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ORIGINAL PAPER

Molecular cloning and identification of a putative tomato cationic amino acid transporter-2 gene that is highly expressed in stamens

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Abstract Amino acid transporters are critical components of amino acid allocation processes in plants. However, they are not well known in tomato, a model for studying biological processes of fleshy fruits. In this study, a putative cationic amino acid transporter gene-2 (*SlCAT2*) from tomato was isolated and identified. The *SlCAT2* gene encodes a deduced 650-amino-acid protein that shares high similarity to *Arabidopsis* AtCAT2 and poplar PtCAT2. The SlCAT2 protein contains 14 putative transmembrane domains and is targeted to the tonoplast. Transcripts of *SlCAT2* are present in major reproductive tissues (floral buds, flowers, fruits) and vegetative tissues (roots, stems, leaves), but accumulate at high levels in flowers. Within the flowers, *SlCAT2* transcript levels in stamens are especially high and increase dramatically during bud-to-anthesis transition, when *SlCAT2* expression elevates slightly in ovaries. The abundance of *SlCAT2* transcripts in ovaries declines substantially from anthesis to postanthesis, when fruit set is expected to occur. Subsequently, *SlCAT2* expression in fruits increases moderately during very early fruit development and keeps steady levels until mature green stage, and then gradually increases as fruit ripening. Expression of *SlCAT2* is negatively regulated by ethylene and auxin. The dynamic transitions in the expression of *SlCAT2* suggest its roles in the crucial stages of flower and fruit development, especially in the stamen. These findings should be helpful in guiding further investigation of the physiological role of SlCAT2 in tomato.

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Abbreviations

dpa	Days postanthesis
EST	Expressed sequence tag
ORF	Open reading frame
RACE	Rapid amplification of cDNA ends
GFP	Green fluorescent protein
bp	Base pair
IAA	Indole-3-acetic acid

Introduction

Amino acids are essential for plant cells in respect that they are the indispensable constituents of proteins and play many metabolic and signaling roles (Coruzzi and Zhou 2001). Amino acid transport is mediated by multiple amino acid transporters, which can be classified into two major superfamilies: the amino acid transporter (ATF) family and the amino acid polyamine choline transporter (APC) family (Wipf et al. 2002b; Lalonde et al. 2004). Whereas ATF genes have been identified in animals and plants but not in bacteria, APC genes exist in almost all organisms (Fischer et al. 2002). Amino acid transport in yeast and human is dominated mainly by APC-type transporters. Our understanding of plant amino acid transporters is restricted almost exclusively to members of the ATF superfamily, which are best characterized in *Arabidopsis*, with far less

known about plant APC transporters (Su et al. 2004; Hammes et al. 2006).

In *Arabidopsis*, the majority of putative amino acid transporters belong to the ATF superfamily, which has a 9- to 11-transmembrane (TM) domain topology. Several of these members have been characterized as broad-specificity amino acid transporters when they are heterologously expressed in yeast (*Saccharomyces cerevisiae*) and *Xenopus laevis* oocytes (Fischer et al. 2002). Su et al. (2004) identified 14 APC-type genes from *Arabidopsis* consisting of the L-type amino acid transporter (LAT) subfamily (five members) with a 12-TM domain topology and the cationic amino acid transporter (CAT) subfamily (nine members) with 14 putative TM domains. The LAT-type transporters have not been characterized, and only a few members of the CAT family have been studied (Frommer et al. 1995; Su et al. 2004; Hammes et al. 2006). The AtCAT1 and AtCAT5 proteins function as specific, high-affinity basic amino acid transporters at the plasma membrane (Wipf et al. 2002a; Su et al. 2004). When expressed in *Arabidopsis* protoplasts, AtCAT8 localizes mainly to the plasma membrane and AtCAT2 localizes primarily to the tonoplast (Su et al. 2004). The expression profiles of *AtCAT5* suggest its function in reuptake of leaking amino acids at the leaf margin, whereas *AtCAT8* is expressed in young and rapidly dividing tissues, such as young leaves and the root apical meristem (Su et al. 2004). The preference of AtCAT6 to transport large, neutral, and cationic amino acids suggests its potential role in supplying amino acids to sink tissues and nematode-induced feeding structures (Hammes et al. 2006).

Tomato (*Solamum lycopersicum*) is one of the most widely cultivated vegetable fruit crops, with well-documented health benefits associated with consumption of its fruits. The short life cycle, small and fully sequenced genome, availability of efficient transformation systems, and typical character of fruit development process together make tomato an ideal model system for studying the biological bases of fleshy fruit development and ripening (Afroz et al. 2011). Fruit development is dependent on the translocation of amino acids to support processes such as the synthesis of enzymes needed for development and ripening (Boggio et al. 2000). The content of free amino acids increases markedly during the transition of tomato fruit to the ripening stage, and also plays an important role in determining the characteristics of tomato fruits. For example, elevated levels of L-glutamate confer the characteristic "umami" flavor (Sorrequieta et al. 2010). Notwithstanding the importance of regulated amino acid transport for the development of tomato fruits, the identities and modes of action of amino acid transporters remain completely unknown in tomato.

This study reports the isolation and identification of a putative tomato cationic amino acid transporter-2 gene, named *SlCAT2*. We examined *SlCAT2* expression in different tomato tissues and at various stages of flower and fruit development, and demonstrated that the gene is regulated by auxin and ethylene treatment. The roles of SlCAT2 in flower and fruit development are also discussed.

