Characterization and Expression Analysis of the Ca²⁺/Cation Antiporter Gene Family in Tomatoes

Kayoko Amagaya 1, Tomoki Shibuya 2, Manabu Nishiyama 1, Kazuhisa Kato 1,∗ and Yoshinori Kanayama 1,∗

1 Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai 980-8572, Japan
2 Faculty of Life and Environmental Science, Shimane University, Matsue 690-8504, Japan
* Correspondence: kazuhisa.kato.d8@tohoku.ac.jp (K.K); yoshinori.kanayama.a7@tohoku.ac.jp (Y.K.)

Received: 31 October 2019; Accepted: 18 December 2019; Published: 23 December 2019

Abstract: The Ca²⁺/cation antiporter (CaCA) superfamily plays an important role in the regulation of the essential element Ca²⁺ and cation concentrations. Characterization and expression analyses of CaCA superfamily genes were performed in the tomato (Solanum lycopersicum) as a representative of dicotyledonous plants and fruit crops. Sixteen CaCA candidate genes were found and identified as tomato CaCA, SlCaCA, by a domain search. In a phylogenetic analysis of the SlCaCA superfamily, the 16 genes were classified into SlCAX, SlNCL, SlCCX, and SlMHX families. Among them, Solyc12g011070, belonging to the SlCAX family, had four splice variants, three of which were predicted to be nonfunctional because of a lack of important motifs. EF-hand domains were only found in SlNCL, in addition to consensus Na_Ca_ex domains, and the region containing EF-hand domains was characteristically long in some members of SlNCL. Furthermore, four genes of the SlCCX family were found to be intronless. As for intracellular localization, one SlCCX member was predicted to be localized to the plasma membrane, while other SlCCXs, SlCAXs, and SlMHXs were predicted to be localized to the vacuolar membrane. The expression patterns of SlCaCAs in various organs, including during several developmental stages of fruit, were classified into four groups. Genes involved in each of the SlCAX, SlNCL, and SlCCX gene families were categorized into three or four groups according to expression patterns, suggesting role sharing within each family. The main member in each subfamily and the members with characteristic fruit expression patterns included genes whose expression was regulated by sugar or auxin and that were highly expressed in a line having metabolite-rich fruit.

Keywords: Solanum lycopersicum; Solanaceae; CaCA superfamily; Ca²⁺/Cation antiporter

1. Introduction

Ca²⁺ is an essential element in plants and is important for growth and development, functioning as a second messenger in response to extracellular signaling molecules. The Ca²⁺/cation antiporter (CaCA) superfamily plays an important role in the regulation of Ca²⁺ and cation concentrations in plant cells [1–6]. Recently, Singh et al. [7] performed a phylogenetic analysis of CaCA proteins in rice and Arabidopsis and proposed that all CaCA proteins should be classified as Na⁺/Ca²⁺ exchangers (NCXs). However, Pittman and Hirschi [8] showed, by phylogenetic analysis and structural modeling, that CaCA contains distinctly different groups with different phylogenies, structures, and functional characteristics. Therefore, not all CaCA proteins can be classified as NCXs. At present, CaCA superfamily proteins in plants are classified into four families: H⁺/cation exchangers (CAXs), Na⁺/Ca²⁺ exchanger-like proteins (NCLs), cation/Ca²⁺ exchangers (CCXs), and Mg²⁺/H⁺ exchangers (MHXs). Taneja et al. [9] showed that CaCA superfamily proteins are classified into the above four families in bread wheat.
Of these four CaCA families, CAX and NCL may play roles in responses to abiotic stress [6,10], and plant hormone signaling and flowering [11,12]. Hocking et al. [13] reported that CAXs may form heteromeric transporters and affect functions of guard cells and mesophyll cells in relation to environmental plasticity. Furthermore, CaCA proteins are considered useful for the production of biofortified crops and phytoremediation because they are involved in metal ion absorption [2,14–16]. In addition, several recent reports have focused on functional analyses of CAX1 in the CaCA family. Navarro-Leon et al. [17] and Qiao et al. [18] reported that CAX1 is involved in the transport of Ca\(^{2+}\) and tolerance for heavy metals in Brassica plants and diploid wheat relatives by using mutants and overexpressors. Ahmadi et al. [19] showed that CAX1 suppresses the formation of Cd-induced reactive oxygen species as a stress tolerance mechanism. These various reports indicate the multifunctionality of the CaCA family genes in plants.

Tomatoes (Solanum lycopersicum) are an important crop worldwide because of their high economic and nutritional value. They are also scientifically useful as an experimental model species of the Solanaceae family and of fleshy-fruited plants. Regarding tomato CaCA members, it has been reported that the tomato CAX, LeCAX2, transports Ca\(^{2+}\) and Mn\(^{2+}\) [20], and its expression is increased by gibberellin (GA4+7) [21]. Ca\(^{2+}\) is closely related to the occurrence of a serious physiological disorder called blossom-end rot in tomato plants, and the relationship between this physiological disorder and CaCA may be interesting [22,23]. However, to our knowledge, tomato NCL and CCX gene families have not yet been reported, and information on tomato CaCA is lacking. In addition, the CaCA superfamily has only been comprehensively characterized in Arabidopsis and cereals such as rice and bread wheat [7–9], while information on dicotyledonous plants and fruit crops is lacking. Therefore, in order to obtain basic knowledge of CaCA, comprehensive characterization of the CaCA superfamily was performed with expression analysis in tomato plants. Together with previous reports, the present study shows the general and unique properties of the CaCA gene family. In addition, for the first time, by using bioinformatics, we use the results of this study to show analysis examples of the main member in each CaCA subfamily and the members with characteristic fruit expression patterns.

2. Results and Discussion

2.1. Phylogenetic Analysis of the Tomato CaCA (SlCaCA) Gene Family

Based on a BLAST search using the CaCA sequences of Arabidopsis thaliana, it was estimated that 16 CaCA genes exist in the tomato plant. Phylogenetic analysis showed that the 16 putative CaCA proteins could be classified into four families: six into the CAX family (Solyc03g123790, Solyc06g006110, Solyc07g056110, Solyc09g005260, Solyc12g011070, and Solyc12g055750), four into the NCL family (Solyc02g077270, Solyc03g006260, Solyc07g062700, and Solyc12g014110), five into the CCX family (Solyc01g098800, Solyc02g069710, Solyc07g006370, Solyc07g042000, and Solyc09g072690), and one into the MHX family (Solyc06g009130). The Sol Genomics Network has published the nucleotide sequence and amino acid sequence of cv. Heinz 1706, although Saand et al. [24] reported sequence errors in the Sol Genomics Network database and the sequence is considered to differ between cultivars [25]. Therefore, the nucleotide sequence of the open reading frame of each CaCA gene was determined in the present study based on cDNA from cv. M82 with primers designed using the nucleotide sequence of each putative CaCA.

