UNIVERSIDADE DE SÃO PAULO
CENTRO DE ENERGIA NUCLEAR NA AGRICULTURA

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Characterization of natural genetic variation in tomato (*Solanum lycopersicum*) for root ammonium uptake

Piracicaba
2021

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Revised version according to Resolution CoPGr 5018 from 2011

Dissertation presented to the Center of Nuclear Energy in Agriculture from University of São Paulo to obtain the Master of Science degree

Concentration Area: Biology in Agriculture and Environment

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Co-advisor: Prof. Dr. Joni Esrom Lima

Piracicaba
2021

Dados Internacionais de Catalogação na Publicação (CIP)
Técnica de Biblioteca - CENA/USP

de Oliveira Ragazzo, Gabriel

Caracterização da variação genética natural para a absorção de amônio em raiz de tomateiro (*Solanum lycopersicum*) / Characterization of natural genetic variation in tomato (*Solanum lycopersicum*) for root ammonium uptake / Gabriel de Oliveira Ragazzo; Antonio Vargas de Oliveira Figueira; coorientador Joni Esrom Lima. - - Versão revisada de acordo com a Resolução CoPGr 6018 de 2011. - - Piracicaba, 2021.

121 p.

Dissertação (Mestrado - Programa de Pós-Graduação em Ciências. Área de Concentração: Biologia na Agricultura e no Ambiente) - Centro de Energia Nuclear Agricultura, Universidade de São Paulo, Piracicaba, 2021.

1. Amônio 2. Cinética de absorção 3. Expressão gênica 4. Fertilizantes nitrogenados 5. Regulação gênica 6. Tomate I. Título

CDU (579.258 + 581.133.1) : 635.64

Elaborada por:
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CRB-8-3631
Resolução CFB No 184/2017

I dedicate this dissertation to my family and friends,
for all support, presence, affection, and good times
during the preparation of this work.

ACKNOWLEDGEMENTS

To God, for health, opportunities, and mercy towards me.

To the University of São Paulo, the Center of Nuclear Energy in Agriculture, its faculty, direction, and administration.

To my advisor, Prof. Dr. Antonio Vargas de Oliveira Figueira, for guidance, opportunity, provided structure, corrections, and confidence in my work.

To my co-advisor, Prof. Dr. Joni Esrom Lima, for all the availability, instructions, encouragement, and knowledge that were essential for the conduction of this work.

To CAPES (process 88882.328960/2019-01), for granting scholarship and financial support necessary to carry out this work.

To Prof. Dr. Lázaro Eustáquio Pereira Peres, for all plant material made available for use. To Prof. Dr. Diego Mauricio Riaño-Pachón and Prof. Dr. Tetsu Sakamoto for helping me with bioinformatics analysis. To Prof. Dr. José Albertino Bendassolli for the availability of the use of mass spectrometer equipment for ^{15}N analysis.

To my lab mates, Albania Patiño, André Yuji, Angélica Vargas, Anieli Baldo, Bárbara Gonçalves, Bruna Factor, Bruna Queiroz, Bruno Bachiega, Daniele Paschoal, Danielle Scotton, Eder Marques, Eduardo Bressan, Gabriel Silvino, Gyanlucca Martins, Hugo Corocher, João Mendes, Laecio Sampaio, Larissa Costa, Leonardo Zimmermann, Lorena Reis, Lucas Melotto, Luciana Chiba, Luis Serezino, Marília Morandi, Rafael Monteiro, Rafael Rosada, Rodolfo Maniero, Verusca Rossi, and Vitor Ometto, for the inspiring discussions and friendship in the last years.

To laboratory technicians Felippe Campana, Paulo Cassieri, and Wlamir Godoy, for the attention and help given.

To my parents, Danilo and Priscila, my warm gratitude for the love, support, patience, and sacrifice. I also express my thanks to my siblings, Larissa and Matheus, for their smiles, enthusiasm, and affection.

To my family, for all the encouragement, care, and companionship.

To everyone who has contributed to this work, directly or indirectly.

“Thus, the task is, not so much to see what no one has seen yet; but to think what nobody has thought yet, about what everybody sees.”

Arthur Schopenhauer

ABSTRACT

DE OLIVEIRA RAGAZZO, G. **Characterization of natural genetic variation in tomato (*Solanum lycopersicum*) for root ammonium uptake**. 2021. 121 p. Dissertação (Mestrado em Ciências) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2021.

