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Review

Ubiquitin related enzymes and plant-specific ubiquitin ligase ATL family in tomato plants

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Abstract Ubiquitination is one of the fundamental post-translational modifications of proteins with ubiquitin, a conserved 76-amino acid protein present in eukaryotes, which is catalyzed by ubiquitin ligase. Compared with humans, the number of ubiquitin ligase genes is nearly double in plant species such as Arabidopsis and rice, suggesting that this enzyme plays critical roles in many aspects of plant growth, including development and abiotic and biotic environmental stress responses. In addition to its fundamental activities in eukaryotic cells, ubiquitin signaling mediates plant specific cellular functions, including phytohormone response, seed and fruit development, and biotic and abiotic stress responses. The ATL family is a RING-H2 type ubiquitin ligase widely conserved in plant species. We previously showed that the plant specific ubiquitin ligase ATL31 regulates the carbon/nitrogen-nutrient response and pathogen resistance in Arabidopsis, and we identified and characterized the basic biochemical function of an ATL31 homologue in tomato plants (*Solanum lycopersicum* L.). This protein, called SlATL31, may act as a ubiquitin ligase in tomato fruit. The tomato is a major crop plant and a model system for fleshy fruit development. This review provides an overview of the ubiquitin ligases and related enzymes, and highlights the ubiquitin ligase ATL family in tomato plants.

Key words: ATL family, post-translational modification, tomato, ubiquitin ligase.

Ubiquitination is a reversible post-translational modification of proteins involved in the control of numerous cellular processes. A ubiquitin tag consists of a single or several linked ubiquitin molecules attached to lysine residues of target proteins. Attachment of a single ubiquitin is called monoubiquitination, whereas attachment of a ubiquitin chain is called polyubiquitination. Ubiquitins are attached to target proteins by three sequential enzymatic steps catalyzed by three classes of enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (Hershko and Ciechanover 1998; Iwai et al. 2014). In the first step, E1 catalyzes the attachment of a cysteine residue to the carboxy-terminal glycine residue of ubiquitin, together with the conversion of ATP to ADP. In the second step, the activated ubiquitin molecule is transferred to E2. In the third step, E3 catalyzes the attachment of ubiquitin to a lysine residue on a substrate protein. In polyubiquitination, an incoming ubiquitin molecule is attached to one of the seven lysine residues or the N-terminal methionine of previously attached ubiquitin, indicating that various types of polyubiquitin chains mediate different cellular signals. For example, polyubiquitin chains linked through lysine 48 (K48) are a tag for proteasomal degradation of target proteins, whereas chains linked through lysine 63 (K63) have been reported involved in DNA damage tolerance, immune signaling, endocytic pathways, and synthesis of ribosomal proteins in yeasts and animals (Iwai et al. 2014; Pickart and Fushman 2004).

The *Arabidopsis thaliana* genome has been found to encode two E1, 37 E2, and more than 1,300 E3 proteins (Callis 1995; Vierstra 2009). The various types of E3 enable ubiquitin signaling in various cellular processes, as these enzymes target specific proteins. By regulating multiple phytohormones, ubiquitin signal plays essential roles in multiple aspects of plant development (Callis 2014; Vierstra 2009). Ubiquitin is also involved in adaptations to various environmental stresses, as ubiquitination regulates the turnover of membrane proteins, such as receptors and transporters, which mediate environmental signal transduction and nutrition homeostasis (Callis 2014; Stone 2014; Vierstra 2009). Ubiquitination has also been associated with biotic stress responses; e.g., plant immunity through the degradation of flagellin-sensing 2 (FLS2) receptor, which binds the microbe-associated molecular pattern, flagellin (Callis 2014; Lu et al. 2011; Trujillo and Shirasu 2010). Ubiquitin signaling in tomato plants has shown physiological significance, mediating biotic and abiotic environmental stress responses and plant development. In addition, the plant specific E3 ATL family has been reported to play essential roles in responses to multiple environmental stresses in Arabidopsis, rice and other plant species including tomato plants. Findings related to ubiquitin signal in tomato plants (Table 1) and the function of ATL family in plants as well as identification of tomato ATL proteins are described below (Figure 1–4).