Phylogenetic tree analysis was performed using the amino acid sequences of CaCA genes from the tomato, which were determined here, with those previously determined from rice and Arabidopsis [8,26], as shown in Figure 1. As a result, 16 CaCAs were grouped into four families: six CAXs, four NCLs, five CCXs, and one MHX, similar to the results of putative tomato CaCAs. The CAXs were further classified into Type1A and Type1B. According to Emery et al. [26] and Shigaki et al. [27], plant CAXs can be classified as Type1A and plant and moss CAXs can be classified as Type2B, suggesting that there are functional differences between Type1A and Type1B. According to Hirschi et al. [2], Shigaki et al. [28], and Edmond et al. [20], Type1B AtCAX2 and AtCAX5 transport various cations, including Ca\(^{2+}\), Cd\(^{2+}\), and Mn\(^{2+}\) [2,20,28], and Type1A AtCAX1 and AtCAX3 specifically transport Ca\(^{2+}\).
in *Arabidopsis* [28,29]. However, there are other reports that Type1A CAXs have a broad cation specificity [3,27,30].

According to Emery et al. [26], the average number of CaCA genes per species is 5.8 in algae, while it is 13.25 in land plants. Therefore, the tomato, with its 16 genes, has an above average number of CaCA genes for land plants. As shown in Figure 1, the number of genes in each subfamily was similar in the three compared species. On the other hand, according to Emery et al. [26], NCL (EFCAX) shows the most diversity in the number of genes, with 1 to 5 genes in land plants. In the species used in this study, the number of NCL genes was 2 to 4 and was largest in the tomato, one of which was *Solyc07g062700*. As discussed later, this gene is of interest because of its expression pattern in fruit, the fact that it exhibits the highest expression among SINCL genes, its response to sugar signals, and its high expression in fruit rich in metabolites. Functional analysis is necessary for both plant physiology and agricultural science.

**Figure 1.** Phylogenetic tree based on amino acid sequences in the Ca\(^{2+}\)/cation antiporter (CaCA) superfamily of the tomato, *Arabidopsis*, and rice. The phylogenetic tree was built using the neighbor-joining method by MEGA7. Branch numbers are the percentages of replicates that support the branch using the bootstrap method (1000 replicates). The scale bar corresponds to 0.2 amino acid substitutions per residue. The sequences from *Arabidopsis* and rice were obtained from Emery et al. [26] and Pittman and Hirschi [8].
2.2. SlCaCA Protein and Gene Characterization

In order to confirm whether the putative SlCaCAs of the tomato function as CaCAs, a domain search was performed on the obtained amino acid sequence using TMHMM (ver. 2.0) and InterPro of the EMBL-EBI. The four mRNA variants of Solyc12g011070 were referred to as Solyc12g011070a, b, c, and d. CaCA has transmembrane domains (TMs) because it performs the countertransport of cations through the membrane. According to previous reports, CaCA has an average of 10 TMs [8,9,26]. However, in the amino acid sequence translated from Solyc12g011070b, c, and d, there were only five TMs (Figure S1). Excluding these variants, the number of TMs in the estimated CaCA was 9 to 13, and the average was 10.9, which is, in general, in agreement with the previous report (Figures S1–S3; Table 1).

When the domain was searched with InterPro of the EMBL-EBI, the amino acid sequences translated from Solyc12g011070b, c, and d had structures different from those documented in previous reports and other putative tomato CaCAs (Figure 2). That is, only one Na_Ca_ex domain (Pfam ID, Pf01699; [9]), which is characteristic of CaCA, was present in Solyc12g011070b, c, and d, while two were present in other CAXs, NCLs, CCXs, and the MHX. In addition, only NCLs contained EF-hand domains (Pfam ID: PF13499): two in Solyc02g077270 and Solyc03g006260 and one in Solyc07g027700 and Solyc12g014110. According to Taneja et al. [9], CaCAs in bread wheat have two Na_Ca_ex (Pfam ID: Pf01699) domains, and only TaNCL has one EF-hand domain (Pfam ID: PF13499). As described above, Solyc12g011070b, c, and d did not have domains of importance to CaCA, so they were not considered to be functional, unlike CaCA, and were excluded from expression analysis. However, since expression regulation by selective splicing has been reported in the stress response [31], it may be necessary to examine the role of variant generations of this gene. Despite this, since important domains were conserved in other putative CaCAs, it was concluded that there were 16 functional CaCAs in the tomato, and these were categorized as SICAX, SINCL, SICCX, or SIMHX.

Emery et al. [26] examined a wide range of organisms, including plants, and found that, although not classified as NCL, some CaCA members in mainly land plants have an additional EF-hand domain with a Ca-binding motif to Na_Ca_ex domains, which is a consensus sequence. Taneja et al. [9] showed that in TaNCL of wheat TaCaCA, the EF-hand domain exists as a loop that is a long amino acid sequence between two TMs near the center, and it is longer than those of members in other subfamilies. This also matched the tomato CaCA superfamily; additional EF-hand domains, specific to SINCL, were found in long amino acid sequences between the specific TMs (Figure 2 and Figures S1–S3). However, members with one or two EF-hand domains were found in the tomato, and among them, members with two were particularly characteristic because the amino acid sequence between the corresponding TMs was nearly twice the length of that of TaNCL. Therefore, future studies should demonstrate the significance of this in function and evolution.

To examine the genomic structure of each CaCA, schematic diagrams of exons and introns were made, based on the genomic DNA sequences of the corresponding genes (European Nucleotide Archive, https://www.ebi.ac.uk/ena) (Figure 3). The second exons of Solyc12g011070b, c, and d contained part or all of the second intron of Solyc12g011070a. Because a stop codon appears in the second intron of Solyc12g011070a (523 bases from the start codon), the translated regions of Solyc12g011070b, c, and d were half the length of the functional sequence of Solyc12g011070a, and consequently contained only one Na_Ca_ex (Pfam ID: Pf01699) domain. Solyc09g005260 and Solyc02g069710 were different in sequence from cv. Heinz 1706. However, it was likely that there was no difference in their functions as CAXs because the difference in the sequence was not within the important motif. Solyc02g069710, Solyc07g006370, Solyc07g042000, and Solyc09g072690, belonging to CCX, had no introns. Three of five TaCCXs also lack introns in bread wheat [9], suggesting that this may be a feature of CCX that is conserved across species and across dicotyledonous and monocotyledonous plants. Because intronless genes form mature mRNAs without splicing, they can respond quickly to stress [32,33]. This suggests that CCX is involved in the stress response, and that transcription and translation of these genes may occur rapidly due to the absence of introns. On the other hand, since there are many intronless genes involved in basic cellular processes [33,34], the...
intronless genes may have significance, such as avoiding energy loss during the transcription process, other than the quick stress response.