Most cultivated plants, and especially tomato (*Solanum lycopersicum*), require large amounts of nitrogen (N) to achieve high commercial yields. Consequently, excessive quantities of the costly N-based fertilizers are supplied during crop cultivation. The investigation of the natural genetic variability for N uptake may help to design genetic strategies to improve N use efficiency (NUE) in tomato and, ultimately, decrease the amount of applied fertilizers. In plants, ammonium is the preferred source of N and is transported through proteins of the AMMONIUM TRANSPORTERS family (AMTs). Thus, the objective of this work was to identify and characterize AMTs, as well as possible regulations that alter the ammonium uptake in tomato. In a genomic survey, eight *AMT*s were identified in tomato, of which four had not been described before. The *SlAMT*s showed significant differences in expression between the plant tissues, indicating possible specific functions for each gene. In root, *SlAMT1.1* was the most expressed and is described as the main responsible for ammonium uptake in several species. Diversity analysis indicated the presence of great variability in the *SlAMT1.1* sequence between tomato accessions and wild *Solanum* species (section *Lycopersicon*). A study of ^{15}N-labeled ammonium uptake kinetics under different N availability was carried out among the genotypes *S. lycopersicum* cv. M82, *S. pimpinellifolium*, *S. habrochaites*, and *S. chmielewskii*. When N was supplied at sufficient levels, *S. chmielewskii* showed a greater ^{15}N-ammonium influx, which correlated with a higher level of *SlAMT1.1* expression in this genotype. During ammonium resupply, *S. habrochaites* and *S. chmielewskii* genotypes showed less inhibition of the ^{15}N-ammonium uptake process when compared to *S. lycopersicum* cv. M82 and *S. pimpinellifolium*. The expression levels suggest a differential allosteric regulation of SlAMT1.1 in these genotypes. These results suggest that variability in the *SlAMT1.1* gene among tomato genotypes can provide different patterns of protein activity and gene expression under different N conditions. Finally, a genomic wide association study (GWAS) using 31 tomato accessions was carried out and indicated the presence of a SNP (G→C) in the 3' UTR region of a *14-3-3* gene, which seem

necessary to modulate the expression of *SlAMT1.1*. Further evidences implied that this locus is associated with the signaling by brassinosteroids, although further studies are necessary to better describe this mechanism. In summary, natural genetic variation in tomato has great potential for breeding new cultivars with greater efficiency in the use of nitrogen.

Keywords: Nitrogen fertilization. Ammonium transporters. Nitrogen use efficiency. Uptake kinetic. Gene regulation.

RESUMO

DE OLIVEIRA RAGAZZO, G. **Caracterização da variação genética natural para a absorção de amônio em raiz de tomateiro (*Solanum lycopersicum*)**. 2021. 121 p. Dissertação (Mestrado em Ciências) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2021.

A maioria das plantas cultivadas, e especialmente o tomateiro (*Solanum lycopersicum*), requer grandes quantidades de nitrogênio (N) para atingir altos índices de produtividade. Consequentemente, quantidades excessivas de fertilizantes nitrogenados são fornecidas durante o cultivo. A investigação da variabilidade genética natural para absorção de N pode ajudar a projetar estratégias genéticas para melhorar a eficiência do uso de N (EUN) em tomateiro e, em última instância, diminuir a quantidade de fertilizantes aplicados. Para plantas, o amônio é a fonte preferencial de N e é transportado através de proteínas da família AMMONIUM TRANSPORTERS (AMTs). Assim, o objetivo deste trabalho foi identificar e caracterizar AMTs, bem como possíveis regulações que alteram a absorção de amônio em tomateiro. Em uma análise genômica, oito *AMT*s foram identificados em tomateiro, dos quais quatro não haviam sido descritos antes. Os *SlAMT*s apresentaram diferenças significativas na expressão entre os tecidos vegetais, indicando possíveis funções específicas para cada gene. Na raiz, *SlAMT1.1* foi o mais expresso e é descrito como o principal responsável pela absorção de amônio em diversas espécies. Uma análise de diversidade indicou a presença de grande variabilidade na sequência de *SlAMT1.1* entre acessos de tomateiros cultivados e espécies selvagens de *Solanum* (seção *Lycopersicon*). Um estudo da cinética de absorção de amônio marcado com ^{15}N sob diferentes disponibilidades de N foi realizado entre os genótipos *S. lycopersicum* cv. M82, *S. pimpinellifolium*, *S. habrochaites* e *S. chmielewskii*. Quando o N foi fornecido em suficiência, *S. chmielewskii* apresentou um maior influxo de ^{15}N-amônio, o que se correlacionou com um nível mais alto de expressão de *SlAMT1.1* neste genótipo. Durante a ressuplementação de amônio, os genótipos *S. habrochaites* e *S. chmielewskii* apresentaram menor inibição do processo de absorção de ^{15}N-amônio quando comparados com *S. lycopersicum* cv. M82 e *S. pimpinellifolium*. Os níveis de expressão sugerem uma regulação alostérica diferencial de *SlAMT1.1* nesses genótipos. Esses resultados sugerem que a variabilidade no gene *SlAMT1.1* entre genótipos de tomateiro pode fornecer diferentes padrões de atividade proteica e

expressão gênica sob diferentes condições de N. Finalmente, um estudo de associação ampla genômica (GWAS) usando 31 acessos de tomateiro foi realizado e indicou a presença de um SNP (G→C) na região 3' UTR de um gene *14-3-3*, que parece necessário para modular o expressão de *SlAMT1.1*. Evidências adicionais sugerem que esse locus está associado à sinalização por brassinosteroides, embora mais estudos sejam necessários para melhor descrever esse mecanismo. Em suma, a variação genética natural em tomateiro apresenta grande potencial para o melhoramento de novas cultivares com maior eficiência no uso do nitrogênio.

Palavras-chave: Fertilização nitrogenada. Transportadores de amônio. Eficiência no uso de nitrogênio. Cinética de Absorção. Regulação gênica.