Materials and methods

Plant materials and phytohormone treatment

Tomato (*Solamum lycopersicum* cv. MicroTom) plants were grown in a controlled environment chamber as described by Yang et al. (2010), with a 14 h day/10 h night cycle (250 μmol m^{-2} s^{-1} light intensity), 25 °C/20 °C day/night temperature, and 80 % humidity. The tissues of roots, stems, leaves, buds, flowers, and fruits were collected from 10-week-old tomato plants. Samples of ovaries, stamens, petals, and sepals were harvested at the bud (−2 dpa, days postanthesis), anthesis (0 dpa), and postanthesis (4 dpa) stages. The fruit stages covered by our analysis spanned the entire ontogeny of fruit development, from the ovary of the floral bud to the ripening fruit.

For phytohormone treatment, tomato seeds were germinated and grown in Murashige and Skoog (MS) culture medium, as described by Girhepuje and Shinde (2011). The 21-day-old seedlings light-grown were treated with 50 μl l^{-1} ethylene for 1 h or 50 % MS buffer containing 20 μM indole-3-acetic acid (IAA) for 3 h. Corresponding mock treatments performed concomitantly provided suitable controls.

Gene cloning and sequence analysis

Based on the tomato expressed sequence tag (EST) SGN-U573591 containing the partial *CAT2-like* open reading frame (ORF), the full-length cDNA of this gene was isolated from tomato by using a BD SMART™ RACE cDNA Amplification kit (BD Biosciences) according to the manufacturer's instructions. The primers used for PCR-mediated amplification were the BD UMP primers provided by the kit and gene-specific primers GSP1 5′-GATAAA AGGAGGGACTGGAACT-3′ (for 5′-RACE) and GSP2 5′-GGTCCCAGTTCCCTCCTCTTAT-3′ (for 3′-RACE).

The encoding sequence of this isolated gene was analyzed using a translation tool (http://www.expasy.org/translate/). Amino acid sequence alignments were performed using ClustalX 2.0.10, and the result was displayed using Genedoc 2.7.0. Transmembrane domains were predicted using TM prediction programs (TMHMM 2.0) (http://www.cbs.dtu.dk/services/TMHMM/).

Phylogenetic analysis

Phylogenetic analysis was performed using MEGA5.0 (http://www.megasoftware.net). The amino acid sequences were aligned by default parameters and the tree was constructed by using the neighbor-joining method with 1,000 bootstrap replicates. The names and GenBank accession numbers of proteins used for the sequences analysis are *Arabidopsis thaliana* AtCAT1 (AAP21253), AtCAT2 (ACI49773.1), AtCAT3 (BAC42395.1), AtCAT4 (NP_187022.5), AtCAT5 (NP_181041), AtCAT6 (AAK25896_1), AtCAT7 (NP_187671.1), AtCAT8 (AAL60003.1), AtCAT9 (NP_563754.1), *Populus trichocarpa* PtCAT1 (EEE94966), PtCAT2 (EEE95112), PtCAT3 (EEE99819), PtCAT4 (EEE 99818), PtCAT5 (EEE78693), PtCAT6 (EEF07438), PtCA T7 (EEE72281), PtCAT8 (EEE85237), PtCAT9 (EEE 81763), PtCAT10 (EEF02662), PtCAT11 (EEE96485), PtCAT12 (EEF05986), and *Schizosaccharomyces pombe* SpCAT1 (CAB63555).

Subcellular localization in tobacco protoplast

The coding sequence of *SlCAT2* was amplified by PCR using the primers F (5′-AAGATTCAATTTTTCCAACAC CCA-3′) and R (5′-ACATGCAAGGGAGATGGCTGA TG-3′). The PCR product was cloned as a C-terminal fusion in frame with the green fluorescent protein (GFP) into the pGreen vector under the transcriptional control of the cauliflower mosaic virus 35S promoter and the nopaline synthase (nos) terminator. Protoplasts of tobacco (*Nicotiana tabacum*) BY-2 cells were isolated and transfected according to the method described by Wang et al. (2005). A 0.2 ml protoplast suspension was transfected with 50 μg shared salmon sperm carrier DNA and 30 μg of either 35S:SlCAT2-GFP or 35S:GFP (control) plasmids. Transfected protoplasts were incubated for 16 h at 25 °C, and fluorescence signals were detected by confocal microscopy, as described by Xuan et al. (2011). All transient expression assays were performed at least three times.

Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen), and the first-strand cDNA synthesis using Omniscript® Reverse Transcription (QiaGen) was performed as described previously (Yang et al. 2010). The constitutively expressed *Slactin-51* gene (GenBank accession number Q96483) was used as a reference in the various samples analyzed. The gene-specific primers were Slactin-51_F (5′-TGTCCCTA TTTACGAGGGTTATGC-3′), Slactin-51_R (5′-CAGTTA AATCACGACCAGCAAGAT-3′), SlCAT2_F (5′-GTTGT CAGGAATGGTCAGTGTT-3′), and SlCAT2_R (5′-CCT CGTCTGGTGGTACGTATCG-3′). Quantitative PCR analysis was performed using the SYBR GREEN PCR master mix (Applied Biosystems) on ABI PRISM 7900HT sequence detection system. The reaction conditions were as follow: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and finally one cycle of 95 °C for 15 s and 60 °C for 15 s. For all quantitative PCR experiments, three biological replicates were performed, and each reaction was run in triplicate. Relative fold differences were calculated based on the comparative threshold cycle (Ct) value method using the *SlActin-51* gene as an internal standard. To determine relative fold differences for each sample in each experiment, the Ct value for the transcripts *SlCAT2* was normalized to the Ct value for *SlActin-51* and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. The standard error (SE) was used as a measure of confidence in the quantitative PCR results.