Table 1. Characteristic features of tomato CaCA superfamily proteins.

| Family | Locus          | Protein Size | TM | Domain  | α1-Repeat | α2-Repeat | Localization      |
|--------|----------------|--------------|----|---------|-----------|-----------|------------------|
| CAX    | Solyc03g123790 | 383          | 11 | Na_Ca_ex| TM3-4     | TM8-9     | vacuole          |
|        | Solyc06g006110 | 423          | 11 | Na_Ca_ex| TM3-4     | TM8-9     | vacuole          |
|        | Solyc07g056110 | 452          | 10 | Na_Ca_ex| TM2-3     | TM7-8     | vacuole          |
|        | Solyc09g005260 | 456          | 10 | Na_Ca_ex| TM2-3     | TM7-8     | vacuole          |
|        | Solyc12g011070a| 342          | 9  | Na_Ca_ex| TM1-2     | TM6-7     | vacuole          |
|        | Solyc12g011070b| 175          | 5  | Na_Ca_ex| TM1-2     |           | vacuole          |
|        | Solyc12g011070c| 175          | 5  | Na_Ca_ex| TM1-2     |           | vacuole          |
|        | Solyc12g011070d| 175          | 5  | Na_Ca_ex| TM1-2     |           | vacuole          |
|        | Solyc12g055750 | 434          | 11 | Na_Ca_ex| TM3-4     | TM8-9     | vacuole          |
| NCL    | Solyc02g077270 | 716          | 10 | Na_Ca_ex| TM7-8     |           |                  |
|        | Solyc03g006260 | 694          | 10 | Na_Ca_ex| TM7-8     |           |                  |
|        | Solyc07g062700 | 589          | 11 | Na_Ca_ex| TM8-9     |           |                  |
|        | Solyc12g014110 | 578          | 10 | Na_Ca_ex| TM7-8     |           |                  |
| CCX    | Solyc01g098800 | 555          | 11 | Na_Ca_ex| TM2-3     | TM9-10    | plasma membrane  |
|        | Solyc02g069710 | 646          | 11 | Na_Ca_ex| TM2-3     | TM9-10    | vacuole          |
|        | Solyc07g006370 | 567          | 13 | Na_Ca_ex| TM3-4     | TM10-11   | vacuole          |
|        | Solyc07g042000 | 623          | 13 | Na_Ca_ex| TM3-4     | TM10-11   | vacuole          |
|        | Solyc09g072690 | 568          | 12 | Na_Ca_ex| TM2-3     | TM9-10    | vacuole          |
| MHX    | Solyc06g009130 | 540          | 11 | Na_Ca_ex| TM2-3     | TM7-8     | vacuole          |

a Sol Genomics Network database. b Amino acids. c Number of transmembrane domains and α-repeat regions (TMHMM). d EMBL-EBI. e ProtComp. f According to Taneja et al. [9].

Figure 2. Domain architecture of tomato CaCA (SiCaCA) proteins. Gray rectangles and white ovals represent Na_Ca_ex and EF-hand domains, respectively.
2.3. Protein Structure and Localization

The amino acid sequences of the tomato α1-repeat and α2-repeat regions were compared with those of rice and Arabidopsis, with reference to Kamiya et al. [3] and Taneja et al. [9]. The regions called α1-repeat and α2-repeat conserved in rice and Arabidopsis are motifs characteristic of CaCA, and they are considered to be cation-binding regions [35–38]. The α1-repeat and α2-repeat sequences of SICAX, SICCX, and SIMHX were very common among species (Figures S4–S6). According to previous reports, however, NCL seems to contain the α2-repeat, but not the α1-repeat [8,9,25]. In the present study, the sequences of the region possibly corresponding to the α1-repeat of SlNCL were compared among species, and common sequences were found, in particular, in OsEFCAX1/NCL1, OsEFCAX2/NCL2, Solyc07g062700, Solyc12g01411, and AtNCL1/EFCAX1, which clustered together in the phylogenetic tree (Figure 1 and Figure S7). The function of this region remains to be established.

An α1-repeat was found in the region containing TM1-2, TM2-3, or TM3-4, and an α2-repeat was found in the region containing TM6-7, TM7-8, TM8-9, or TM9-10 (Table 1). When the number of TMs was 10, an α1-repeat was found in the region of TM2-3 and an α2-repeat was found in the region of TM7-8, which is in agreement with previous reports [8,9,25]. According to TMHMM, Solyc01g098800 (SICCX) and Solyc02g069710 (SICCX) contain an α1-repeat in the region containing only TM3, whereas, according to Taneja et al. [9], an α1-repeat is present in the region containing TM2-3. According to TMHMM, Solyc06g009130 (SIMHX) contains an α2-repeat in the region containing TM9-10, whereas, according to Taneja et al. [9], an α2-repeat is present in the region containing TM7-8. In other CaCAs with two α-repeats, these had TM helices facing each side of the membrane. In
summary, the α-repeat regions of Solyc01g098800, Solyc02g069710, and Solyc06g009130 were determined with reference to Taneja et al. [9].

Identifying intracellular localization is important to understand how CaCA regulates the cation concentration in cells. Therefore, the intracellular localization of CaCAs was predicted by ProtComp (Table 1). It was predicted that all SICAXs localized to the vacuolar membrane, while the localization of SINCL could not be predicted. Among SICCXs, Solyc01g098800 was predicted to be localized to the plasma membrane, while other SICCXs were predicted to be localized to the vacuolar membrane. It has been reported that CaCAs localize to vacuolar and plasma membranes [7,39]; the localization prediction here is consistent with those reported previously. In addition, AtCAX1, AtCAX2, AtMHX, and OsCCX2 have been experimentally confirmed to be localized to the vacuolar membrane [2,5,40,41], and tomato CaCAs contained in the same cluster as AtCAX1 and AtMHX in the phylogenetic tree were also predicted to be localized to the vacuolar membrane in the present study. On the other hand, AtCCX5, which is classified as being in the same CCX group as Solyc01g098800, has been experimentally confirmed to be localized to the plasma membrane [42].