SUMMARY

1 INTRODUCTION

The growing world demand for food in the first half of the 20th century led to the intensification of agriculture through the use of chemical inputs, such as fertilizers and agrochemicals (FOLEY et al., 2011). The excessive use of nitrogen-based fertilizers, combined with a loss of 50-70% of the added nitrogen (N) (MASCLAUX-DAUBRESSE et al., 2010), has increasingly introduced reactive N into the environment (GALLOWAY et al., 2008; AUSTIN et al., 2013). Excessive N became a threat to the quality of air, water and soil, generating severe environmental problems (ZHANG et al., 2015).

Nitrogen is often a limiting nutrient and a major determinant for plant growth and yield. To ensure adequate N uptake, induction of molecular responses, such as the expression of nutrient transporters at the root plasma membrane, and recycling and remobilization mechanisms, enable plants to adapt to frequent natural conditions of poor N availabilities (JACKSON; CALDWELL, 1993; BOUWMAN et al., 2017).

Tomato (*Solanum lycopersicum*) is the most cultivated horticultural crop in the world (FAO, 2018). Tomato crops produce low biomass and yield when grown upon soil N concentrations below 7-8 mM of N indicating that N is a limiting factor to maintain high productivity (SIDDIQI et al., 1998; FERREIRA et al., 2006). Improving the N use efficiency (NUE) under low N availability became a current major challenge (HIREL et al., 2007; VON WIRÉN, 2011). Selection of plants with high NUE can be a relevant alternative to avoid the excessive use of N fertilizers in agricultural systems. NUE can be defined as the maximum economic yield of a crop per unit of applied N (MOLL; KAMPRATH; JACKSON, 1982). In a broader sense, NUE describes the crop ability to convert N available in the soil into biomass for growth and development. To reduce environmental N losses and target high NUE simultaneously, it is necessary to characterize the crop capacity to use the available N efficiently, leading to the development of cultivars more adapted to low N environments (HIREL et al., 2007) while reducing agronomic N inputs (BOUWMAN et al., 2017).

Ammonium is the preferential N source for several plant species (BRITTO; KRONZUCKER, 2013; GU et al., 2013). The AMMONIUM TRANSPORTERS (AMT) family is responsible for ammonium uptake in several species (GAZZARRINI et al., 1999; VON WIRÉN et al., 2000a; ROBINSON et al., 2011; GU et al., 2013; KOEGEL et al., 2013). In the model plant *Arabidopsis thaliana*, studies demonstrated that AtAMT1.1 and AtAMT1.3 are responsible for 70% of the total ammonium uptake

(LOQUÉ et al., 2006). In the tomato genome, four genes belonging to the AMT family have been identified to date: *SlAMT1.1*, *SlAMT1.2*, *SlAMT1.3*, and *SlAMT2.1* (VON WIRÉN et al., 2000a; LUDEWIG; VON WIREN; FROMMER, 2002). Understanding the mechanism and regulation of N uptake is an important step towards obtaining plants with higher NUE, leading to a more productive crop.

Despite the significant knowledge accumulated over the last decades about mechanisms underlying N starvation responses, the genetic diversity present in the *Solanum* section *Lycopersicon* for key regulators in coordinating responses to N signaling and adaptive response associated with low N stress remains poorly understood.

Here, we identified and characterized AMTs, as well as possible regulatory components that alter ammonium uptake in tomato. In a genomic survey, eight *AMT*s genes were identified, from which four have not been previously described; all showed variability in expression among plant tissues and between cultivars and wild *Solanum* species. *SlAMT1.1* was the mostly expressed *AMT* gene in tomato roots, appearing to be the most important in ammonium uptake. Variability in the *SlAMT1.1* coding sequence among accessions of *Solanum* section *Lycopersicon* was detected. Considering the presence of natural variability in the *SlAMT1.1*, analyses of ^{15}N-ammonium uptake kinetics upon contrasting N availability was carried out among *S. lycopersicum* cv. M82, *S. pimpinellifolium*, *S. habrochaites* and *S. chmielewskii* genotypes, and indicated that the variability in *SlAMT1.1* coding sequence can result in different patterns of protein activity and gene expression among genotypes. Finally, a genomic wide association study (GWAS) was carried out and indicated that the presence of an SNP (G→C) in the 3' UTR region of a *14-3-3* gene appears to be related to the modulation of *SlAMT1.1* expression, probably associated with brassinosteroid signaling pathway, but further studies should be carried out to better describe this mechanism.

2 LITERATURE REVIEW

2.1 Importance of nitrogen for plant development

Nitrogen (N) is an abundant element in plant tissues and an essential mineral nutrient for plant growth and development (LUO; ZHANG; XU, 2020). N is an important component of many essential structural, genetic and metabolic compounds in plant cells, such as DNA, RNA, amino acids, proteins, enzymes, chlorophyll, and some hormones (HIREL et al., 2007; YANG et al., 2015; LEE et al., 2020b). Besides, N-containing molecules, such as nitrate and ammonium, can also act as signaling molecules regulating essential physiological processes, such as root development, flowering time, leaf expansion, as well as uptake of other nutrients (BOUGUYON; GOJON; NACRY, 2012; LÓPEZ-ARREDONDO et al., 2013).