Results

Cloning and in silico analysis of SlCAT2

Based on the SGN-U573591 sequence, a 2,646-bp full-length cDNA of this gene was isolated from tomato flowers using RACE PCR. The cDNA contains a 1,950-bp ORF sequence that encodes a polypeptide of 650 amino acids with a molecular weight of about 68 kDa (Fig. 1). The deduced amino acid sequence shares high identity with AtCAT2 (66.92 %) and AtCAT3 (65.44 %) of *Arabidopsis*, as well as PtCAT2 (70.95 %) and PtCAT3 (70.34 %) of poplar, slightly lower identity to AtCAT4 (57.69 %) and PtCAT4 (59.69 %), and relatively low similarity to other CAT proteins (Table 1). Owing to its closest homology to AtCAT2 and PtCAT2, the protein was designated as SlCAT2 (GenBank accession number JQ991012).

In order to assess the relationship between tomato SlCAT2 and CATs from *Arabidopsis* and poplar, phylogenetic analysis was conducted using their amino acid sequences. As shown in Fig. 2, phylogenetic analysis classified the CAT proteins of *Arabidopsis* and poplar as four subgroups. Clustering of SlCAT2 in subgroup 4 of plant CAT proteins suggests that it is an ortholog of AtCAT2 and PtCAT2.

To determine the transmembrane domains of the SlCAT2 protein, its amino acid sequence was analyzed using the TMHMM tool. As shown in Fig. 3, the SlCAT2 polypeptide contains 14 putative transmembrane domains that coincide with the positions of the corresponding domains of AtCAT2 (Fig. 1), and the predicted locations of both termini are on the inside of membrane. The data supports the proposal that the deduced SlCAT2 is a putative transmembrane protein.

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SlCAT2 MGFVGDGSCSSSGGDKGGCFIGGMKSLVRRKQVDSANSKSSSTSGSSHQLAKALTIPHLITIGVGSTIGAGVYILVGTVARE  82
AtCAT2 MGFLVD-----TQKEGGGHSWGYVRSLVRRKQVDSANGQS-----HGHQLARALTVPHLVAIGVGATIGAGVYILVGTVARE  72
                                                                                TM 1

SlCAT2 HSGPALTISFLIAGIAAALSAFCYAELASRCPSAGSAYHYSYICVGEGVAWLIGWALVLEYTIGRSAVARGISPNLAMLFGS 164
AtCAT2 HSGPSLALSFLIAGIAAGLSAFCYAELSSRCPSAGSAYHYSYICVGEGVAWIIGWALILEYTIGGSAVARGISPNLALIFGG 154
                  TM 2                                 TM 3

SlCAT2 PDSLPSFLARHTIPGLNITVDPCAAILVFLVTGLLCVGIKESTVVQGFVTSVNVCVMAFVIIAGGYLGYKAGWPGYELPVGY 246
AtCAT2 EDGLPAILARHQIPGLDIVVDPCAAILVFVVTGLLCMGIKESTFAQGIVTAVNVCVLLFVIVAGSYLGFKTGWPGYELPTGF 236
                              TM 4                          TM 5

SlCAT2 FPYGVDGMLAGASTVFFAYIGFDSVASTAEEVKNPQRDLPMGIGFALSICCSLYMLVSAVIVGLVPYYAMDPDTPISSAFAS 328
AtCAT2 FPFGVDGMFAGSATVFFAFIGFDSVASTAEEVRNPQRDLPIGIGLALLLCCSLYMMVSIVIVGLIPYYAMDPDTPISSAFAS 318
                TM 6                                          TM 7

SlCAT2 HGINWAAYIITIGACTSLCSTLMGSIMPQPRILMAMARDGLLPSFFSDVNKRTQVPIKGTIATGLLSGTLAFFMNVEQLSGM 410
AtCAT2 HDMQWAVYLITLGAVMALCSALMGALLPQPRILMAMARDGLLPSIFSDINKRTQVPVKATVATGLCAATLAFFMDVSQLAGM 400
                    TM 8                                                  TM 9

SlCAT2 VSVGTLLAFTMVAISVLILRYVPPDEVPVPSSYQEAIDSVRLRRSSCSSSSDMDVEKTKIPAVTSGDSTPLLGEISVGHPLA 492
AtCAT2 VSVGTLLAFTMVAISVLILRYVPPDEQPLPSSLQERIDSVSFICGETTSSGHVGTSDSSHQPLIVNN------DALVDVPLI 476
          TM 10

SlCAT2 EKAAAKLSYLVSQ--RRKVAGCTILFICIGVCIVTSAASIVNLSNPARYALSGIGGLLLISGLIILTCIDQDDARHSFGHTG 572
AtCAT2 KNQEALGCLVLSEETRRIVAGWSIMFTCVGAFLLSYAASSLSFPGLIRYPLCGVGGCLLLAGLIALSSIDQDDARHTFGHSG 558
                             TM 11                         TM 12

SlCAT2 GFTCPFVPLLPIACILINVYLLINLGGETWARVSIWLVIGTCIYALYGRTHSSLKTAVYVPSTHVDEIYETSAISLAC 650
AtCAT2 GYMCPFVPLLPIICILINMYLLVNLGSATWARVSVWLLIGVIVYVFYGRKNSSLANAVYVTTAHAEEIYREHEGSLA- 635
           TM 13                            TM 14
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Fig. 1 Sequence analysis of SlCAT2. The deduced amino acid sequences of tomato SlCAT2 and *Arabidopsis* AtCAT2 were aligned. *Numbers* show the positions of amino acid residues. Conserved residues are *shaded* in *black*. *Underlined* residues indicate the 14 putative transmembrane (TM) domains predicted by the TMNMM tool (http://www.cbs.dtu.dk/services/TMHMM/)