2.4. Expression Profile of SICaCA Genes in Vegetative Organs, Flowers, and Fruit throughout Development

Although the expression profile can be referred to in the web database, the expression differs depending on the variety, cultivation condition, and measuring method. In fact, the expression pattern was similar, but sometimes different, from that of the web database. Therefore, expression analysis was carried out during this study. A heat map of the expression levels of 16 genes in various organs and some developmental stages of fruit is shown in Figure 4A, in which expression levels can be appropriately compared between genes. There were two genes (Solyc12g011070 and Solyc09g072690) for which expression was not detected at all or was lower than the median if detected. However, the mRNA levels of other genes were above the median in one or more of the organs and fruit developmental stages. Therefore, in order to clarify the characteristic expression patterns for each gene, expression levels in organs and fruit developmental stages were normalized, and hierarchical clustering was performed based on the expression patterns (Figure 4B). As a result, expression patterns were divided into two clusters: high expression in flowers and high expression in fruit, stems, or roots with low expression in leaves. The former was further divided into two clusters: high expression specifically in flowers (Pattern1) and relatively high expression in other organs, as well as flowers (Pattern2). The latter was also divided into two clusters: high expression in fruit, roots, and stems (Pattern3) and low expression in fruit, with the highest expression in stems (Pattern4). There were only two genes with a higher than average expression in leaves, suggesting that CaCAs are more important in sink organs in constitutive expression. Since SICAXs, SINCLs, and SICCXs were classified into three, three, and four different patterns, respectively, each gene belonging to the same family may play different roles in various organs and developmental stages of fruit.

For Pattern3 genes, in which the expression is high in fruit, expression was commonly high 10 days after flowering (DAF) and during breaker and ripe stages. The stages 10 DAF, 20 to 30 DAF, and breaker to ripe correspond to cell division, cell expansion, and ripening stages, respectively [43], and Pattern3 genes may play a role in the cell division and ripening stages. TaCAX from bread wheat shows a high expression in grains and seeds [9], and tomato CAX, SICAX, in Pattern3, Solyc03g123790, and Solyc12g055750, also showed a high expression during the fruit ripening stage, suggesting a similar role for those genes. CAX is mainly present in the vacuolar membrane and is important in 

H+/Ca2+ exchange, according to previous reports [44,45]. As for the relationship between fruit ripening and Ca2+, Ca2+-mediated cross-linking of pectin accompanied by demethylation by pectin methyltransferase has been recorded [46], and the transport of Ca2+ into vacuoles by SICAX might affect this ripening-related phenomenon. In fact, it has been reported that overexpression of the Arabidopsis CAX gene in the tomato reduces the Ca2+ concentration in the fruit apoplastic fraction, which contains cell wall pectin [47].

Pattern3 also included Solyc01g098800, belonging to SICCX. CCX localizes to the vacuolar or plasma membrane [4,42,48], and, in Arabidopsis, AtCCX1 and AtCCX3 play roles in Na+/K+ exchange and H+/K+ and Na+ exchange, respectively [4,48]. AtCCX5 plays a role in K+ uptake [42].
Solyc01g098800 was closest to AtCCX5 in the phylogenetic tree (Figure 1) and was predicted to be localized to the plasma membrane like AtCCX5, suggesting its role in K⁺ uptake. Tomato fruits are rich in K⁺. While K⁺ intake is useful for preventing hypertension [49], it is necessary to suppress intake in renal dysfunction [50]. Therefore, control of the K⁺ concentration related to SlCCX is an important issue.

We showed the main members in each CaCA subfamily and members with characteristic fruit expression patterns for the first time by genome-wide studies of the CaCA family in fruit crops; thus, various analyses using this information became possible. We focused on the expression of these genes in response to sugar and auxin, as well as in fruit rich in metabolites, since there is a lack of information on the regulation of expression other than the stress response. Because the highly expressed genes in the three subfamilies were Solyc09g005260 in SICAX, Solyc07g062700 in SINCL, and Solyc07g006370 in SICCX (Figure 4A), these genes were hypothesized to play a central role in each family. Sugar is known to affect the metabolism as an important signal in fruit [51,52]. Therefore, the induction of expression by sugar was examined for these three genes. An enhanced expression of Solyc09g005260 (SlCAX) was induced by fructose, glucose, and mannitol (Figure 5). In contrast, although Solyc07g062700 (SlNCL) and Solyc07g006370 (SlCCX) expression was remarkably upregulated by fructose treatment, it was slightly downregulated or unchanged by glucose and mannitol. If gene expression is affected by mannitol, as well as fructose and glucose, as with Solyc09g005260, it is thought to be a response to osmotic stress; however, the expression of Solyc07g062700 and Solyc07g006370 appeared to occur in response to the sugar signal instead of osmotic stress. Interestingly, Solyc07g062700, an SINCL, belonged to Pattern3 (Figure 4B), which suggests its importance in fruit. Its expression in ripe fruit was the highest of all genes, as shown in Figure 4A. Its expression level increased during fruit development, a pattern consistent with that of sugar accumulation in the tomato fruit [53]. Because fructose and glucose rely on different sensors of hexokinase 1 (HXK1) and fructose-insensitive 1 (FINS1), respectively [54], the response of the gene may be different for fructose and glucose. In fact, glucose is more effective in the enhancement of glutamate synthase gene expression, in contrast to Solyc07g005260 [51]. The role of Solyc07g062700 in fruit metabolism requires further investigation.

Regarding the relationship between auxin and CaCA, Li et al. [12] reported that auxin suppresses NCL expression in Arabidopsis. In this study, SINCL (Solyc07g062700) expression was not affected by auxin (1-naphthylacetic acid, NAA), whereas SICAX (Solyc09g005260) expression was suppressed (Figure 5). Using Arabidopsis mutants, Cho et al. [11] reported that CAX promotes apoplast pH reduction. Since apoplast pH reduction may affect auxin transport and cell elongation, which are related to fruit development, the regulation of SICAX expression by auxin in this study could be interesting. In fact, auxin affects cell division, cell size, and the expression of genes related to them in tomato fruit [55].