Ubiquitin ligases and related enzymes regulating tomato growth

Although relatively few studies have assessed ubiquitin ligase functions in tomato plants, several ubiquitin ligases were found to play essential roles in tomato plant adaptation to biotic stress (Table 1). SINA3 (SEVEN IN ABSENTIA 3) (Solyc05g050580) can ubiquitinate defense-related transcriptional factor NAC1, resulting in its degradation, thereby playing a negative role in defense signaling (Miao et al. 2016). Programmed cell death is often regarded as a hypersensitive immune response, with AdBiL (Adi3 Binding E3 Ligase) (Solyc01g099790) contributing to host defense responses by regulating the stability of the programmed cell death suppressor, Adi3, in tomato plants (Avila and Devarenne 2013). Another E3, SlBAH1 (BENZOIC ACID HYPERSENSITIVE 1) (Solyc09g075040), was recently identified in tomato plants. Plants in which this gene was silenced showed greater resistance to fungal infections, indicating that this protein has a negative impact on defense responses (Zhou et al. 2016). Two other E3 proteins in tomato, SlHUB1 (HISTONE MONOUBIQUITINATION 1) (Solyc11g013370) and SlHUB2 (HISTONE MONOUBIQUITINATION 2) (Solyc01g006030), were found to contribute to resistance to *Botrytis cinerea* by monoubiquitination of histone B3 (Zhang et al. 2015). In addition to E3, ubiquitin conjugating ligases (E2 proteins) also affected immune signaling. For example, UBC13/Fni3 (Fen-interacting protein 3) (Slyc07g062570) catalyzes the formation of K63-linked ubiquitin chains and positively regulates effector-triggered immunity against *Pseudomonas syringae* pv. *tomato* (Mural et al. 2013).

During adaptations to abiotic stress, tomato plants mostly require the function of E3 proteins. When two different tomato genotypes were subjected to cold stress, an ATL78-Like E3 ShATL78L (Solyc11g005280) was found to improve tolerance to cold, drought and oxidative stresses (Liu et al. 2012; Song et al. 2016). SpRing (Solyc12g006230) was identified as a positive regulator of salt-tolerance (Qi et al. 2016). Furthermore, another type of E3, CULLIN4 (CUL4)–DAMAGED DNA BINDING PROTEIN1 (DDB1)-based ubiquitin ligase, which is associated with the substrate receptor protein DDB1-interacting 1 (DDI1), was found to participate in tolerances to salt, mannitol, and UV-C stress (Miao et al. 2014).

CUL4-DDB1-based E3 has also been shown to regulate fruit quality by ubiquitinating SlGLK2 (GOLDEN 2-LIKE 2), a transcription factor that regulates chloroplast development in tomato fruit (Powell et al. 2012; Tang et al. 2016). Tomato fruit ripening was also found to be affected by a specific E2, SlUBC32 (Solyc07g062570) and SlUBC41 (Solyc10g012240) (Wang et al. 2014).

Plant specific ubiquitin ligase ATL family

The ATL (ARABIDOPSIS TOXICOS EN LEVADURA) family is a plant-specific RING-type E3 (Aguilar-

Table 1. Ubiquitin ligases and related enzymes regulating tomato growth.

