.

2.7 RT-qPCR

Total RNA was isolated using RNAiso Reagent (TaKaRa Bio Inc.) and reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Bio Inc.). Specific primers for *BcAMT1;4* (5′-ATCTGCGGGTTTGTAGC-3′ and 5′-CATCGAATTGGAGCTTATC-3′) were designed to perform the RT-qPCR and determine the *BcAMT1;4* expression levels under different treatments, using the LightCycler 480 real-time PCR system (Roche, Basel, Switzerland) with SYBR Premix ExTaq (TaKaRa Bio Inc.) and the cycling profile described by Zhong et al. (2016). The relative expression of *BcAMT1;4* was calculated against the expression levels of the two housekeeping genes *ACTIN* and *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*GAPDH*) using the $2^{-\Delta\Delta CT}$ method, where the cycle threshold (CT) values were obtained from three independent biological replicates (Livak and Schmittgen 2001).

2.8 Statistical and graphical analyses

All data were statistically analysed using one-way analysis of variance (ANOVA) and Duncan's post hoc tests in the statistical package for the social sciences (SPSS) 12.0 (IBM Corp., Armonk, NY, USA). Graphs were constructed using

SigmaPlot 11.1.0 (Systat Software Inc., San Jose, CA, USA) and all graphs and images were arranged using Adobe Photoshop CS5 (Adobe Systems Software Ltd., San Jose, CA, USA).

3 Results

3.1 Isolation and sequence analysis of *BcAMT1;4*

A novel *AMT* gene, *BcAMT1;4* (GenBank accession number MF966939), was isolated from flowering Chinese cabbage. The *BcAMT1;4* complete ORF was 1530 bp long and encoded a 54.38 kDa protein composed of 509 amino acid residues. A phylogenetic analysis based on multiple alignments of 29 AMT proteins from eight plant species clustered BcAMT1;4 within the AMT1 subgroup (Fig. 1); its sequence showed the highest similarity (89.29%) to that of *AtAMT1;4* from Arabidopsis. Based on the protein structure prediction analysis, BcAMT1;4 was identified as a membrane protein with 10 transmembrane domains (TMs) located in the plasma membrane (Fig. 2). The TM6 domain (Fig. 2) contained the motif "DFAGSGMVHMVGGIAGLWGAFIESPR" that corresponded to D-[FYWS]-[AS]-G-[GSC]-x(2)-[IV]-x(3)-[SAG](2)-x(2)-[SAG]-[LIVMF]-x(3)-[LIVMFYWA](2)-x-[GK]-x-R, which is generally considered the hallmark of potential AMT proteins (de Castro et al. 2006).