Plants can obtain N from the soil as nitrate (NO_3^-), ammonium (NH_4^+), urea, amino acids, small peptides and even as proteins (XU; FAN; MILLER, 2012). Of all the essential nutrients, N is the one that is most often limiting for crop growth. Therefore, to expand and maximize crop yield, a large amount of N-based fertilizers is frequently used in agriculture (YANG et al., 2015).

2.2 Challenges in the use of nitrogen fertilizers

The increase in the world population and the growing need for food security is placing unprecedent pressure on agriculture and natural resources (FOLEY et al., 2011). With the ‘Haber-Bosch’ industrial process for N reduction, there has been an 800% increase in the use of N fertilizers in the last 50 years (TILMAN et al., 2001). To meet the current world demand for food, the production of N fertilizers and biological N fixation convert around 120 million tons of atmospheric N_2 into reactive N forms per year, considered beyond the sustainable limit of the planet (ROCKSTRÖM et al., 2009). Moreover, it is estimated that more than half of that N is lost from the plant-soil system, severely contaminating the environment (SCHROEDER et al., 2013).

Intensification of agricultural practices have led to excessive use of fertilizers, causing the degradation of water bodies and marine ecosystems, due to nitrate leaching (VITOUSEK et al., 2009; FOLEY et al., 2011). Atmosphere contamination due to the emission of greenhouse gases, such as nitric oxide and ammonia (TILMAN et

al., 2001; GALLOWAY et al., 2008) and the constant increase of reactive N in terrestrial ecosystems (AUSTIN et al., 2013) are also consequences of today's agriculture. Considering these aspects, there is an urgent need to improve the use efficiency of N fertilizers by cultivated plants, which represents a major challenge for modern agriculture (FOLEY et al., 2011; ZHANG et al., 2015).

2.3 Nitrogen nutrition in tomato yield

Tomato (*Solanum lycopersicum;* Solanaceae) presents great economic and social importance as the world's leading horticulture crop, with a total annual production of 182 million tons, worth over US$ 60 billion in 2018 (FAO, 2018). Brazil is the tenth largest producer, with 2.3% of world production, where approximately 4.1 million tons of tomato are harvested annually (FAO, 2018). Tomato is a functional food due to the high levels of vitamin A and C, in addition to being rich in lycopene, a carotenoid with antioxidant properties associated with reduced development of prostate and ovarian cancer and lower incidence of cardiovascular diseases (CARVALHO; PAGLUICA, 2007).

With the rising tomato production, there is a dramatic increase in N fertilizer use to maintain the required level of yield. However, current tomato cultivars, obtained by an intense and long genetic breeding process, display a high N demand to maintain high growth and fruiting rates (SIDDIQI et al., 2002; RONGA et al., 2019). The N requirement is higher at the early stages of tomato growth, whereas N deficiency can delay growth and decrease yield (FERREIRA et al., 2006). In addition, N fertilization affects processing tomato fruit yield and quality parameters required by the canning industry, such as total solids, soluble solids, pH, and acidity (RONGA et al., 2015).

Solanum lycopersicum genotypes produce low biomass and yield when grown in soil with N concentrations below 7-8 mM, confirming that N is a limiting factor to maintain high yields and demonstrating that most of the improved cultivars have been selected under N rich soils or conditions (SIDDIQI et al., 1998; FERREIRA et al., 2006). As a consequence, improving NUE under low availability is a relevant target of agronomic, economic, and environmental issues in tomato production (HIREL et al., 2007; VON WIRÉN, 2011).

2.4 Tomato natural genetic variability

The Solanaceae encompasses over 3,000 species of flowering plants with great diversity in terms of growth habit, habitat, and morphology, and includes important agricultural crops. Tomato is a member of *Solanum*, the largest genus of the Solanaceae, which also includes several other species of commercial importance, such as potato (*S. tuberosum*) and eggplant (*S. melongena*) (AFLITOS et al., 2014). The tomato clade species originated in the Andean region, including Peru, Bolivia, Ecuador, Colombia and Chile (Figure 1). The Andean region covers a great habitat diversity with environments ranging from near sea level to mountains up to 3,300 m of altitude, and climates ranging from arid to rainy. This peculiar range of ecological conditions may have contributed to the tomato wild species variability in morphology, physiology, sexual and molecular levels (PERALTA; SPOONER, 2005; BAUCHET; CAUSSE, 2012).