Protein name	Type	Locus ID[a]	Function	Substrate	Ref.
Biotic stress					
LeATL6/SlATL31	E3	Solyc03g112340	Positively regulates resistance to oomycetes		Hondo et al. (2007); Takahashi et al. (2010)
SINA3	E3	Solyc05g050580	Negatively regulates defense signaling	NAC1	Miao et al. (2016)
AdBiL	E3	Solyc01g099790	Ubiquitinates tomato PCD[b] suppressor Adi3	Adi3	Avila and Devarenne (2013)
SlBAH1	E3	Solyc09g075040	Negatively regulates resistance to fungi		Zhou et al. (2016)
SlHUB1	E3	Solyc11g013370	Regulation of resistance to *B. cinerea*[c]	Histone H2B	Zhang et al. (2015)
SlHUB2	E3	Solyc01g006030	Regulation of resistance to *B. cinerea*[c]	Histone H2B	Zhang et al. (2015)
Fni3	E2	Solyc07g062570	Positively regulates effector-triggered immunity		Mural et al. (2013)
Abiotic stress					
ShATL78L	E3	Solyc11g005280	Tolerance to cold, drought and oxidative stresses		Liu et al. (2012); Song et al. (2016)
SpRing	E3	Solyc12g006230	Salt-tolerance		Qi et al. (2016)
CUL4-DDB1-DDI1 E3 complex	E3	Solyc02g021470, Solyc02g021650, Solyc09g031610	Tolerance to salt, mamnitol and UV stresses		Miao et al. (2014)
Development					
CUL4-DDB1-DET1 E3 complex	E3	Solyc02g021470, Solyc02g021650, Solyc01g056340	Fruit quality control	SlGLK2	Powell et al. (2012); Tang et al. (2016)
SlUBC32	E2	Solyc07g062570	Fruit ripening control		Wang et al. (2014)
SlUBC41	E2	Solyc10g012240	Fruit ripening control		Wang et al. (2014)

[a] ITAG accession number of *Solanum lycopersicum* genes. [b] Programmed cell death. [c] *Botrytis cinerea*

Hernández et al. 2011; Martínez-García et al. 1996). To date, 91 members of this family have been identified in Arabidopsis among the 1,347 potential E3 ligases encoded in the genome (Guzmán 2014). ATL family proteins contain a transmembrane-like hydrophobic region at the N-terminus, a basic amino acid rich region, a region with highly conserved amino acid sequences (GLD), a RING-H2 type zinc finger domain and a non-conserved C-terminal region (Aguilar-Hernández et al. 2011) (Figure 1). ATL2 was first identified as a protein that, when overexpressed, results in toxicity in yeast (Martínez-García et al. 1996), but was later reported to be involved in plant defense responses to pathogen attack (Salinas-Mondragón et al. 1999). Several other ATL members in Arabidopsis, including ATL1, ATL6, ATL9 and ATL55/RING1, have been reported to function in plant immunity (Berrocal-Lobo et al. 2010; Lin et al. 2008; Salinas-Mondragón et al. 1999; Serrano et al. 2014). ATL54 has been reported to function in secondary cell wall formation (Noda et al. 2013), and ATL14, also designated IDF1, has been found to regulate Fe uptake via the ubiquitination of the Fe transporter IRON-REGULATED TRANSPORTER1 (IRT1) (Shin et al. 2013). In addition to Arabidopsis, ATL isoforms are involved in defense responses in varied plant species; e.g., EL5 and BIRF1 in rice (Liu et al. 2008; Takai et al. 2002), StRFP1 in potato (Ni et al. 2010) and ACRE132 in tobacco (Durrant et al. 2000). EL5 is also involved in root meristem development via cytokinin-mediated nitrogen effect in rice (Koiwai et al. 2007; Mochizuki et al. 2014).

The biochemical properties of ATL proteins were first characterized in rice EL5. Analysis of the ubiquitin ligase activity of EL5 with several E2 enzymes in vitro showed that EL5 displayed ubiquitination activity in reactions with human UBC4/5 as well as with rice UBC5a/5b (Takai et al. 2002). Moreover, mutant EL5 protein, in which Cys153, the third conserved cysteine residue in the RING domain, was replaced by Ser, does not exhibit E3 activity, indicating that EL5 possesses RING-type E3 activity (Takai et al. 2002). Further analysis of