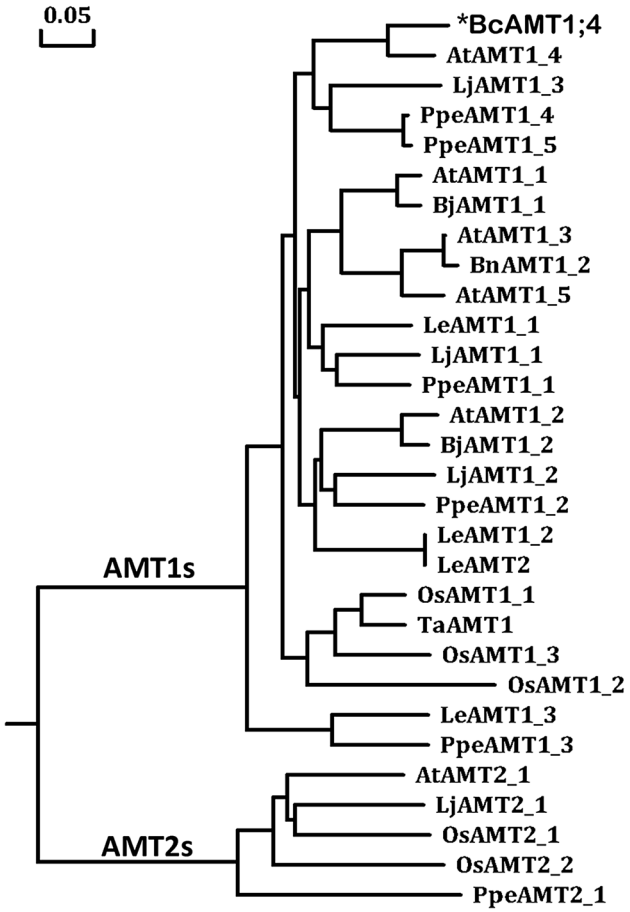

Fig. 1 Phylogenetic tree of *BcAMT1;4* and other *ATM* genes from plants. The full-length amino acid sequences were aligned using DNAMAN 6.0, and the phylogenetic analyses were performed using the neighbour-joining method with 1000 bootstrap replicates. The scale bar indicates the average number of amino acid substitutions per site. *At* (*Arabidopsis thaliana*): *AtAMT1;1* (NP_193087), *AtAMT1;2* (NP_176658), *AtAMT1;3* (NP_189073), *AtAMT1;4* (NM_119012), *AtAMT1;5* (NM_113335), *AtAMT2;1* (NP_973634); *Bn* (*Brassica napus*): *BnAMT1;2* (AF306518); *Bj* (*Brassica juncea*): *BjAMT1;1* (KT119596), *BjAMT1;2* (KT119597); *Le* (*Lycopersicon esculentum*): *LeAMT1;1* (NP_001304667), *LeAMT1;2* (NP_001234253), *LeAMT1;3* (NP_001234216), *LeAMT2* (X95098.1); *Lj* (*Lotus japonicus*): *LjAMT1;1* (CAC10555), *LjAMT1;2* (AAM95453), *LjAMT1;3* (CAE01484), *LjAMT2;1* (AAL08212); *Os* (*Oryza sativa*): *OsAMT1;1* (AAL05612), *OsAMT1;2* (AAL05613), *OsAMT1;3* (AAL05614), *OsAMT2;1* (AB051864), *OsAMT2;2* (AB083582); *Ta* (*Triticum aestivum*): *TaAMT1* (*AY390355*); *Ppe* (*Prunus persica*): *PpeAMT1;1* (KJ598789), *PpeAMT1;2* (KJ598790), *PpeAMT1;3* (KJ598791), *PpeAMT1;4* (KJ598792), *PpeAMT1;5* (KJ598793), *PpeAMT2;1* (KJ598794)

3.2 BcAMT1;4 localizes to the plasma membrane

To determine the localization of BcAMT1;4 in plant cells, the full-length BcAMT1;4 coding sequence was fused in-frame to the N-terminal of *GFP*. The resulting protein-fusion construct and a construct expressing free GFP were individually cloned upstream of the 35S promoter to create pBI121-BcAMT1;4:GFP and pBI121-GFP, respectively. The pBI121-GFP-transformed onion epidermal cells showed GFP fluorescence throughout the cytoplasm and the nucleus under confocal microscopy (Fig. 3). The onion epidermal cells transformed with pBI121-BcAMT1;4:GFP displayed GFP fluorescence predominantly at the plasma membrane (Fig. 3). These results confirmed that BcAMT1;4 is localized to the plasma membrane of plant cells.

3.3 BcAMT1;4 transports NH_4^+ in yeast

We used NH_4^+ uptake-deficient yeast to test the transport activity of BcAMT1;4 when supplied with NH_4^+. The recombinant yeast strain 31019b expressing pYES2-BcAMT1;4 or the empty vector (pYES2) grew on solid medium with 2 mM arginine (Fig. 4). When 2 mM NH_4^+ was added as the N source, the yeast expressing the pYES2 grew weakly or not at all; however, the yeast expressing BcAMT1;4 grew normally on the solid medium supplemented with NH_4^+ (Fig. 4). These results indicated that BcAMT1;4 transported NH_4^+ into yeast cells.