Table 1 Comparative analysis of SlCAT2 amino acid sequence with CAT proteins in *Arabidopsis* and poplar

	Identity (%) SlCAT2		Identity (%) SlCAT2
AtCAT1	26.18	AtCAT7	27.12
AtCAT2	66.92	AtCAT8	22.87
AtCAT3	65.44	AtCAT9	30.95
AtCAT4	57.69	PtCAT2	70.95
AtCAT5	23.91	PtCAT3	70.34
AtCAT6	25.42	PtCAT4	59.69

SlCAT2-GFP localizes to the tonoplast

The deduced SlCAT2 protein is not only likely to be an integral membrane protein, but a protein subcellular localization prediction program (PSORT, http://wolfpsort.org/) further predicted SlCAT2 to localize to the tonoplast. To validate this prediction, a fusion of SlCAT2 to GFP under the control of the CaMV35S promoter was transiently expressed in tobacco protoplasts. Confocal imaging of protein fluorescence showed that control cells transformed with GFP alone fluoresced throughout the cytoplasm and nuclei (Fig. 4a), whereas the SlCAT2-GFP fusion protein localized to the tonoplast (Fig. 4b-1). Localization in the tonoplast suggests that SlCAT2 functions in amino acid storage or export from the vacuole. At the same time, a punctuate fluorescence was additionally observed in a few SlCAT2-GFP expressing cells (Fig. 4b-2). Although the contour of the vacuole is not clear in Fig. 4b-2, we believe that the circular part encircled by fluorescence is the vacuole based on its large volume.

SlCAT2 transcripts are ubiquitous in tomato tissues but are most abundant in flowers

Uncovering the tissue-specific and developmentally regulated patterns of a particular gene expression can provide important clues about its physiological function. Therefore, quantitative RT-PCR was used to examine the expression profile of *SlCAT2* in different tissues of tomato. As shown in Fig. 4a, *SlCAT2* transcripts were present in all tested tissues, with the highest levels in flowers, where levels were more than fivefold higher than in other tissues, and the lowest abundance in floral buds.

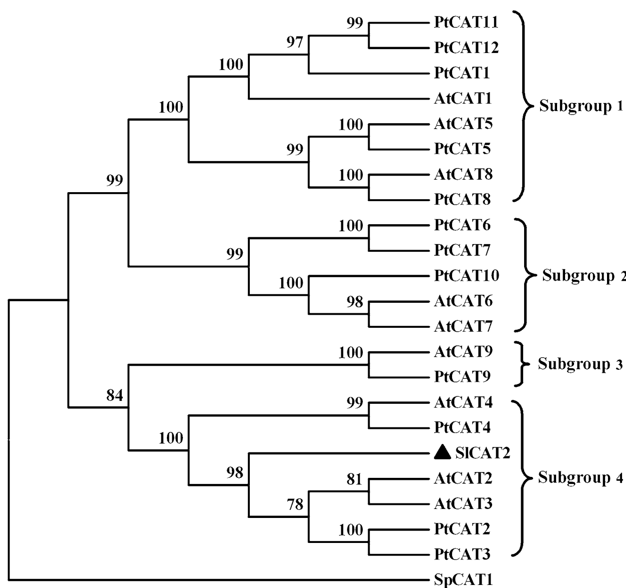

Fig. 2 SlCAT2 and its homologs belong to a distinct subgroup of the CAT family. The phylogenetic tree was obtained using the Neighbor-Joining approach provided as part of the MEGA5 software package. SpCAT1 was used as outgroup because of its relative isolation following preliminary calculations. Values above the branches are bootstrap percentages (1,000 replicates)

Compared with flowers, other tissues had only basal mRNA levels of *SlCAT2*. The transcripts of *SlCAT2* in flowers increase 9.7-fold during the transition from floral buds to fully formed flowers. These expression profiles suggest that *SlCAT2* functions ubiquitously in tomato, in plant parts as diverse as roots, stems, leaves, floral buds, flowers and fruits.

SlCAT2 expression shows dynamic transition during fruit initiation and increases gradually with fruit ripening

Given the established role of the increase amino acid levels during tomato-fruit ripening and the presumed function of CAT-like protein in amino acids transport,the expression of *SlCAT2* was analyzed using quantitative RT-PCR throughout the ontogeny of tomato fruit development, from ovary to ripening fruit (Fig. 5b). In ovaries, levels of *SlCAT2* transcript increase obviously from the bud stage (−2 dpa) to the time of anthesis, and decline rapidly (by more than 70 %) from the time of anthesis to the postanthesis period (4 dpa) when the ovary begin to develop into a fruit. Abundance of *SlCAT2* transcript increases slightly during the very early stage of fruit development (8 dpa), and then reaches a stable level until mature green fruits are formed (about 40 dpa). *SlCAT2* transcript levels increase further at the break stage (about 42 dpa), which coincides with the onset of ripening, and are twofold higher in ripening fruits (about 50 dpa) than their in mature green fruits. These data demonstrate that *SlCAT2* expression is regulated by fruit developmental process, and may be related to the increase in amino acid content during tomato fruit ripening.