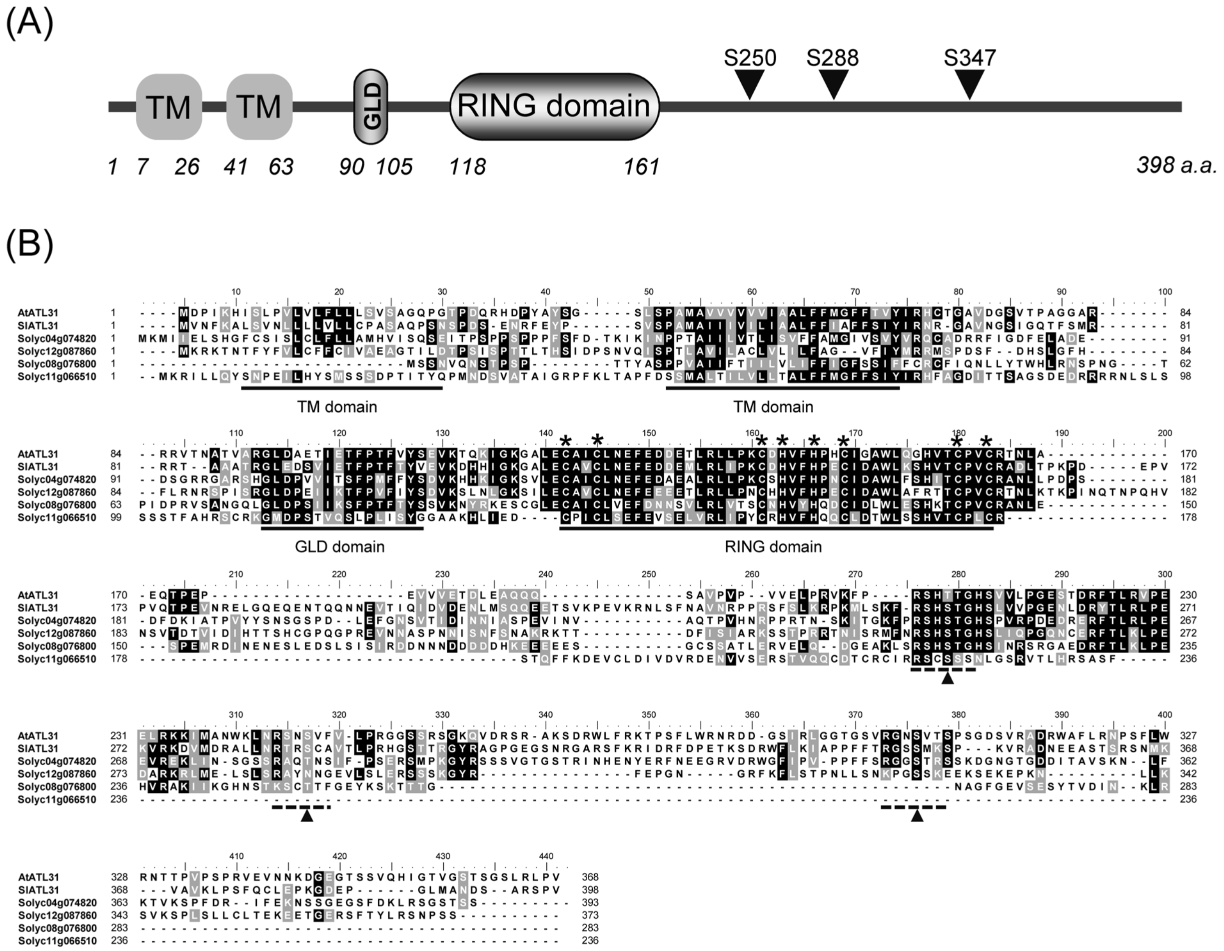

Figure 1. Schematic diagram and alignment of SlATL31 and homologous proteins in tomato. (A) Schematic representation of the tomato SlATL31 protein. TM, transmembrane-like hydrophobic region. GLD, highly conserved motif containing Gly-Leu-Asp residues. RING, RING-H2 type zinc finger domain. Arrowheads indicate putative phosphorylated Ser and Thr residues at the putative 14-3-3 biding sites. (B) Alignment of Arabidopsis ATL31 and tomato ATL31 homologous proteins. Asterisks indicate conserved Cys and His residues in the RING domain. Dashed lines indicate the putative 14-3-3 binding sites.

the biochemical properties and structural features of the EL5 RING domain, using NMR spectroscopy and titration experiments, identified residues in this domain critical for E2 binding (Katoh et al. 2003). As expected from its N-terminal hydrophobic domain, EL5 localizes to the plasma membrane, and membrane localization is required for EL5 to function physiologically in root development of rice (Koiwai et al. 2007). In addition to localizing to plasma membranes, ATL proteins localize to endomembrane compartments; for example, Arabidopsis ATL1 localizes to the *trans*-Golgi network/early endosome and ATL9 localizes to endoplasmic reticulum (Berrocal-Lobo et al. 2010; Serrano et al. 2014).