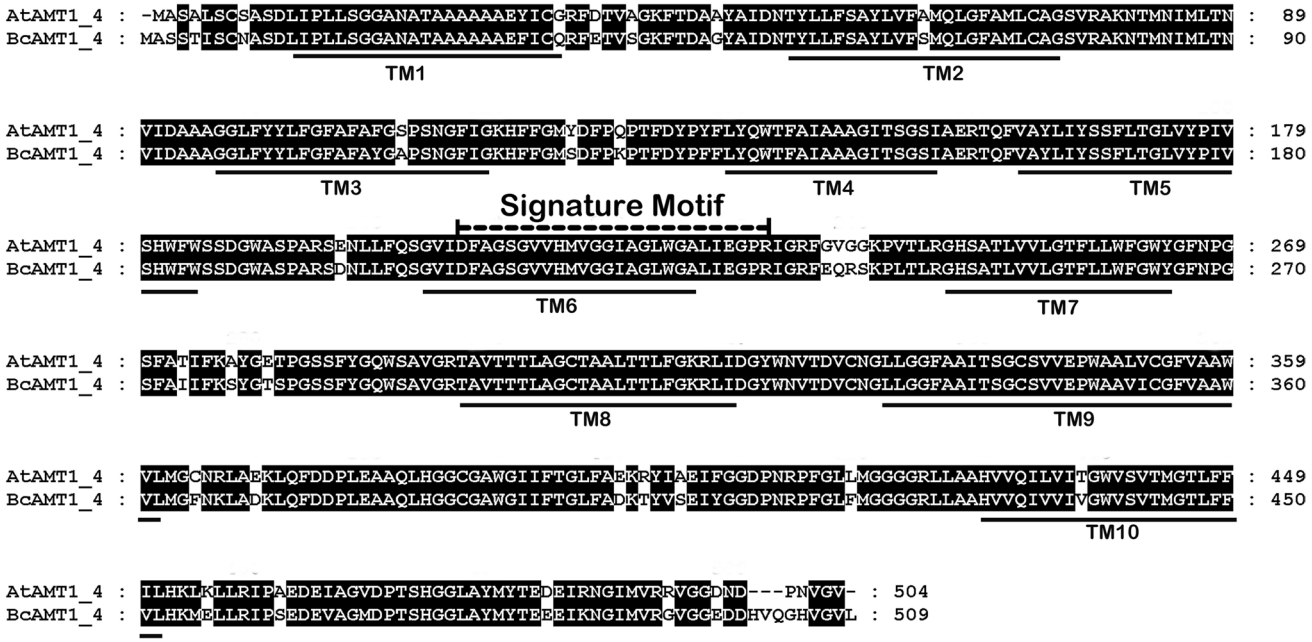

Fig. 2 Amino acid sequence alignment of *BcAMT1;4* and *AtAMT1;4*. Predicted transmembrane domains (TMs) are underlined, and the signature motif is indicated with a dotted line above the sequence. Shading indicates identical amino acids