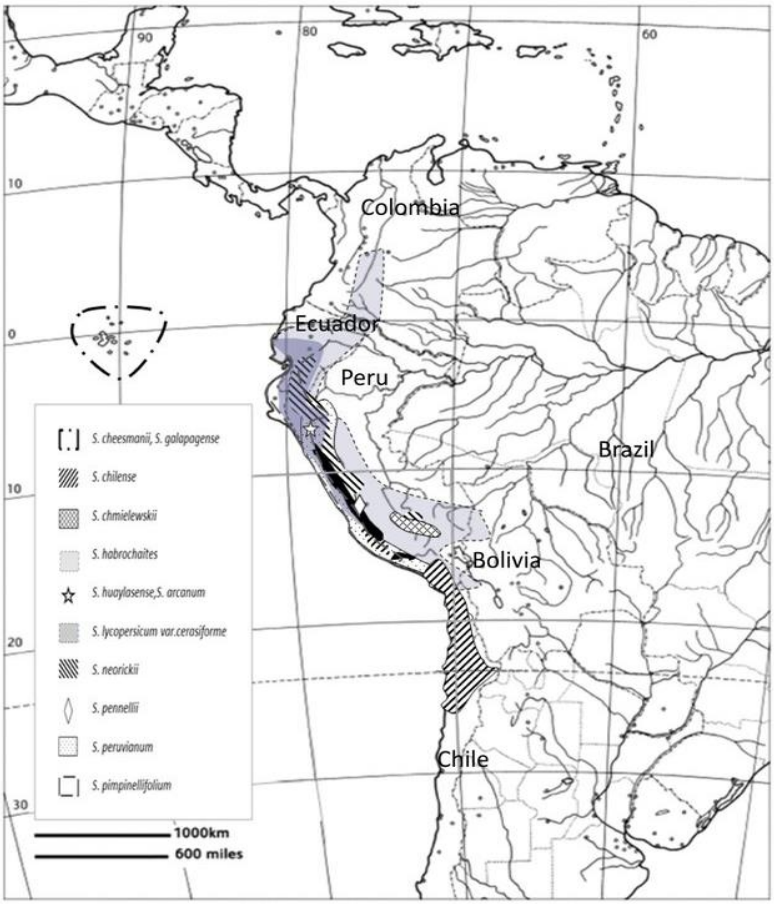


Figure 1 - Geographic distribution of wild species in *Solanum* sect. *Lycopersicon* (BAUCHET; CAUSSE, 2012).

Over time, several classifications have been proposed to tomato and related species (Figure 2). In the first edition of *Species plantarum* (LINNAEUS, 1753), tomatoes were classified into the genus *Solanum*, but the tomato group turned to a specific genus, *Lycopersicon* (MILLER, 1754). Müller (1940) and Luckwill (1943) produced the most complete and diverse analyses based on morphological concepts of wild tomatoes, and recognized them under the *Lycopersicon* genus. Later studies adapted and updated the classification of the genus *Lycopersicon*, including other species such as *L. pennellii* and *L. chilense* (RICK, 1979). Child (1990) returned tomato to the genus *Solanum*, dividing it into three series: Lycopersicon, Neolycopersicon and Eriopersicon. Later, molecular data confirmed tomato membership of Linnaeus classification, but also improved the classification (PERALTA; SPOONER; KNAPP, 2008).

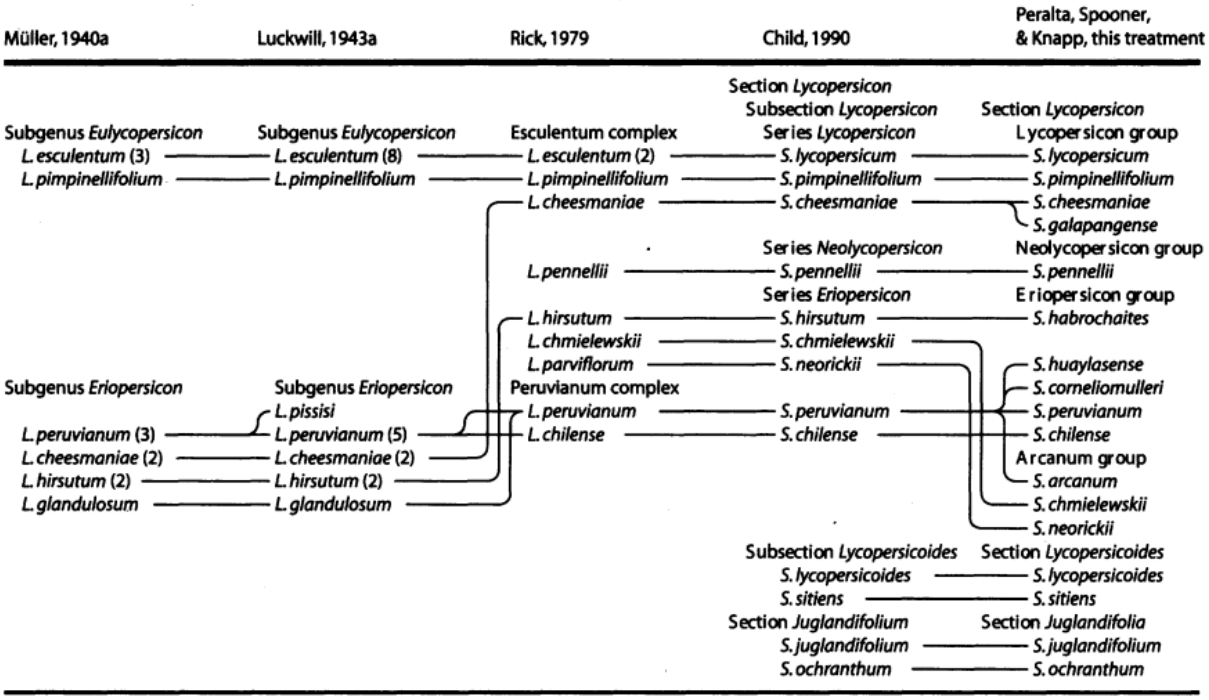


Figure 2 - Chronological flow chart of hypothesis of species boundaries and relationships of *Solanum* sect. *Lycopersicon* (PERALTA; SPOONER; KNAPP, 2008).