The C/N regulatory ubiquitin ligase ATL31

We have identified ATL31/CNI1 (CARBON/NITROGEN INSENSITIVE 1) as a novel regulator of Arabidopsis plant responses to the availability of carbon and nitrogen nutrients, called the C/N balance (Sato et al. 2009). Overexpression of ATL31 results in enhanced resistance to disrupted high C/low N stress conditions, whereas its loss of function resulted in a hypersensitive phenotype during post-germinative growth (Sato et al. 2009). Biochemical and genetic analyses confirmed that ATL31 functions as a RING-type ubiquitin ligase at membrane compartments to regulate plant C/N response (Sato et al. 2009). Subsequent studies revealed that ATL31 directly interact with 14-3-3 protein at C-terminal region and negatively regulate 14-3-3 protein stability via ubiquitination (Sato et al. 2011; Yasuda et al. 2014). The 14-3-3 protein is a versatile regulator that binds to a phosphorylated target and regulates targets function, such as enzymatic activity, localization, and protein–protein interactions (Chevalier et al. 2009; Obsil and Obsilova 2011; Roberts 2003). Thus, by regulating the stability of 14-3-3 protein, ATL31 regulates the function of 14-3-3 targets, a regulation essential for plant adaptation to disruptions in their C/N-nutrient balance.

Because atmospheric CO_2 is the sole C source for plants in nature, cellular C/N balance is affected by the balance between atmospheric CO_2 and the available amount of N taken up from the soil (CO_2/N). Our previous study demonstrated that the combination of elevated CO_2 and limited N promotes the progression of plant senescence, such as leaf yellowing and anthocyanin accumulation (Aoyama et al. 2014). Under these conditions, the expression of *ATL31* in senescent leaves, which is under the control of the transcription factor WRKY53, was transcriptionally up-regulated. Furthermore, the promotion of senescence under conditions of elevated CO_2 and limited N was suppressed in *ATL31* overexpressors, but enhanced in loss-of-function mutant plants (Aoyama et al. 2014). These results indicate that ATL31 play essential roles not only during the post-germinative growth stage but in the progression of plant senescence in response to changes in CO_2/N conditions.

SlATL31 and 14-3-3 proteins in tomato plants

The reproductive growth of plants is also highly dependent on the availability of carbon and nitrogen nutrients, suggesting the importance of ATL31 and 14-3-3 in tomato fruit development. We recently identified the tomato protein SlATL31 (Solyc03g112340), the closest homologue of the Arabidopsis ATL31 (AtATL31) protein (Lu et al. 2016) (Figure 1). The RING-H2 domain is well conserved in these two proteins, with the E3 activity of SlATL31 confirmed by in vitro analysis (Lu et al. 2016). In addition, the C-terminal region of SlATL31 contains several putative 14-3-3 binding motifs, with both SlATL31 and AtATL31 interacting similarly with 14-3-3 protein, suggesting that SlATL31 functions as an E3 for 14-3-3 (Lu et al. 2016). In addition, proteomic analysis using co-IP and MS/MS analysis identified 106 proteins as putative 14-3-3 targets in tomato fruit, including key enzymes involved in sugar metabolism, cell wall biosynthesis and photosynthesis (Lu et al. 2016).

Both AtATL31 and its closest homologue AtATL6 are involved in plant defense responses and positively regulate pathogen resistance in Arabidopsis (Huarancca Reyes et al. 2015; Maekawa et al. 2012; Maekawa et al. 2014). SlATL31, previously designated LeATL6, was also reported to regulate tomato plant immunity (Hondo et al. 2007; Takahashi et al. 2010). Although AtATL31 was shown to interact with SNARE protein SYP121, a regulator of membrane trafficking (Maekawa et al. 2014), and to be involved in pathogen-associated cell wall formation in Arabidopsis, the detailed molecular function of SlATL31 in tomato defense responses remains unclear.