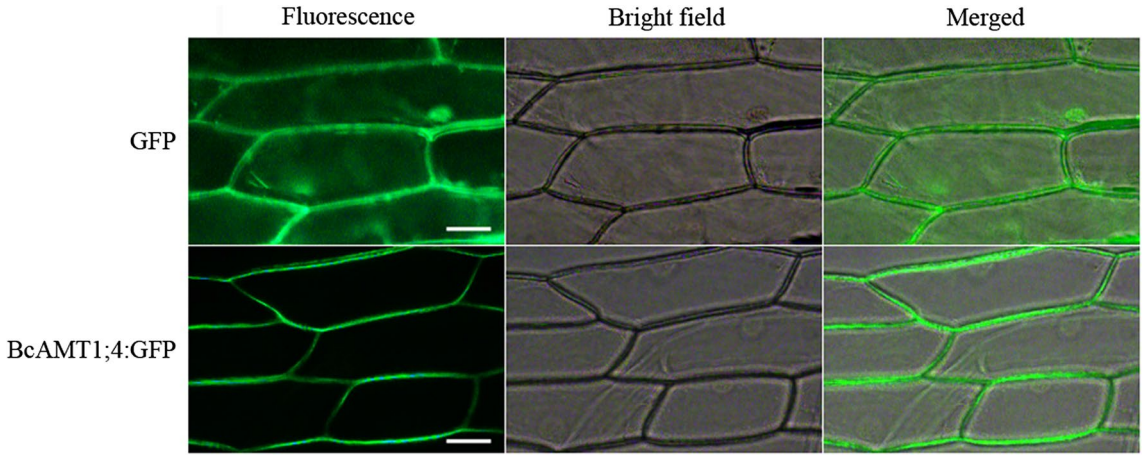

Fig. 3 Subcellular localization of transiently expressed BcAMT1;4:GFP in onion epidermal cells. GFP: pBI121-GFP; BcAMT1;4:GFP: pBI121-BcAMT1;4:GFP. Fluorescence: image of GFP expression under confocal microscopy; Bright-field: image of GFP expression under bright-field microscopy; Merged: overlaid image of corresponding bright-field and fluorescence images. Scale bar, 50 μm

3.4 Organ-dependent expression of *BcAMT1;4*

The abundance of *BcAMT1;4* transcripts in the root, leaf, petiole, stem, and flower tissues of flowering Chinese cabbage was investigated to determine its physiological function. The highest expression levels of *BcAMT1;4* were detected in the flower, followed by that in the leaf (Fig. 5). Extremely low transcription levels were detected in the root, stem, and petiole. Considering that flowering Chinese cabbage plants consist mostly of leaves and that *BcAMT1;4* was highly expressed in the leaf, we focused on the expression of *BcAMT1;4* in leaf tissue under different N treatments in the subsequent experiments.

3.5 Effect of N deficiency on *BcAMT1;4* expression

Flowering Chinese cabbage plants were pre-cultured in hydroponic nutrient solutions supplemented with 4 mM

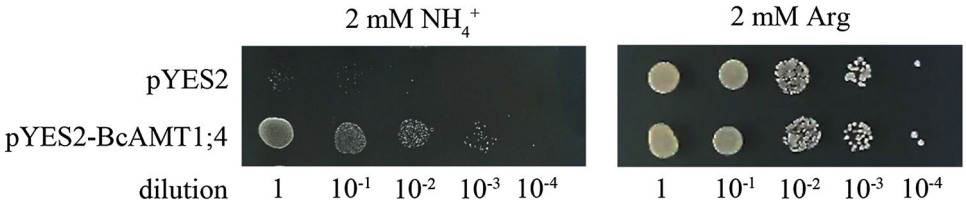

Fig. 4 Complementation of the NH_4^+ uptake-defective yeast strain 31019b (*Δmep1*, *Δmep2*, *Δmep3*, and *ura3*) by BcAMT1;4. Yeast cells were transformed with pYES2:BcAMT1;4 or an empty vector control (pYES2) and their growth was assessed on yeast N base plates supplemented with 2 mM NH_4Cl or 2 mM arginine (Arg). Yeast expressing pYES2:BcAMT1;4 grew in the presence of NH_4Cl but the control yeast did not