IL8-3 is a near-isogenic line in which a chromosomal fragment of tomato’s wild relative is introduced into chromosome 8 [56]. The fruit of IL8-3 shows a useful phenotype with high sugar and amino acid concentrations, which is derived from metabolism in the fruit at 20 DAF [51]. Therefore, we compared the expression of the Pattern3 genes, which were predicted herein to play a role in the fruit, between IL8-3 and the parental cultivar M82 at 20 DAF using the published omics data [51]. The mRNA levels of the three genes analyzed were higher in IL8-3 (Figure 5), suggesting a relationship between the CaCA genes and the active metabolism of IL8-3 fruit.

MHX is thought to be highly expressed in stems and leaves as it transports Mg²⁺, which is important for chlorophyll synthesis [9]. In the organs analyzed in the present study, the expression of SIMHX was indeed lowest in roots and ripe fruit, which did not contain chlorophyll (Figure 4B), and lower than the median (Figure 4A). All other organs, including flowers with green sepals, contained chlorophyll, supporting the previous report. However, since AtMHX in Arabidopsis transports Zn²⁺ and Fe²⁺, as well as Mg²⁺ [5], it is necessary to consider whether it has roles other than in chlorophyll synthesis.
Figure 4. Expression profile of SICaCA superfamily genes in various organs containing fruit from 10 days after flowering (DAF) to ripeness.YL and ML represent young and mature leaves, respectively. (A) In order to accurately compare the expression levels between genes, the copy number corresponding to the amount of each mRNA was determined using the standard curve. The expression level was expressed as log2, and the heat map was prepared by subtracting the expression level of each gene from the median; red represents the highest expression levels, white indicates median expression, and blue shows the lowest expression levels. Gray represents undetectable expression. Each log2 expression value was based on the mean of three biological replicates. (B) To compare the expression levels among organs for each gene, expression levels were normalized in each gene. Color bar denotes Z-score: red represents the highest expression levels, white indicates mean expression, and blue shows the lowest expression levels in each gene. Gray represents undetectable expression. Hierarchical clustering was performed using Ward’s method. Each expression value was based on the mean of three biological replicates.
3. Materials and Methods

3.1. Plant Materials

*Solanum lycopersicum* cv. M82 plants were grown in a greenhouse, as described previously [51], and their roots, stems, young leaves, mature leaves, flowers, and fruit were used for analysis of the sequence of *SlCaCA* cDNA and its expression (Figure 4). Fruit was sampled at 10 DAF, 20 DAF, 30 DAF, the breaker stage, and the ripe stage, respectively. *Solanum lycopersicum* cv. Ailsa Craig plants were used to investigate the effects of sugar and auxin on gene expression (Figure 5). For sugar treatment, cotyledons 5 days after germination were incubated with 300 mM glucose, fructose, or mannitol and sampled as described previously [51]. For auxin treatment, tomato plants were grown in a phytotron, and fruit was dipped in 0.1 mM or 1 mM NAA every day from 4 DAF, as described previously, and used at 15 DAF for expression analysis [55].

3.2. RNA Extraction and cDNA Synthesis

RNA was extracted using a Cica Geneus RNA Prep Kit (for Plant, Kanto Chemical). ReverTra Ace® qPCR RT Master Mix (TOYOBO) was used for genomic DNA removal and the reverse transcription reaction to prepare cDNA.

![Figure 5](image)

**Figure 5.** In the top two rows, the effect of sugar and auxin (NAA) on gene expression is shown for *Solyc09g005260* (*SlCAX*), *Solyc07g062700* (*SlNCL*), and *Solyc07g006370* (*SlCCX*). Relative expression levels are shown, with error bars indicating the standard error of the mean of three biological replicates. Values with different letters are significantly different at $P < 0.05$ according to the Tukey–Kramer test. In the bottom rows, the expression of *Solyc03g123790* (*SlCAX*), *Solyc12g055750* (*SlCAX*),
and Solyc07g062700 (SINCL) is shown for fruits of parental tomato cultivar M82 and its near-isogenic line IL8-3 containing a chromosome segment from tomato’s wild relative. Relative expression levels from the microarray data [51] are shown, with error bars indicating the standard error of the mean of three biological replicates. ** P < 0.01 between M82 and IL8-3, as revealed by a t-test.

### 3.3. SlCaCA Sequence Analysis

A BLAST search was performed in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the Sol Genomics Network (http://solgenomics.net/) to search for putative SlCaCA proteins using Arabidopsis thaliana CaCA (AtCaCA) amino acid sequences identified by Emery et al. [26] and Pittman and Hirschi [8]. Using the sequences of Arabidopsis CaCAs, homologous sequences having a query cover of approximately 100 and the highest identity were selected. Furthermore, the BLAST search was performed again using the obtained sequence as a query to confirm the presence or absence of similar sequences. The presence or absence of similar sequences was also checked, taking into account the possible presence of tomato CaCA that does not correspond to each member of Arabidopsis CaCA. Specific primers were designed for each putative SlCaCA gene based on the sequence of S. lycopersicum cv. Heinz 1706 to amplify the cDNA by PCR with Q5 High-Fidelity DNA Polymerase (New England Biolabs, Japan). Primer sequences and annealing temperatures are shown in Table S1. The PCR product was electrophoresed on 1% agarose gel and extracted using NucleoSpin® Gel and PCR Clean-up (Mahala Nagel). Extracted DNA and primers were submitted to Macrogen, Japan, for general sequence analysis.

Multiple sequence alignments using amino acid sequences of SlCaCA and A. thaliana and Oryza sativa CaCA identified by Emery et al. [26] and Pittman and Hirschi [8] were made using Clustal W and an unrooted neighbor-joining tree was constructed based on a full-length protein sequence with 1000 bootstrap replications by MEGA7 software [57]. A domain search was performed using TMHMM (ver. 2.0, http://www.cbs.dtu.dk/services/TMHMM/) and EMBL-EBI InterPro (https://www.ebi.nih.gov) on SlCaCA amino acid sequences to define CaCA proteins based on important conserved domains. Subcellular localization was examined using ProtComp (ver. 9.0, http://www.softberry.com/berry.phtml?Topic=protcomppl&group=programs&subgroup=proloc). The protein and genomic structures of Figures 2 and 3 were constructed with PowerPoint (Microsoft) based on length after analyzing the sequence with the software described above and Genetyx (ver. 10; https://www.genetyx.co.jp/).

### 3.4. Expression Analysis

For real-time PCR, KOD SYBR qPCR Mix (TOYobo) was used with primers at the annealing temperatures described in Table S2. PCR was performed using a PTC-2000 DNA Engine Cycler CFX Connect Real-Time Detection System (Bio Rad), according to the following protocol: 98 °C for 2 min; 39 cycles of 98 °C for 15 s, X °C for 30 s, and 68 °C for 30 s, plate read; melting curve from 65 °C to 95 °C, read every 0.2 °C, hold for 10 s. A melting curve was used to confirm the presence of single products.