SlCAT2 expression is the highest in stamens

Considering that the levels of *SlCAT2* transcript were higher in flowers than any of the other tissues tested (Fig. 5a), we further examined its expression profile in different parts of flowers at three developmental stages (Fig. 6). Generally, *SlCAT2* transcripts are found at

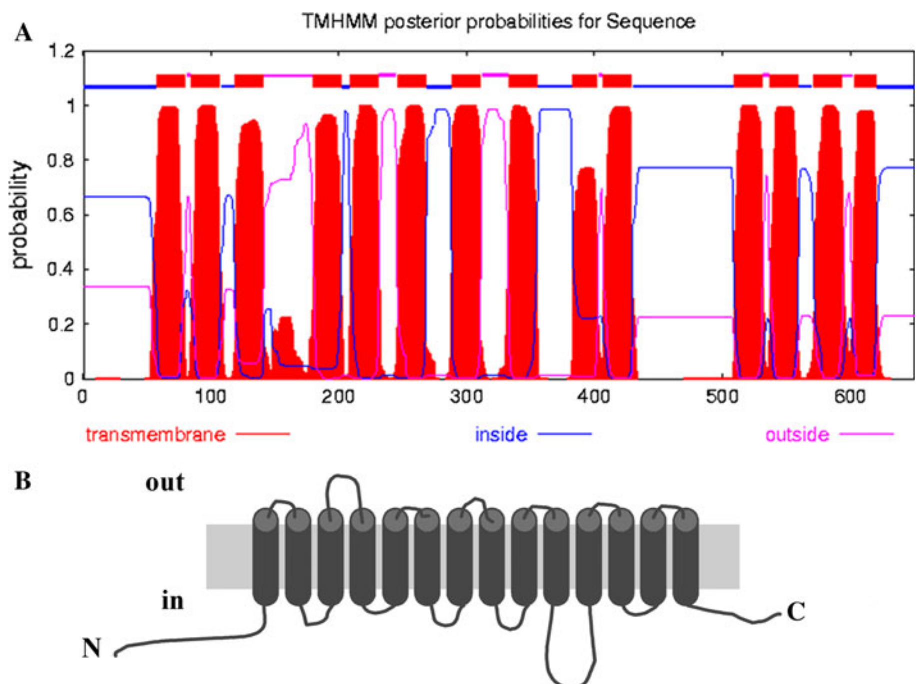

Fig. 3 The transmembrane domains of SlCAT2. **a** Prediction of the TM domains of SlCAT2 using the TMNMM tool. **b** A cartoon sketch of the deduced two-dimensional structure

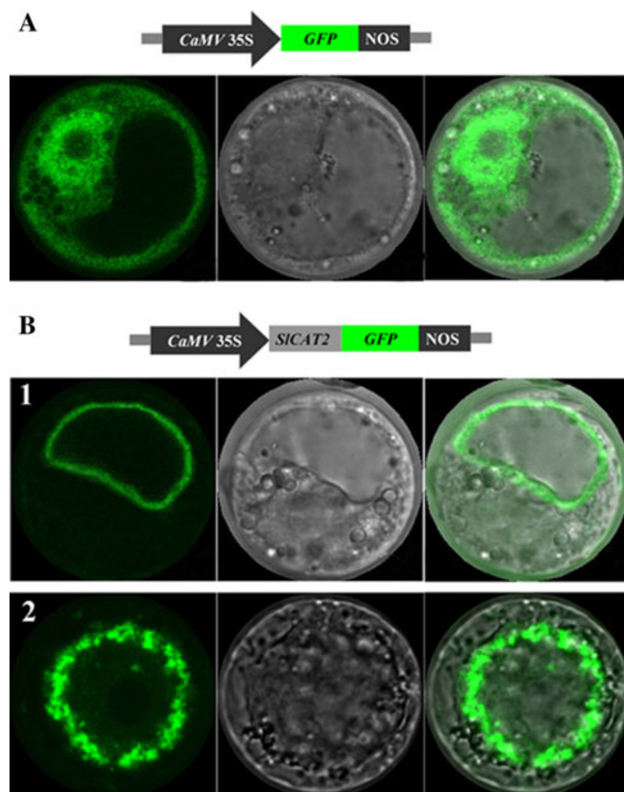

Fig. 4 Subcellular localization of SlCAT2-GFP. **a** Expression of GFP alone as control in the cytosol and nuclei of tobacco protoplasts. **b** Localization of SlCAT2-GFP to the tonoplast (B1), with some staining in dot-like structures observed in some of the protoplasts that express SlCAT2-GFP (B2). The subcellular localization of fluorescent proteins was determined using confocal laser scanning microscopy (*left panel*). Light micrographs (*middle panel*) and fluorescence (*left panel*) images are merged (*right panel*) to illustrate the different locations of the two proteins