In the most recent classification, *Solanum* section *Lycopersicon* includes the common cultivated tomato (*S. lycopersicum*) and 13 wild relatives: *S. arcanum*, *S. chmielewskii*, *S. neorickii*, *S. pennellii*, *S. habrochaites*, *S. chilense*, *S. huaylasense*, *S. peruvianum*, *S. corneliomulleri*, *S. cheesmaniae*, *S. galapagense*, *S. pimpinellifolium,* and *S. lycopersicum* var. *cerasiforme*. Tomato wild relatives are divided into four groups: Arcanum, Neolycopersicon, Eriopersicon and Lycopersicon

(Figure 2; Table 1) (PERALTA; SPOONER, 2007; PERALTA; SPOONER; KNAPP, 2008; LIN et al., 2014). The tomato clade is an interesting example for research on plant biodiversity, notably, on evolution, adaptation, domestication and nutrition (PERALTA; SPOONER, 2007).

Table 1 - Main features of *Solanum* sect. *Lycopersicon.*

Subsection	Species	Habitat	Genetic polymorphism
Arcanum	*S. arcanum*	Dry valleys, dry rocky slopes	Intermediate
	S. chmielewskii	Dry and drained areas	Intermediate
	S. neorickii	Humid and well drained areas	Low
Neolycopersicon	*S. pennellii*	Dry and rocky hillsides	High
Eriopersicon	*S. habrochaites*	Forest regions	High
	S. chilense	Dry river bed	High
	S. huaylasense	Rocky slopes	High
	S. peruvianum	Coastal deserts	High
	S. corneliomulleri	Lower slopes on the edges of landslides	High
Lycopersicon	*S. cheesmaniae*	Galapagos island endemic, from sea shore to volcanic areas	Low
	S. galapagense	Galapagos island endemic, sea shore	Low
	S. pimpinellifolium	South valleys of the pacific coast	Intermediate
	S. lycopersicum var. cerasiforme	Adventive worldwide in tropics and subtropics	Low
Cultivars	*S. lycopersicum*	Various range of habitats	Very Low

Data: (BAUCHET; CAUSSE, 2012).

Tomato domestication was conditioned to a narrow and specific selection of traits, especially those related to fruit size and shape (RODRÍGUEZ et al., 2011; AFLITOS et al., 2014). Previous studies have revealed that the wild red-fruited *S. pimpinellifolium* was domesticated in South America to give rise to *S. lycopersicum* var. *cerasiforme* (cherry tomato), and later, the big-fruited cultivated tomatoes arose in Mesoamerica through subsequent breeding (BLANCA et al., 2012; 2015; LIN et al., 2014). As a result of continuous selection, domestication and breeding, increasing tomato yield, the genetic basis narrowed with only extremely reduced variability remaining in modern cultivars (DOEBLEY; GAUT; SMITH, 2006; BAI; LINDHOUT, 2007; BAUCHET; CAUSSE, 2012; RAZIFARD et al., 2020). Therefore, in recent

decades, wild germplasm has been increasingly used as a source of new alleles for conventional and modern tomato breeding to increase the genetic variation of desirable traits (BAI; LINDHOUT, 2007; AFLITOS et al., 2014; SOYK et al., 2017; ZSÖGÖN et al., 2018).

2.5 Nitrogen Use Efficiency (NUE) in plants

By definition, NUE is determined by the accumulation of total shoot biomass or grain/fruit yield accumulated per unit of available N in the soil (MOLL; KAMPRATH; JACKSON, 1982). NUE is dependent on the uptake efficiency of different sources of N available in the soil, together with the efficiency of N use, which comprises the assimilation and remobilization processes during plant development (GOOD; SHRAWAT; MUENCH, 2004; HIREL et al., 2007). To improve NUE, plants need to coordinate the transporter systems responsible for the N uptake from the soil, relocation, and assimilation according to N demand over all developmental stages (MASCLAUX-DAUBRESSE et al., 2010; XU; FAN; MILLER, 2012). Previous studies have shown that species- and genotype-dependent processes offer large potential in the improvement of N uptake or utilization efficiency (LOQUÉ; VON WIRÉN, 2004; MASCLAUX-DAUBRESSE et al., 2010; GUTIÉRREZ, 2012; RISTOVA; BUSCH, 2014). Thus, identification of the genetic basis regulating N uptake and assimilation, which differ according to plant species, is fundamental for improving NUE.

The ecosystem directly affects the N forms present in the soil, which, in general, include inorganic and organic N forms (CRAWFORD; GLASS, 1998). In agricultural soils, the inorganic forms of N, ammonium ($N\text{-}NH_4^+$) and nitrate ($N\text{-}NO_3^-$), are predominant and preferentially acquired by plant roots (MILLER et al., 2007). The transport of ammonium and nitrate in plants occurs through high affinity transport systems (HATS; when N<1mM) or low affinity transport systems (LATS, when N>1mM) (GLASS, 2003; LOQUÉ; VON WIRÉN, 2004).