To accurately compare the expression levels between genes in Figure 4A, the copy number corresponding to the amount of each mRNA was determined using a standard curve obtained by employing the copy number calculated from the cDNA fragment of each SlCaCA and SlUbiquitin of known concentrations. The cDNA fragments were extracted from the agarose gel mentioned in Section 3.3, and the standard curves were prepared based on the calculated copy numbers and the results of real-time PCR using a series of cDNA with a known copy number in each gene. The amount of mRNA was shown as the ratio of the copy number of the target gene to that of SlUbiquitin. To compare the expression levels among organs for each gene in Figure 4B, expression levels were normalized in each gene.

For expression analysis of the sugar and auxin response in Figure 5, calibration using the standard curves was not performed. Threshold cycles were determined, and the constitutively expressed ubiquitin gene was used as a reference for the normalization of gene expression. The levels of DNA microarray signals [51] were used to show the relative abundance of transcripts of Pattern3.
genes (Solyc03g123790, Solyc12g055750, and Solyc07g062700) in M82 and its near-isogenic line IL8-3 in Figure 5. The microarray was designed for 43,803 tomato probe sets based on the tomato whole-genome sequences. The data for Solyc01g098800, another gene in Pattern3, was not shown in Figure 5 because the gene had been removed in a false discovery rate selection. The data were analyzed with the Tukey–Kramer test or Student’s t test using the Excel Toukei ver. 3.0 (Social Survey Research Information Co., https://www.ssri.com/).

4. Conclusions

The structure and expression of CaCA superfamily genes were examined in the tomato, as a representative of dicotyledonous plants and fruit crops. Sixteen CaCA candidate genes were found, identified as CaCA, and classified into four subfamilies, and we identified their general and unique properties. The expression patterns of CaCAs were classified into four groups. The main members in each subfamily and the members with characteristic fruit expression patterns were revealed for further studies to elucidate the roles of CaCA family genes. Similar bioinformatics reports on other tomato transporters have had a significant impact on the many citations [58,59], and by integrating these types of studies and functional genomics, we may reveal the whole picture of fruit metabolism in the future.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1: Figure S1: Transmembrane domains of SlCAX searched using TMHMM; Figure S2: Transmembrane domains of SINCL searched using TMHMM; Figure S3: Transmembrane domains of SlCCX and SIMHX searched using TMHMM; Figure S4: Multiple alignments of conserved $\alpha_1$-repeat and $\alpha_2$-repeat regions in CAX proteins; Figure S5: Multiple alignments of conserved $\alpha_1$-repeat and $\alpha_2$-repeat regions in CCX proteins; Figure S6: Multiple alignments of conserved $\alpha_1$-repeat and $\alpha_2$-repeat regions in MHX proteins; Figure S7: Multiple alignments of conserved $\alpha_1$-repeat and $\alpha_2$-repeat regions in NCL proteins; Table S1: List of primers used for sequence analysis of SlCaCA; Table S2: List of primers used for expression analysis of SlCaCA.

Author Contributions: Conceptualization, K.A., K.K., and Y.K.; methodology, K.A., M.N., and Y.K.; investigation, K.A. and T.S.; formal analysis, K.A. and T.S.; resources, M.N.; data curation, K.A.; writing—original draft preparation, K.A.; writing—review and editing, K.K. and Y.K.; visualization, K.A.; supervision, K.K. and Y.K.; project administration, Y.K.; funding acquisition, Y.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by Grants-in-Aid for Scientific Research [16H02534].

Acknowledgments: The authors thank the National BioResource Project tomato (NBRP tomato) for information.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Hirschi, K.D.; Zhen, R.G.; Cunningham, K.W.; Rea, P.A.; Fink, G.R. CAX1, an H+/Ca2+ antiporter from Arabidopsis. Proc. Natl. Acad. Sci. USA 1996, 93, 8782–8786.
2. Hirschi, K.D.; Korenkov, V.D.; Wilganowski, N.L.; Wagner, G.J. Expression of Arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiol. 2000, 124, 125–133.
3. Kamiya, T.; Akahori, T.; Maeshima, M. Expression profile of the genes for rice cation/H+ exchanger family and functional analysis in yeast. Plant Cell Physiol. 2005, 46, 1735–1740.
4. Morris, J.; Tian, H.; Park, S.; Sreevidya, C.S.; Ward, J.M.; Hirschi, K.D. AtCCX3 is an Arabidopsis endomembrane H+-dependent K+ transporter. Plant Physiol. 2008, 148, 1474–1486.
5. Shaul, O.; Hilgemann, D.W.; de Almeida Engler, J.; Montagu, M.V.; Inzé, D.; Galili, G. Cloning and characterization of a novel Mg2+/H+ exchanger. EMBO J. 1999, 18, 3973–3980.
6. Wang, P.; Li, Z.; Wei, J.; Zhao, Z.; Sun, D.; Cui, S. A Na+/Ca2+ exchanger-like protein (AtNCL) involved in salt stress in Arabidopsis. J. Biol. Chem. 2012, 287, 44062–44070.
7. Singh, A.K.; Kumar, R.; Tripathi, A.K.; Gupta, B.K.; Pareek, A.; Singla-Pareek, S.L. Genome-wide investigation and expression analysis of Sodium/Calcium exchanger gene family in rice and Arabidopsis. Rice 2015, 8, 21.
8. Pittman, J.K.; Hirschi, K.D. Phylogenetic analysis and protein structure modelling identifies distinct Ca²⁺/Cation antiporters and conservation of gene family structure within Arabidopsis and rice species. Rice 2016, 9, 3.

9. Taneya, M.; Tyagi, S.; Sharma, S.; Upadhyay, S. Ca²⁺/Cation antiporters (CaCA): Identification, characterization and expression profiling in bread wheat (Triticum aestivum L.). Front. Plant Sci. 2016, 7, 1775.

10. Yamada, N.; Therewaitya, C.; Chauw, S.; Kirdmanee, C.; Takabe, T. Expression and functional analysis of putative vacuolar Ca²⁺-transporters (CAXs and ACAs) in roots of salt tolerant and sensitive rice cultivars. Protoplasma 2014, 251, 1067–1075.