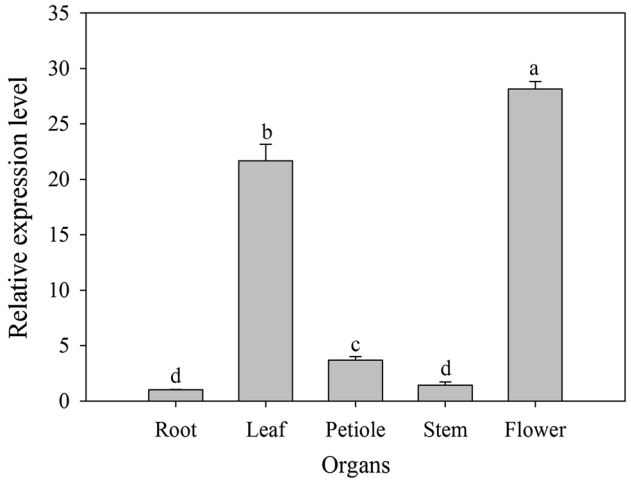

Fig. 5 Tissue-dependent expression of *BcAMT1;4* in flowering Chinese cabbage. Values for *BcAMT1;4* gene expression are expressed as means ± standard deviations (SD, error bars) of three biological and three technical replicates. Values with different letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests

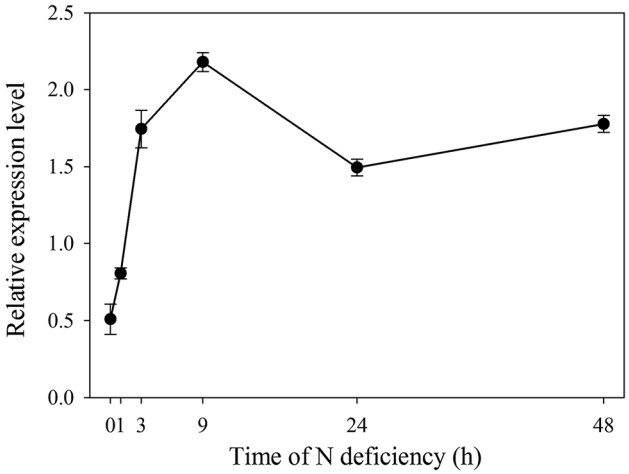

Fig. 6 Effects of N deficiency on *BcAMT1;4* expression. Values for *BcAMT1;4* gene expression are expressed as means ± standard deviations (SD) of three biological and three technical replicates

$NaNO_3$ as the sole N source for 12 days and then transferred to N-free nutrient solutions. The N-deficiency treatment increased the *BcAMT1;4* transcription level in the leaves, with the highest level occurring 9 h after the N-deficiency treatment, and this was almost fourfold higher than that at 0 h. When measured 24 and 48 h after the treatment, the transcription of *BcAMT1;4* decreased to levels that were still threefold higher than those at 0 h (Fig. 6).

3.6 Effect of N supply on *BcAMT1;4* expression

To investigate the effect of the external NH_4^+ supply and whole-plant N nutrition status on *BcAMT1;4* expression, N-deficient and N-sufficient flowering Chinese cabbage plants were supplied with 4 mM NH_4Cl or 2 mM NH_4NO_3 for 24 h. The external supply of NH_4^+ to plants in the different N nutrition status groups affected *BcAMT1;4* expression in the leaves. In N-deficient plants, supplying NH_4Cl or NH_4NO_3 significantly repressed *BcAMT1;4* expression in the leaves (Fig. 7), whereas N-sufficient plants supplied with NH_4NO_3 showed significantly upregulated *BcAMT1;4* expression levels in the leaves (Fig. 7).

3.7 Effects of alterations of the circadian rhythm on *BcAMT1;4* expression

We observed diurnal variation in the expression of *BcAMT1;4* in the leaves of flowering Chinese cabbage plants (Fig. 8). The transcription of *BcAMT1;4* exhibited a clear diurnal rhythm; transcription levels were higher during the light period than during the dark period, and the highest transcript levels were observed at 18:00, 1 h before the dark period.

4 Discussion

The AMT transporters mediate NH_4^+ transmembrane uptake, serving as the major high-affinity NH_4^+ transporters in plants (Ninnemann et al. 1994; Loqué et al. 2006).