Genome-wide identification of tomato ATL proteins

A recent analysis of the ATL family in several plant species, including both monocot and eudicot species, was recently reported (Aguilar-Hernández et al. 2011), as was a genome wide characterization of the ATL family in grapevine (Ariani et al. 2016). Tomato is a major crop plant and a model system for fleshy fruit development (Shikata et al. 2015; Tohge et al. 2014). The entire genome sequence of this species was published in 2012 (The Tomato Genome Consortium). Although a few studies have focused on specific ATL proteins in tomatoes, including ShATL78L and SlATL31/LeATL6, comprehensive knowledge of the tomato ATL family is still unknown. We therefore performed a complete

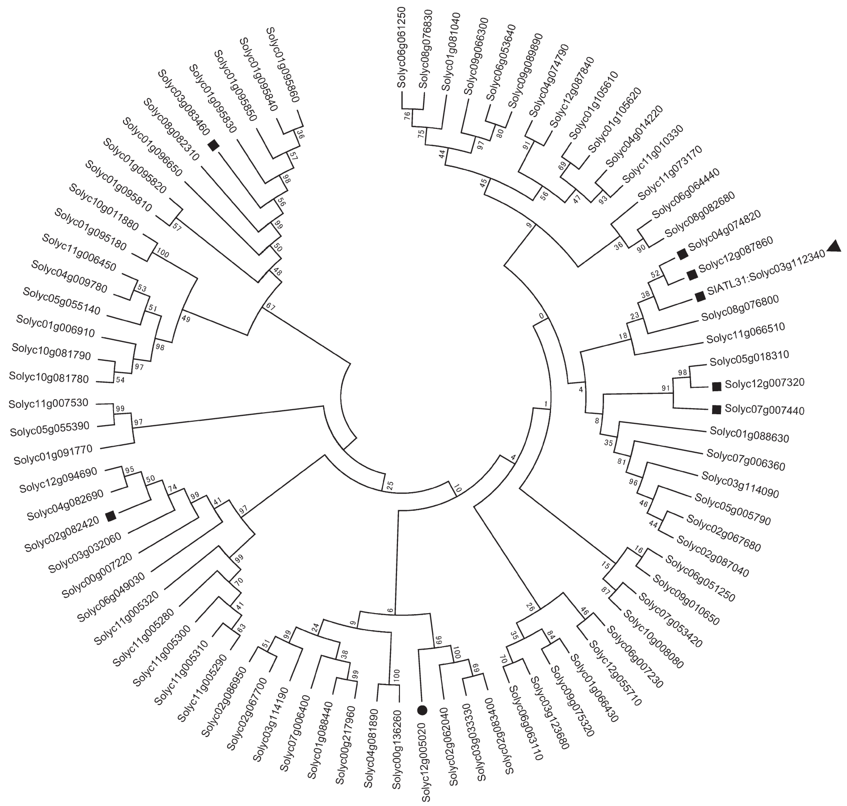

Figure 2. Phylogenetic tree of the ATL family in tomato. A unrooted phylogenetic tree was generated using MEGA7 software from the full-length amino acid sequences of 82 tomato ATLs aligned by the MUSCLE method (Edgar 2004). The Neighbor-Join phylogenetic tree was generated with 1,000 bootstrap replicates using the Jones-Taylor-Thornton model and a 0.95 gamma parameter (Aguilar-Hernández et al. 2011). The arrowhead indicates the position of SlATL31. The closed black squares or black circle indicate ATL members possess two or three transmembrane-like hydrophobic domains, respectively.