11. Cho, D.; Villieres, F.; Kroniewicz, L.; Lee, S.; Seo, Y.J.; Hirschi, K.D.; Leonardt, N.; Kwak, J.M. Vacuolar CAX1 and CAX3 influence auxin transport in guard cells via regulation of apoplastic pH. Plant Physiol. 2012, 160, 1293–1302.

12. Li, P.; Zhang, G.; Gonzales, N.; Guo, Y.; Hu, H.; Park, S.; Zhao, J. Ca²⁺-regulated and diurnal rhythm-regulated Na⁺/Ca²⁺ exchanger AtNCL affects flowering time and auxin signalling in Arabidopsis. Plant Cell Environ. 2016, 39, 377–392.

13. Hocking, B.; Conn, S.J.; Manohar, M.; Xu, B.; Athman, A.; Stancombe, M.A.; Webb, A.R.; Hirschi, K.D.; Gilliham, M. Heterodimerization of Arabidopsis calcium/proton exchangers contributes to regulation of guard cell dynamics and plant defense responses. J. Exp. Bot. 2017, 68, 4171–4183.

14. Morris, J.; Hawthorne, K.; Hotze, T.; Abrams, S.; Hirschi, K.D. Nutritional impact of elevated calcium transport activity in carrots. Proc. Natl. Acad. Sci. USA 2008, 105, 431–4315.

15. Navarro-León, E.; Ruiz, J.; Graham, N.; Blasco, B. Physiological profile of CAX1a TILLING mutants of Brassica rapa exposed to different calcium doses. Plant Sci. 2018, 272, 164–172.

16. Park, S.; Elless, M.P.; Park, J.; Jenkins, A.; Lim, W.; Edgar, C.I.V.; Hirschi, K.D. Sensory analysis of calcium-biofortified lettuce. Plant Biotechnol. J. 2009, 7, 106–117.

17. Navarro-León, E.; Ruiz, J.M.; Albacete, A.; Blasco, B. Effect of CAX1a TILLING mutations and calcium concentration on some primary metabolism processes in Brassica rapa plants. J. Plant Physiol. 2019, 237, 51–60.

18. Qiao, K.; Wang, F.; Liang, S.; Hu, Z.; Chai, T. Heterologous expression of TuCAX1a and TuCAX1b enhances Ca²⁺ and Zr²⁺ translocation in Arabidopsis. Plant Cell Rep. 2019, 38, 597–607.

19. Ahmadi, H.; Corso, M.; Weber, M.; Verbruggen, N.; Clemens, S. CAX1 suppresses Cd-induced generation of reactive oxygen species in Arabidopsis halleri. Plant Cell Environ. 2018, 41, 2435–2448.

20. Edmond, C.; Shigaki, T.; Ewert, S.; Nelson, M.D.; Connon, J.M.; Chalova, V.; Noordally, Z.; Pittman, J.K. Comparative analysis of CAX2-like cation transporters indicates functional and regulatory diversity. Biochem. J. 2009, 418, 145–154.

21. De Freitas, S.T.; Jiang, C.Z.; Mitcham, E.J. Mechanisms involved in calcium deficiency development in tomato fruit in response to gibberellins. J. Plant Growth Regul. 2012, 31, 221–234.

22. Ikeda, H.; Shibuya, T.; Nishiyama, M.; Nakata, Y.; Kanayama, Y. Physiological mechanisms accounting for the lower incidence of blossom-end rot in tomato introgression line IL8-3 fruit. Hort. J. 2017, 86, 327–333.

23. Kanayama, Y.; Kochetov, A.V. Abiotic Stress Biology in Horticultural Plants; Springer: New York, NY, USA, 2015.

24. Saand, M.; Xu, Y.P.; Li, W.; Wang, J.P.; Cai, X.Z. Cyclic nucleotide gated channel gene family in tomato: Genome-wide identification and functional analyses in disease resistance. Front. Plant Sci. 2015, 6, 303.

25. Hirakawa, H.; Shirasawa, K.; Ohyama, A.; Fukuoka, H.; Aoki, K.; Rothan, C.; Sato, S.; Isobe, S.; Tabata, S. Genome-wide SNP genotyping to infer the effects on gene functions in tomato. DNA Res. 2013, 20, 221–233.

26. Emery, L.; Whelan, S.; Hirschi, K.D.; Pittman, J.K. Protein phylogenetic analysis of Ca²⁺/cation antiporters and insights into their evolution in plants. Front. Plant Sci. 2012, 3, 1.

27. Shigaki, T.; Barkla, B.J.; Miranda-Vergara, M.C.; Zhao, J.; Pantoja, O.; Hirschi, K.D. Identification of a crucial histidine involved in metal transport activity in the Arabidopsis cation/H⁺ exchanger CAX1. J. Biol. Chem. 2005, 280, 30136–30142.

28. Shigaki, T.; Pittman, J.K.; Hirschi, K.D. Manganese specificity determinants in the Arabidopsis metal/H⁺ antiporter CAX2. J. Biol. Chem. 2003, 278, 6610–6617.