Inorganic N is frequently found in limited concentrations in natural environments, which is favorable for plant species to develop more efficient mechanisms for N uptake from soil and/or for N assimilation (BERNARD; HABASH, 2009; MÜLLER et al., 2015). In general, part of the absorbed nitrate is assimilated directly in the roots, while the vast majority is transported to shoots, where nitrate is reduced to nitrite by nitrate reductase (NR) and to ammonium by nitrite reductase (NiR) and glutamine

synthetase (GS). Conversely, ammonium acquired by ammonium transporters (AMTs) or derived from nitrate reduction is assimilated into amino acids via the GS/glutamine-2-oxoglutarate aminotransferase (GOGAT) cycle (XU; FAN; MILLER, 2012).

Previous studies have provided evidence for significant natural genetic variation for NUE in *Solanum* species (OLIVEIRA, 2004). The commercially-grown cultivars *S. lycopersicum* cv. Sol da Manhã and cv. Santa Clara, as well as the wild genotypes *S. lycopersicum* var. cerasiforme, *S. pimpinellifolium*, *S. galapagense*, *S. neorickii*, *S. habrochaites*, and *S. pennellii*, were grown under contrasting N levels and evaluated for total biomass production and total N concentration to determine NUE (OLIVEIRA, 2004). Santa Clara exhibited greater NUE under high N availability, while the wild species *S. pimpinellifolium* conferred better NUE when cultivated under limited N availability. Further, the wild genotype *S. habrochaites* showed no alteration in NUE under either N supply (OLIVEIRA, 2004). Short-term uptake analysis using ^{15}N-labeled NH_4^+ in N-deficient or sufficiency showed that *S. pimpinellifolium* absorbed 35-80% more ammonium than cultivar cv. Santa Clara or *S. habrochaites* (TAGLIAFERRO, 2015). These results indicate that roots of the *S. pimpinellifolium* possess a higher ammonium uptake capacity compared to the *S. lycopersicum* cv. ‘Santa Clara’ and *S. habrochaites* genotypes, and confirms the presence of natural genetic variation for the ammonium uptake (TAGLIAFERRO, 2015).

2.6 Ammonium Transporters

Ammonium is the preferential N source for several plant species (GAZZARRINI et al., 1999; VON WIRÉN et al., 2000a; BRITTO; KRONZUCKER, 2013; GU et al., 2013). The AMT protein family is part of the AMT/MEP/Rh superfamily, which is found in all life domains, including bacteria, archaea, yeasts, plants and animals (VON WIRÉN et al., 2000a; LUDEWIG et al., 2001). Ammonium uptake and transport in plants is mainly mediated by membrane proteins of the AMT (AMMONIUM TRANSPORTER) family, which have been identified in many monocotyledonous and dicotyledonous species (GAZZARRINI et al., 1999; VON WIRÉN et al., 2000a; D’APUZZO et al., 2004; GU et al., 2013; WITTGENSTEIN et al., 2014). The number of *AMT* genes present in the genome of plant species differs considerably. While *Arabidopsis thaliana* contains six genes and *Lotus japonicus* and *S. lycopersicum* four *AMTs* (D’APUZZO et al., 2004), in the *Oryza sativa* genome, more than 10 orthologous

have been identified, subdivided into subfamilies AMT1 and AMT2 (LOQUÉ; VON WIRÉN, 2004; WITTGENSTEIN et al., 2014). Phylogenetic analysis revealed that plant AMT1 subfamily clustered with cyanobacterial ammonium transporters, while AMT2 was more closely related to the MEP subfamily from prokaryotic homologues (LUDEWIG et al., 2001).

AMT/MEP/Rh are highly hydrophobic integral membrane proteins with 400-500 amino acids, with predicted molecular mass of 45–55 kDa and 11-12 transmembrane helices (LUDEWIG et al., 2003). Biochemical studies provided evidence that plant AMTs form trimers with extra-cytosolic orientation of the N-terminal and intra-cytosolic of the C-terminal (LUDEWIG et al., 2003; GRAFF et al., 2011). AMT require a productive interaction between all three subunits to work (LANQUAR et al., 2009). The C-terminal region contains an important regulatory function of these membrane transporters. A Gly456Asp mutation at the SlAMT1.1 C-terminal causes the inactivation of transport in SlAMT1.1 and in its paralogous SlAMT1.2, being an evidence that AMTs form oligomers. Cross-inhibition by mutant subunits was taken as evidence for homooligomerization and possibly heterooligomerization by plant AMTs (LUDEWIG et al., 2003), whereas each subunit in the oligomer forms a functional pore (LOQUÉ et al., 2007).

Functional studies have identified the main membrane transporters responsible for ammonium uptake. In *A. thaliana*, *AtAMT1.1* and *AtAMT1.3* are mainly expressed in epidermis cells and root cortex, including root hairs (LOQUÉ et al., 2006). Uptake evaluation by short-term influx using ^{15}N-labeled ammonium ($^{15}N\text{-}NH_4^+$) in roots of double mutant demonstrated that AtAMT1.1 and AtAMT1.3 are responsible for 70% of the total ammonium uptake (LOQUÉ et al., 2006). The *AtAMT1.2* defective mutant shows 26% less influx of $^{15}N\text{-}NH_4^+$ in roots (YUAN et al., 2007a). The analysis of the *AtAMT1.2* gene promoter indicates its expression in the root endoderm and cortex, suggesting the role of this carrier in the apoplastic transport of ammonium (YUAN et al., 2007a). The *A. thaliana* quadruple knock-out mutant (*qko*), defective in *AtAMT1.1*, *AtAMT1.2*, *AtAMT1.3*, and *AtAMT2.1* maintains 10% root uptake of ammonium when compared to wild type (YUAN et al., 2007a). This remaining ability to acquire ammonium is due to the activity of the transmembrane protein AtAMT1.5, located in the cells of the rhizodermis and root hairs (YUAN et al., 2007a).