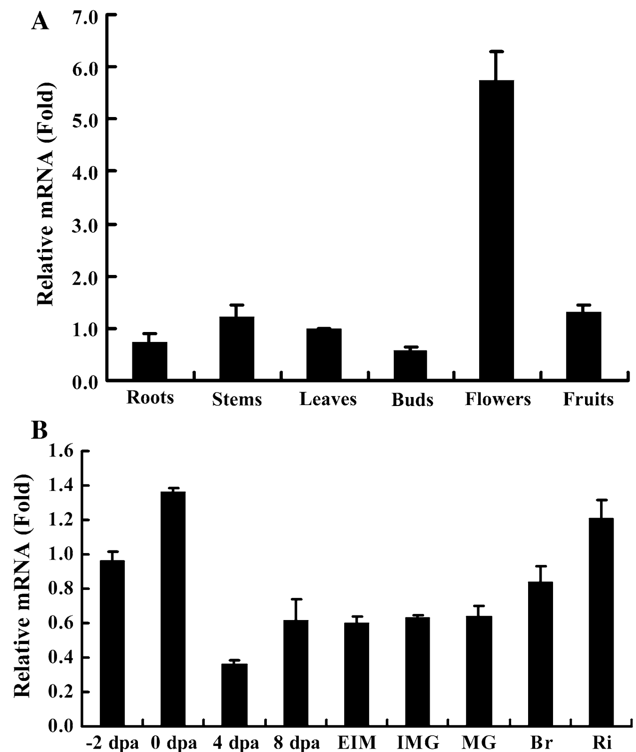

Fig. 5 Quantitative RT-PCR analysis of *SlCAT2* expression patterns in tomato. **a** Expression of *SlCAT2* in different tissues. **b**. Expression of *SlCAT2* at different stages of fruit development. The −2, 0 and 4 dpa samples represent the ovaries of floral bud, ovaries at the time of anthesis, and ovaries just after fruit set, respectively. Fruit stages contain early young (8 dpa), early immature green (EIM, about 20 dpa), immature green (IMG, about 30 dpa), mature green (MG, about 40 dpa), breaker (Br, about 42 dpa) and ripening (Ri, about 50 dpa). Data are expressed as relative values, based on the values of leaves taken as reference sample set to 1. Each value represents the mean ± SE of more than 10 plants and three independent replicates

moderate levels in ovaries, petals and sepals, and at dramatically higher levels in stamens. *SlCAT2* transcript levels in stamens are 11.5-fold and 34.8-fold higher than its levels in ovaries at the bud and anthesis stages, respectively. However, ovaries at the time of anthesis have the highest expression of *SlCAT2* throughout the whole tomato fruit ontogeny (Fig. 5b), and *SlCAT2* transcripts are most abundant in flowers among all of the tissues tested (Fig. 5a). Taken together, these data indicate that the stamens have the highest abundance of *SlCAT2* transcripts among all tomato samples studied, including the different tissues (Fig. 5a), fruits at different developmental stages (Fig. 5b), and different parts of flowers (Fig. 6).

From bud period to anthesis stage, *SlCAT2* transcript levels increased only slightly (by less than 0.5-fold) in ovaries, petals, and sepals, in contrast with a more remarkable 4.3-fold increase in stamens. *SlCAT2* transcript accumulation in ovaries decreased obviously by more than 73 % from the time of anthesis to the postanthesis phase. This dynamic expression pattern suggests that *SlCAT2* may be involved in controlling flower development and the flower-to-fruit transition triggered by pollination, while potentially playing an especially important role in regulating stamen development.

Accumulation of *SlCAT2* transcripts is reduced by ethylene or auxin treatment

It is well established that ethylene stimulates flower opening and fruit ripening, and that auxin induces fruit initiation. Fluctuations in the expression of *SlCAT2* during these processes prompted us to use quantitative RT-PCR to determine the potential responsiveness of *SlCAT2* mRNA levels to ethylene and auxin treatment. A 1-h exposure to exogenous ethylene reduced *SlCAT2* transcript levels in light-grown tomato seedlings (Fig. 7a). Similarly, expression of *SlCAT2* was negatively regulated by exogenous treatment for 3 h with IAA, the major auxin compound (Fig. 7b).

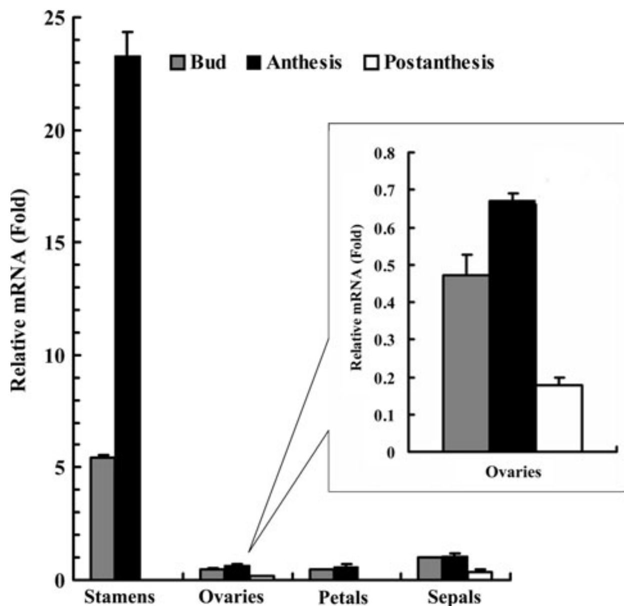

Fig. 6 Levels of *SlCAT2* transcript are especially high in stamens and during the developmental transitions that result in flower opening and fruit set. Expression of *SlCAT2* was detected by quantitative RT-PCR in different parts of flowers at different developmental stages. Given that stamens and petals are shed during the postanthesis stage, no data are shown for these two plant parts at this stage. Relative expression level refers to the fold difference in *SlCAT2* transcript levels relative to the sepals of floral buds. The data are the mean ± SE of more than 10 plants and three independent replicates