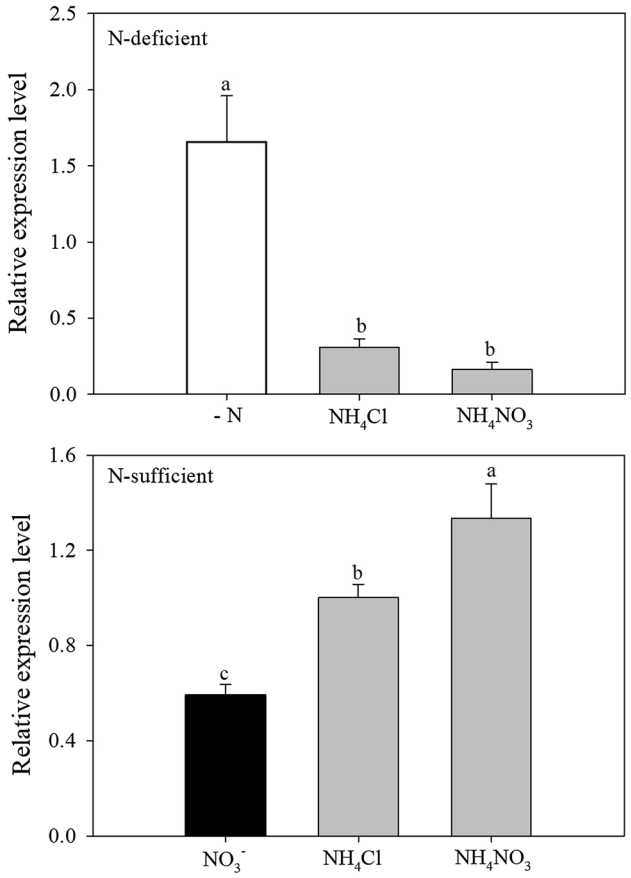

Fig. 7 Effects of N supply on *BcAMT1;4* expression in leaves. Flowering Chinese cabbage plants were grown under different N nutritional conditions (N-deficient and N-sufficient) and then transferred into nutrient solutions containing 4 mM NH_4Cl or 2 mM NH_4NO_3 for 24 h. N-deficient: plants were cultured in the normal nutrient solution for 12 days and then transferred to N-free nutrient solution (normal nutrient solution without $NaNO_3$) for another 2 days (represented as '–N'). N-sufficient: plants were continuously cultured under NO_3^- nutrition for 14 days (represented by 'NO_3^-'). Values of *BcAMT1;4* gene expression are means ± standard deviations (SD) of three biological and three technical replicates. Values with different letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests

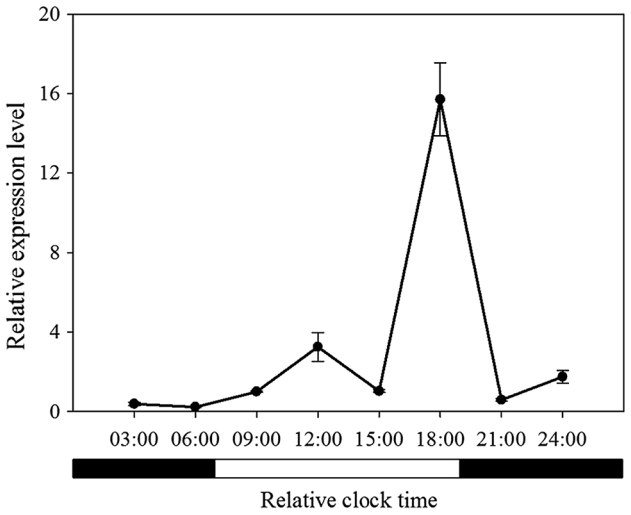

Fig. 8 Effect of the light–dark cycle on *BcAMT1;4* expression in flowering Chinese cabbage leaves. Black and white bars represent dark and light periods, respectively. Values of *BcAMT1;4* gene expression are means ± standard deviations (SD) of three biological and three technical replicates