29. Conn, S.; Gilliham, M.; Athman, A.; Schreiber, A.W.; Baumann, U.; Moller, I.; Cheng, N.H.; Stancombe, M.; Hirschi, K.D.; Webb, A.; et al. Cell-specific vacuolar calcium storage mediated by CAX1 regulates
apoplastic calcium concentration, gas exchange, and plant productivity in *Arabidopsis*. *Plant Cell* 2011, 23, 240–257.
30. Mei, H.; Cheng, N.H.; Zhao, J. Root development under metal stress in *Arabidopsis thaliana* requires the H+/cation antiporter CAX4. *New Phytol.* 2009, 183, 95–105.
31. Shang, X.; Cao, Y.; Ma, L. Alternative splicing in plant genes: A means of regulating the environmental fitness of plants. *Int. J. Mol. Sci.* 2017, 18, 432.
32. Jeffares, D.C.; Penkett, C.J.; Bähler, J. Rapidly regulated genes are intron poor. *Trends Genet.* 2008, 24, 375–378.
33. Yan, H.; Zhang, W.; Lin, Y.; Dong, Q.; Peng, X.; Jiang, H.; Zhu, S.; Cheng, B. Different evolutionary patterns among intronless genes in maize genome. *Biochem. Biophys. Res. Commun.* 2014, 449, 146–150.
34. Jain, M.; Khurana, P.; Tyagi, A.K.; Khurana, J.P. Genome-wide analysis of intronless genes in rice and *Arabidopsis*. *Funct. Integr. Genom.* 2008, 8, 69–78.
35. Iwamoto, T.; Nakamura, T.; Pan, Y.; Uehara, A.; Imanaga, I.; Shigekawa, M. Unique topology of the internal repeats in the cardiac Na+/Ca2+ exchanger. *FEBS Lett.* 1999, 446, 264–268.
36. Nishizawa, T.; Kita, S.; Maturana, A.D.D.; Furuya, N.; Hirata, K.; Kasuya, G.; Ogawara, S.; Dohmae, N.; Iwamoto, T.; Ishitani, R.; et al. Structural basis for the counter-transport mechanism of a H+/Ca2+ exchanger. *Science* 2013, 341, 168–172.
37. Philipson, K.D.; Nicoll, D.A. Sodium-calcium exchange: A molecular perspective. *Ann. Rev. Physiol.* 2000, 62, 111–133.
38. Waight, A.B.; Pedersen, B.; Schlessinger, A.; Bonomi, M.; Chau, B.H.; Roe-Zurz, Z.; Risenmay, A.J.; Sali, A.; Stroud, R.M. Structural basis for alternating access of a eukaryotic calcium/proton exchanger. *Nature* 2013, 499, 107–110.
39. Bickerton, P.D.; Pittman, J.K. Role of cation/proton exchangers in abiotic stress signaling and stress tolerance in plants. In *Elucidation of Abiotic Stress Signaling in Plants: Functional Genomics Perspectives*; Springer: New York, NY, USA, 2015; Volume 1, pp. 95–117.
40. Kamiya, T.; Akahori, T.; Ashikari, M.; Maeshima, M. Expression of the vacuolar Ca2+/H+ exchanger, OsCAX1a, in rice: Cell and age specificity of expression, and enhancement by Ca2+. *Plant Cell Physiol.* 2006, 47, 96–106.
41. Yadav, A.K.; Shankar, A.; Jha, S.K.; Kanwar, P.; Pandey, A.; Pandey, G.K. A rice tonoplastic calcium exchanger, OsCCX2 mediates Ca2+/cation transport in yeast. *FEBS Lett.* 2015, 5, 17117.
42. Zhang, X.; Zhang, M.; Takano, T.; Liu, S. Characterization of an AtCCX5 gene from *Arabidopsis thaliana* that involves in high-affinity K+ uptake and Na+ transport in yeast. *Biochem. Biophys. Res. Commun.* 2011, 414, 96–101.
43. Nishio, S.; Shigaki, T.; Hirshi, K.D. Plant cation/H+ exchangers (CAXs): Biological functions and genetic manipulations. *Plant Biol.* 2011, 13, 561–569.
44. Pittman, J.K. Vacuolar Ca2+ uptake. *Cell Calcium* 2011, 50, 139–146.
45. Hyodo, H.; Terao, A.; Furukawa, J.; Sakamoto, N.; Yurimoto, H.; Satoh, S.; Iwai, H. Tissue specific localization of pectin-Ca2+ cross-linkages and pectin methyl-esterification during fruit ripening in tomato (*Solanum lycopersicum*). *PLoS ONE* 2013, 8, e78949.
46. De Freitas, S.T.; Padda, M.; Wu, Q.; Park, S.; Mitcham, E.J. Dynamic alternations in cellular and molecular components during blossom-end rot development in tomatoes expressing sCAX1, a constitutively active Ca2+/H+ antiporter from *Arabidopsis*. *Plant Physiol.* 2011, 156, 844–855.
47. Chen, Z.; Wu, Y.; Di, L.; Wang, G.; Shen, Y. The AtCCX1 transporter mediates salinity tolerance in both *Arabidopsis* and yeast. *Plant Cell Tissue Organ Cult.* 2012, 109, 91–99.
48. Perez, V.; Chang, E.T. Sodium-to-potassium ratio and blood pressure, hypertension, and related factors. *Adv. Nutr.* 2014, 5, 712–741.
49. Picq, C.; Asplanato, M.; Bernillon, N.; Fabre, C.; Roubeix, M.; Ricort, J.-M. Effects of water soaking and/or sodium polystyrene sulfonate addition on potassium content of foods. *Int. J. Food Sci. Nutr.* 2014, 65, 673–677.
51. Ikeda, H.; Shibuya, T.; Imanishi, S.; Aso, H.; Nishiyama, M.; Kanayama, Y. Dynamic metabolic regulation by a chromosome segment from a wild relative during fruit development in a tomato introgression line, IL8-3. *Plant Cell Physiol.* 2016, 57, 1257–1270.

52. Sagor, G.H.M.; Berberich, T.; Tanaka, S.; Nishiyama, M.; Kanayama, Y.; Kojima, S.; Muramoto, K.; Kusano, T. A novel strategy to produce sweeter tomato fruits with high sugar contents by fruit-specific expression of a single bZIP transcription factor gene. *Plant Biotechnol. J.* 2016, 14, 1116–1126.

53. Beckles, D.M.; Hong, N.; Stamova, L.; Luengwilai, K. Biochemical factors contributing to tomato fruit sugar content: A review. *Fruits* 2012, 67, 49–64.

54. Cho, Y.H.; Yoo, S.D. Signaling role of fructose mediated by FINS1/FBP in *Arabidopsis thaliana*. *PLoS Genet.* 2011, 7, e1001263.

55. Nariyama, H.; Sugiyama, Y.; Shibuya, T.; Hayashi, K.; Kanayama, Y. HPY2 gene expression analysis and the role of auxin in early fruit development in tomato. *Acta Hortic.* 2018, 1206, 247–252.

56. Ikeda, H.; Hiraga, M.; Shirasawa, K.; Nishiyama, M.; Kanahama, K.; Kanayama, Y. Analysis of a tomato introgression line, IL8-3, with increased Brix content. *Sci. Hortic.* 2013, 153, 103–108.

57. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 2016, 33, 1870–1874.

58. Ofori, P.A.; Mizuno, A.; Suzuki, M.; Martinoia, E.; Reuscher, S.; Aoki, K.; Shibata, D.; Otagaki, S.; Matsumoto, S.; Shiratake, K. Genome-wide analysis of ATP binding cassette (ABC) transporters in tomato. *PLoS ONE* 2018, 13, e0200854.

59. Reuscher, S.; Akiyama, M.; Yasuda, T.; Makino, H.; Aoki, K.; Shibata, D.; Shiratake, K. The sugar transporter inventory of tomato: Genome-wide identification and expression analysis. *Plant Cell Physiol.* 2014, 55, 1123–1141.

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).