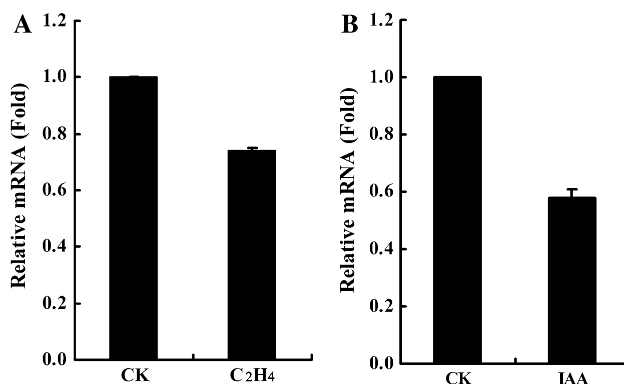

Fig. 7 *SlCAT2* is negatively regulated by ethylene and auxin. Light-grown tomato seedlings were treated with 50 μl l^{-1} ethylene for 1 h (**a**) or 20 μM IAA for 3 h (**b**). Relative mRNA accumulation of *SlCAT2* was tested using quantitative RT-PCR. The values of control taken as reference sample were set to 1. Each value represents mean ± SE of 30 plants and is representative of three independent replicates

Discussion

As the major form of easily mobilized organic nitrogen in plants, amino acids play fundamental roles in a multitude of processes in plants (Coruzzi and Zhou 2001). Amino acid transporters are used by plants to transport these metabolites from source tissues to sink tissues during development and under changing environmental conditions (Hammes et al. 2006). In order to respond to a wide range of physiological needs and environmental changes, plants have evolved a substantial number of amino acid transporters. For example, the demonstration that *Arabidopsis* contains more than 50 distinct amino acid transporter genes (Su et al. 2004) underscores the complexity of regulated amino-acid transport in plants. Nonetheless, little is known about amino acid transporter genes in tomato, a model system for understanding plants with fleshly fruits.

The present study involved isolation and characterization of a putative tomato cationic amino acid transporter gene, named *SlCAT2*. The deduced SlCAT2 protein bears strong sequence and structural similarity with its CAT2-4 homologs from *Arabidopsis* and poplar, with especially high similarity to the plant CAT2 and CAT3 proteins. As described for the *Arabidopsis* and poplar CAT family, plant CAT members can be phylogenetically classified into four small subgroups (Su et al. 2004; Couturier et al. 2010). The tomato putative SlCAT2 protein, *Arabidopsis* AtCAT2 and poplar PtCAT2 all belong to subgroup 4 of plant CAT proteins (Fig. 2). Moreover, both SlCAT2-GFP (Fig. 4) and AtCAT2-GFP (Su et al. 2004) localize to the tonoplast. Considered together, these data suggest that *SlCAT2* is an ortholog of *Arabidopsis AtCAT2*.

Given their essential roles in cell expansion, storage of biochemicals, and metabolite partitioning with the cytosol, plant vacuoles are important regulators of plant growth (Martinoia et al. 2007). As described previously by Su et al. (2004), concentrations of most amino acids are similar to or lower than the cytosolic concentrations in many plants. However, the total amino acid content in the vacuole is often higher than that in the cytosol. Although it is controversial whether plant vacuoles are storage compartments for amino acids, the exchange of amino acids between the vacuolar and cytosolic is critical for plant survival (Okumoto and Pilot 2011). In *Arabidopsis*, the vacuole proteome includes the AtCAT2, AtCAT4, AtCAT8, and AtCAT9 proteins (Carter et al. 2004; Jaquinod et al. 2007). Localization of AtCAT2 to the tonoplast (Su et al. 2004) suggests that plant CAT members may be involved in transporting amino acids into and out of the vacuole. Similarly, the tonoplast localization of SlCAT2 suggests that it is a vacuolar amino acid transporter in tomato.

Transport of amino acids to developing flowers has significant consequences for flower set, levels of floral abortion, pollen and embryo development, and seed production (Lee and Tegeder 2004). Among the tissues tested, flowers had the highest levels of *SlCAT2* transcript (Fig. 5a). This suggests that SlCAT2 plays an important role in flowers. Within the flowers, large amounts of viable pollen need to be produced in stamens to fertilize the ovules and guarantee sexual reproduction. Pollen development requires plentiful amounts of amino acids to

synthesize a range of proteins, including enzymes, hydroxyproline-rich glycoproteins, storage proteins, protease inhibitors, and many others (Agyare-Tabbi et al. 2011). Given that the transport of amino acids to flowers is essential for the maturation of microspores to pollen grains in stamens, stamens often represent a major sink for amino acids (Goldberg et al. 1993; Lee and Tegeder 2004). The importance of an adequate supply of amino acids to the successful development of pollen underscores the value of investigating the role of amino acid transporters in regulating amino acid levels in stamens. The *SlCAT2* gene is highly expressed in stamens and substantially up-regulated from the time of bud formation to the time of anthesis (Fig. 6), when pollen grains are expected to mature. The expression profiles of *SlCAT2* in stamens indicate its putative important function in transporting amino acids to stamens for pollen development and maturation.

The dynamic expression of *SlCAT2* in flower organs during the bud-to-anthesis and anthesis-to-postanthesis transitions reflects the apparent importance of its regulated expression both during flower development and fruit initiation. The gradual increase in the abundance of *SlCAT2* transcripts from the mature green stage to the ripening stage in fruits (Fig. 5b) suggests an important role for SlCAT2 in the fruit ripening process, when marked accumulation of amino acids occurs in fruit (Boggio et al. 2000; Sorrequieta et al. 2010). It is well known that ethylene stimulates flower opening and fruit ripening, and that auxin controls fruit set. The observation that levels of *SlCAT2* transcript were negatively regulated by both ethylene and auxin treatment of tomato seedlings (Fig. 7) suggests that *SlCAT2* expression may be related to ethylene and auxin signaling.