survey of the tomato genome (*Solanum lycopersicum* L.) to identify tomato ATL proteins. Members of this family have N-terminal transmembrane-like hydrophobic domains and a RING-H2 domain containing a highly conserved zinc-binding motif, consisting of six cysteine and two histidine residues (Figure 1A). The signature of the RING-H2 motif is the highly conserved distances between the second and third and between the sixth and seventh cysteine residues, distances corresponding to 15 and 10 amino acid residues, respectively (Guzmán 2012). As a first step in identifying tomato ATL proteins, we isolated canonical ATL-type RING-H2 proteins with a consensus sequence, Cys-X(2)-Cys-X(15)-Cys-X(1)-His-X(2)-His-X(2)-Cys-X(10)-Cys-X(2)-Cys (Aguilar-Hernández et al. 2011), from the tomato genome database (http://solgenomics.net/). After inspecting visualized sequences and excluding nonspecific protein, we further extracted the proteins containing transmembrane-like hydrophobic domains at the N-terminal region. We identified 82 proteins with a conserved ATL-type RING-H2 domain and an N-terminal transmembrane-like region as tomato ATL proteins (Figure 2). These tomato proteins also contained a tryptophan residue spaced out three amino acids downstream from the sixth cysteine and a proline residue next to the seventh cysteine, with both residues conserved in the RING-H2 domain (Figure 3A). Importantly, most of these proteins contained a proline residue before the third cysteine, which is well conserved in ATL proteins in other plant species (Figure 3A). Of the 82 tomato ATLs, 65 had the GLD motif, which is also highly conserved across all plant ATL families (Figure 3B). Interestingly, 74 of the 82 proteins were found to contain a single transmembrane-like hydrophobic region, but only eight, including SlATL31 and its close homologues, contained two or three hydrophobic regions (Figure 2).

Construction of a phylogenetic tree showed that the identified SlATL31, along with four other proteins, belong to a distinct clade (Figure 2). To determine whether SlATL31 related proteins were biochemically redundant, the sequences of the proteins included in the clade containing SlATL31 were aligned along with AtATL31 (Figure 1B and 2). These alignments indicate that the second transmembrane-like hydrophobic domain, the GLD motif, the RING domain, and a specific region in the C-terminal domain were conserved (Figure 1B). Our previous study identified 14-3-3 binding sites containing phosphorylated Ser/Thr residues in the C-terminal region of AtATL31, with

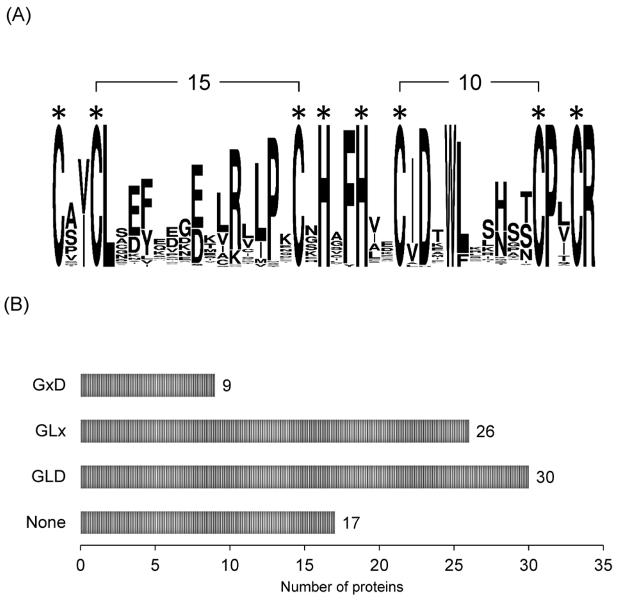

Figure 3. Domain architecture of the RING-H2 zinc finger and presence of the GLD motif in the tomato ATL family. (A) The sequence LOGO represents the RING-H2 zinc finger domain of tomato ATLs. The conserved 43 amino residues of the RING-H2 zinc finger domain from all 82 tomato ATL family proteins were used for the construction of LOGO. Asterisks indicate the conserved cysteine and histidine residues of the zinc finger. Numbers indicate the number of residues between the cysteine residues. (B) Number of conserved varieties of the GLD motif in the tomato ATL family.

the Thr209 residue being the predominant binding site of 14-3-3 (Yasuda et al. 2014). This 14-3-3 binding site corresponding to AtATL31Thr209 is well conserved in SlATL31 (SlATL31Ser250) and in four other SlATL homologues, whereas the other 14-3-3 binding sites are partially conserved in SlATL31, Solyc04g074820 and Solyc12g087860 (Lu et al. 2016) (Figure 1B), suggesting that these three proteins bind 14-3-3.