The first plant *AMT* gene was isolated from Arabidopsis and its function was identified by complementation of a yeast mutant (Ninnemann et al. 1994). Several AMTs have been cloned from Arabidopsis (Ninnemann et al. 1994; Yuan et al. 2009), maize (*Zea mays*) (Gu et al. 2013), wheat (*Triticum aestivum*) (Li et al. 2017), and other plant species (Li et al. 2015, 2016; Song et al. 2017a). All these *AMT* genes have AMT signature motifs (Couturier et al. 2007; McDonald et al. 2012) and they can be divided into two distinct subfamilies, *AMT1* and *AMT2* (Loqué and von Wirén 2004; Adetunji et al. 2015). In the present study, *BcAMT1;4* was isolated from flowering Chinese cabbage for the first time and identified as an *AMT1*-type gene. The predicted amino acid sequence of BcAMT1;4 contained one AMT signature motif in the sixth transmembrane domain and it was highly homologous with other *AMT1* genes (Fig. 2). Transforming the fusion expression vector pBI121-BcAMT1;4:GFP into onion epidermal cells showed that the fusion protein BcAMT1;4:GFP was localized to the plasma membrane (Fig. 3). Furthermore, BcAMT1;4 activity was analysed by complementation of the NH_4^+ uptake-deficient yeast strain 31019b. Yeasts of this strain expressing recombinant pYES2-BcAMT1;4 grew normally on solid medium supplemented with 2 mM NH_4^+ as the sole N source, indicating that BcAMT1;4 functions in NH_4^+ transport.

The individual characteristics of the different AMTs from various plant species depend on their physiological roles (Ludewig 2006; Li et al. 2016). In Arabidopsis, approximately 90% of the total high-affinity N uptake in roots is mediated by AtAMT1;1, AtAMT1;2, and AtAMT1;3, and the coding genes of these proteins are strongly and preferentially expressed in roots (Yuan et al. 2007). However, *AtAMT1;4* is specifically expressed in the flower, and AtAMT1;4 mediates high-affinity NH_4^+ transmembrane uptake in pollen, which contributes to pollen N nutrition via NH_4^+ uptake or retrieval (Yuan et al. 2009). BcAMT1;4 shared a high similarity with AtAMT1;4 in its putative amino acid sequence (89%), but, unlike *AtAMT1;4*, *BcAMT1;4* was highly expressed in both flowers and leaves (Fig. 5), suggesting that BcAMT1;4 provides N nutrition to pollen and is involved in N metabolism in the leaf.

Nitrate (NO_3^-) is one of the main N sources in plant tissues and it is accumulated in large amounts in the vacuole under N-sufficient conditions (Bonasia et al. 2008; Miller

and Smith 2008; Ibrahim et al. 2017). Under N-deficient conditions, the vacuole NO_3^- can be transported into the cytoplasm for use (Zhang et al. 2012). One of the primary sites for N assimilation is the chloroplast, but NH_4^+ in the apoplast must cross the plasma membrane before it is transported into the chloroplast (Pearson et al. 2002). Therefore, the significant upregulation of *BcAMT1;4* under N-deficient conditions may contribute to the transport of NH_4^+ into the cell to maintain the N nutrition status of the plant (Fig. 6).