The selectivity of SlCAT2 and the transport mechanism involved in its action have not been identified. Nonetheless, its dynamic expression during key transitions during reproductive growth and development and responsiveness to exogenous ethylene and auxin together suggest roles for SlCAT2 in the stimulation of flower opening and fruit ripening by ethylene, and the induction of fruit initiation by auxin. Nevertheless, further study of the *SlCAT2* gene and its coding protein is required for a better understanding of the physiological role of SlCAT2 in pollen grains, the development of stamens and flowers, and the ripening of fruit.

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References

Afroz A, Chaudhry Z, Rashid U, Ali GM, Nazir F, Iqbal J, Khan MR (2011) Enhanced resistance against bacterial wilt in transgenic tomato (*Lycopersicon esculentum*) lines expressing the *Xa21* gene. Plant Cell Tiss Organ Cult 104:227–237

Agyare-Tabbi A, Zhang J, Xiong A, Atnaseo C, Bahramnejad B, Erickson L (2011) A protein coding for a pollen-specific gene in alfalfa (*Medicago sativa* L.) is localized mainly in the intine of the pollen wall. Plant Cell Tiss Organ Cult 104: 277–280

Boggio SB, Palatnik JF, Heldt HW, Valle EM (2000) Changes in amino acid composition and nitrogen metabolizing enzymes in ripening fruits of *Lycopersicon esculentum* Mill. Plant Sci 159:125–133

Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV (2004) The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell 16:3285–3303

Coruzzi GM, Zhou L (2001) Carbon and nitrogen sensing and signaling in plants: emerging 'matrix effects'. Curr Opin Plant Biol 4:247–253

Couturier J, Doidy J, Guinet F, Wipf D, Blaudez D, Chalot M (2010) Glutamine, arginine and the amino acid transporter Pt-CAT11 play important roles during senescence in poplar. Ann Bot 105: 1159–1169

Fischer WN, Loo DD, Koch W, Ludewig U, Boorer KJ, Tegeder M, Rentsch D, Wright EM, Frommer WB (2002) Low and high affinity amino acid H1-cotransporters for cellular import of neutral and charged amino acids. Plant J 29:717–731

Frommer WB, Hummel S, Unseld M, Ninnemann O (1995) Seed and vascular expression of a high-affinity transporter for cationic amino acids in *Arabisopsis*. Proc Natl Acad Sci USA 92: 12036–12040

Girhepuje PV, Shinde GB (2011) Transgenic tomato plants expressing a wheat endochitinase gene demonstrate enhanced resistance to *Fusarium oxysporum* f. sp. *lycopersici*. Plant Cell Tiss Organ Cult 105:243–251

Goldberg RB, Beals TP, Sanders PM (1993) Anther development: basic principles and practical applications. Plant Cell 5:1217–1229

Hammes UZ, Nielsen E, Honaas LA, Taylor CG, Schachtman DP (2006) AtCAT6, a sink-tissue-localized transporter for essential amino acids in *Arabidopsis*. Plant J 48:414–426

Jaquinod M, Villiers F, Kieffer-Jaquinod S, Hugouvieux V, Bruley C, Garin J, Bourguignon J (2007) A proteomics dissection of *Arabidopsis thaliana* vacuoles isolated from cell culture. Mol Cell Proteomics 6:394–412

Lalonde S, Wipf D, Frommer WB (2004) Transport mechanisms for organic forms of carbon and nitrogen between source and sink. Annu Rev Plant Biol 55:341–372

Lee Y, Tegeder M (2004) Selective expression of a novel high-affinity transport system for acidic and neutral amino acids in the tapetum cells of *Arabidopsis* flowers. Plant J 40:60–74

Martinoia E, Maeshima M, Neuhaus HE (2007) Vacuolar transporters and their essential role in plant metabolism. J Exp Bot 58:83–102

Okumoto S, Pilot G (2011) Amino acid export in plants: a missing link in nitrogen cycling. Mol Plant 4:453–463

Sorrequieta A, Ferraro G, Boggio SB, Valle EM (2010) Free amino acid production during tomato fruit ripening: a focus on L-glutamate. Amino Acids 38:1523–1532

Su YH, Frommer WB, Ludewig U (2004) Molecular and functional characterization of a family of amino acid transporters from *Arabidopsis*. Plant Physiol 136:3104–3113

Wang H, Jones B, Li Z, Frasse P, Delalande C, Regad F, Chaabouni S, Latché A, Pech JC, Bouzayen M (2005) The tomato *Aux/IAA* transcription factor *IAA9* is involved in fruit development and leaf morphogenesis. Plant Cell 17:2676–2692

Wipf D, Benjdia M, Tegeder M, Frommer WB (2002a) Characterization of a general amino acid permease from *Hebeloma cylindrosporum*. FEBS Lett 528:119–124

Wipf D, Ludewig U, Tegeder M, Rentsch D, Koch W, Frommer WB (2002b) Conservation of amino acid transporters in fungi, plants and animals. Trends Biochem Sci 27:139–147

Xuan N, Jin Y, Zhang H, Xie Y, Liu Y, Wang G (2011) A putative maize zinc-finger protein gene, *ZmAN13*, participates in abiotic stress response. Plant Cell Tiss Organ Cult 107:101–112

Yang Y, Wu Y, Pirrello J, Regad F, Bouzayen M, Deng W, Li Z (2010) Silencing *Sl-EBF1* and *Sl-EBF2* expression causes constitutive ethylene response phenotype, accelerated plant senescence, and fruit ripening in tomato. J Exp Bot 61:697–708