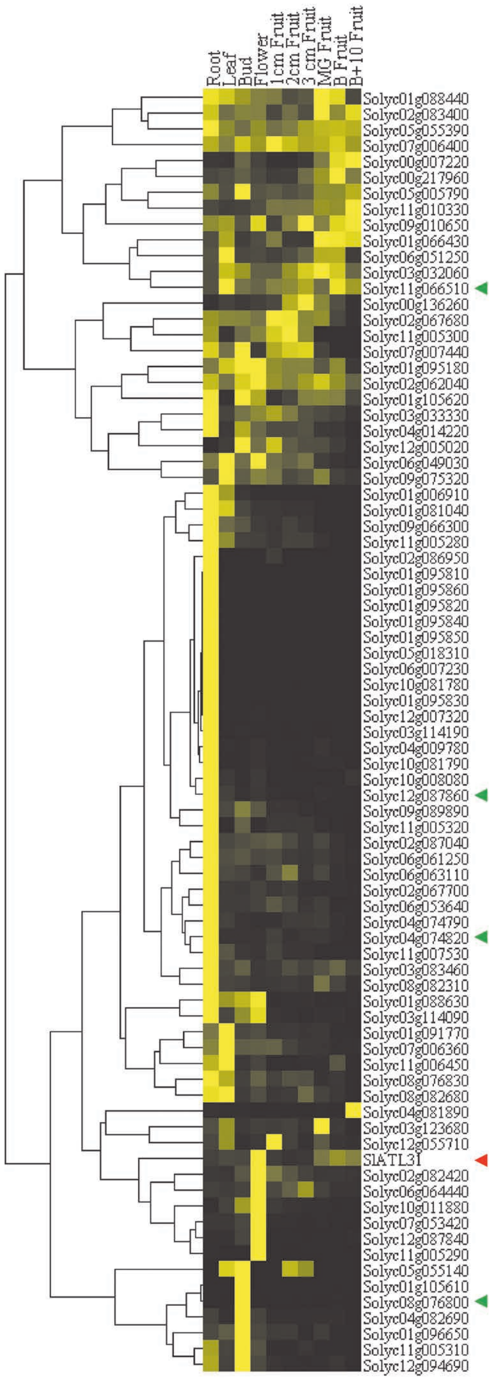

Figure 4. Transcriptome profile of tomato ATL genes. The normalized expression values relative to the highest level of expression of each tomato ATL gene in the Tomato Genome Consortium (Nature 2012, Vol: 485 635-641) were used for hierarchical cluster analysis based on the city-block distance metric. The brightness of the yellow color represents the level of expression. The red arrowhead indicates SlATL31, the green arrowhead indicates the ATL member belonging to the same clade as SlATL31. MG, mature green. B, beaker. B+10, beaker +10 days.

To comprehensively determine the transcription pattern of the tomato ATL family, the tissue-specific levels of expression of all ATL genes were extracted from the microarray data of the Tomato Genome Consortium (The Tomato Genome Consortium) and clustered (Figure 4). One gene, Solyc011g073170, was not expressed, at least in these microarrays, whereas the levels of expression of the remaining genes could be classified into several clusters. Although one subset of ATL genes was broadly expressed across tissues, many other genes showed tissue-specific expression patterns. SlATL31 was highly expressed in flowers and mature fruit, whereas the cluster that included Solyc04g074820 and Solyc12g087860, the closest SlATL31 homologues (Figure 1 and Figure 2), showed strong expression in roots. Another SlATL31 homologue, Solyc08g076800, with one conserved 14-3-3 binding motif, was specifically expressed in bud tissue. These data suggest transcriptional control of the physiological function of ATL family in combination with biochemical feature of each ATL protein. Together with our previous findings and other studies, these results suggest that the physiological significance of ATL proteins in tomato plant tissue, including fruit, differs markedly. Moreover, AtATL31 gene expression is modulated in response to C/N-nutrient condition as well as pathogen attack signal (Aoyama et al. 2014; Maekawa et al. 2012). Global transcriptome profiles in response to environmental cues and genetic investigations with tomato mutant lines may further reveal the biological functions and agricultural impact of the ATL family in tomatoes.

Conflicts of interest

The authors declare that they have no conflicts of interest associated with the submission of this manuscript. The manuscript has been approved for publication by all authors.

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