The regulatory mechanisms that control plant responses to N are the local cellular and systemic signalling pathways that communicate the internal nutrient status across the different tissues and plant organs (Gojon et al. 2009; Alvarez et al. 2012). In the present study, the external supply of NH_4^+ to N-deficient and N-sufficient plants affected *BcAMT1;4* expression in leaves; *BcAMT1;4* expression was significantly repressed by the supply of both NH_4Cl and NH_4NO_3 in N-deficient plants, whereas the expression was significantly upregulated in the N-sufficient plants by the supply of NH_4NO_3 (Fig. 7). This observation suggests that *BcAMT1;4* transcription is regulated by both the external N supply and the whole-plant N status. Plants can experience NH_4^+ toxicity and display symptoms such as leaf chlorosis, growth suppression, and yield depression, particularly when NH_4^+ is supplied as the sole N source (Wang et al. 2016; Jian et al. 2017). In contrast, plants usually exhibit better growth when cultured in a mixture of N forms (Ahmed and Johnson 2000; Bybordi 2012; Hu et al. 2015), as NO_3^- taken up by the cell alkalinizes the cellular environment and counterbalances the harmful effects of NH_4^+ (Bijlsma et al. 2000; Britto and Kronzucker 2002). In addition, the NH_4NO_3 mixed nutrient solution upregulated the expression of *BcAMT1;4*. In our preliminary study, we found that using a suitable NH_4^+/NO_3^- ratio for N nutrition increased nutrient uptake and enhanced the quality and biomass production of flowering Chinese cabbage (Song et al. 2012, 2017b). However, under N-sufficient conditions, the expression of *BcAMT1;4* in leaves was significantly upregulated by the supply of NH_4NO_3 (Fig. 7). Overall, these results indicate that the involvement of *BcAMT1;4* in the NH_4^+ growth regulation of flowering Chinese cabbage requires further research.

The NH_4^+ production in leaves depends on photorespiration (Kumagai et al. 2011), and it is transported into the chloroplast by transmembrane transport proteins and incorporated into glutamate by the GS/ferredoxin-dependent glutamine-2-oxoglutarate aminotransferase (GS/Fd-GOGAT) via the N metabolic pathway (Guan et al. 2015). Excessive NH_4^+ is transported into the apoplast of the mesophyll cells where it is eventually accumulated in the substomatal cavity in the form of NH_3 and released into the air (Husted et al. 2002; Rolny et al. 2016). This process depends on the amount of NH_4^+ accumulated in the apoplast of leaf mesophylls cells, which is continuously supplied with NH_4^+ from the cytoplasm (Husted and Schjoerring 1995; Husted et al. 2002), but photorespiration inhibitors (pyrid-2-yl hydroxymethane sulphonate) reduce the volatilization of NH_3 (Mattsson et al. 1998) and AMTs participate in NH_4^+ transmembrane transport in this process (Bauwe 2010). Zhang et al. (2018) analysed the expression characteristics of different *AMT* genes of *Camellia sinensis* and showed that *CsAMT1.1* (closely related to *AtAMT1.4*) and *CsAMT3.1* were mainly expressed in the leaves and that these two genes could be involved in the regulation of the photorespiratory ammonium metabolism. In the present study, we observed a diurnal variation in the expression of *BcAMT1;4*, which was higher during the day than at night (Fig. 8). A similar pattern was observed for NH_3 volatilization in rapeseed leaves (Nielsen and Schjoerring 1998; Schjoerring et al. 2000). In particular, its specific expression in leaves and response to different N forms and circadian rhythms in leaves suggest that *BcAMT1;4* is involved in the transport of leaf NH_4^+, although its regulatory mechanisms remain unclear and need further investigation.

5 Conclusions

BcAMT1;4, cloned from flowering Chinese cabbage, encodes an AMT protein with 509 amino acids, 10 transmembrane domains, and one AMT signature motif. BcAMT1;4 is localized to the plasma membrane and transports NH_4^+ in yeast cells. High expression of *BcAMT1;4* was detected in flowering Chinese cabbage leaves and flowers and was significantly correlated with the plant's N nutritional status, the N form supplied, and the circadian rhythm. These findings suggest that *BcAMT1;4* plays an important role in the transport of NH_4^+ in the leaves of flowering Chinese cabbage and future studies should further elucidate the mechanisms underlying this transport and the relationship between NH_4^+ and growth regulation in this crop species.

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Author contribution SS conceived and designed the experiments; LZ and XH performed the experiments and wrote the manuscript; YZ and EK completed the yeast functional complementation test. HL helped analyse the data; GS and RC helped to revise the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no competing interests.

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