Therefore, the spatial organization and activity of the various AMT proteins regulate the homeostasis of ammonium in roots according to the plant N demand.

Membrane transporters of the AMT1 subfamily act as sensors during ammonium uptake (LOQUÉ et al., 2007; LANQUAR et al., 2009) and modulate root development (LIMA et al., 2010), through an unknown signaling mechanism. While AtAMT1.1, AtAMT1.3 and AtAMT1.5 mediate the ammonium uptake in outer root cells, AtAMT1.2 takes up ammonium from the root apoplast, and AtAMT2.1 is involved in the xylem loading process (LOQUÉ et al., 2006; YUAN et al., 2007a, 2013; GIEHL et al., 2017; DUAN et al., 2018).

In tomato, whose N uptake strongly relies on ammonium nutrition, three AMT1s have been investigated so far. *SlAMT1.1* and *SlAMT1.2* are preferentially expressed in root hairs and are upregulated under N deficiency and after ammonium resupply, indicating a role for N uptake at low and moderate external ammonium provision (VON WIRÉN et al., 2000b; LUDEWIG; VON WIREN; FROMMER, 2002). In contrast, *SlAMT1.3* is not mainly expressed in roots, but only in above-ground plant organs (VON WIRÉN et al., 2000b).

Since a recent meta-study has shown that N transporter genes improved yield and NUE parameters more efficiently than did the other gene types (LI et al., 2018), the question that arises is to what extent the modification of *AMT* genes or their expression level can contribute to higher N uptake efficiency.

2.7 Transcriptional and post-transcriptional regulation of AMTs

In general, the expression of genes associated with mineral nutrient transport can be induced or repressed according to the availability of the respective nutrient in the soil, or with the nutrient concentration present within the plant cells (KIBA; KRAPP, 2016). The expression of *AMTs* can be regulated at multiple levels. At the transcriptional level, ammonium supply can modulate the expression of *AMTs* genes, probably triggered by an ammonium metabolite, such as glutamine (GAZZARRINI et al., 1999; RAWAT et al., 1999; VON WIRÉN et al., 2000a; GU et al., 2013; WU et al., 2015, 2019). Some transcription factors, such as NITRATE-INDUCIBLE GARP-TYPE TRANSCRIPTIONAL REPRESSOR 1 (NIGT1), can negatively regulate *AtAMT1.1* expression (KIBA et al., 2018; MAEDA et al., 2018), and possibly act as repressors of N starvation responses under high N availability conditions (KIBA et al., 2018; SAFI et al., 2021). In contrast, GROWTH REGULATION FACTOR 4 (GRF4) and INDETERMINATE DOMAIN 10 (IDD10) can activate expression of *OsAMT1.1* and

OsAMT1.2, respectively, in rice (XUAN et al., 2013; LI et al., 2018). Nevertheless, most studies of transcriptional network regulation related to the use of N have been carried out only in *A. thaliana*. Most of these studies only focused on plant responses after exposure to a N supply, and did not explore transcriptional networks related to NUE responses (UEDA et al., 2020).

In addition to transcriptional regulation, AMT1.1 has also been shown to be post-transcriptionally regulated. In *A. thaliana*, *AtAMT1.1* overexpression resulted in abundant accumulation of *AtAMT1.1* transcripts in shoots, but the transcripts appeared to be degraded in roots (YUAN et al., 2007b). A novel non-coding RNA (ncRNA), which is highly abundant in N-sufficient roots, may target *AtAMT1.1* 3' UTR for transcript degradation (ZHANG; WU; YUAN, 2020). The ectopic overexpression of *AtAMT1.1* in transgenic tobacco lines leads to transcript degradation under high N conditions (YUAN et al., 2007b). The degradation of expressed *AtAMT1.1* resulted from post-transcriptional gene silencing through siRNAs, of which the production was determined by excessive expression of *AtAMT1.1* (ZHANG; WU; YUAN, 2020). Organ- and N-dependent post-transcriptional regulation mechanism, although not fully elucidated yet, is apparently exclusive to *AtAMT1.1* and does not occur with the other *AtAMTs* (YUAN et al., 2007b; ZHANG; WU; YUAN, 2020).

2.8 Post-Translational regulation of AMTs

Post-translational regulation of transporter proteins is another important AMT regulatory mechanism. Post-translational regulation allows plants to have a quicker response to sudden changes in N availability when compared to transcriptional regulation. Protein kinase-mediated phosphorylation modulates ammonium uptake (HO et al., 2009; LANQUAR; FROMMER, 2010; STRAUB; LUDEWIG; NEUHÄUSER, 2017). Calcineurin B-like Interacting Protein Kinase 23 (CIPK23) was described as an inhibitor of ammonium uptake and it modulates growth sensitivity to ammonium by phosphorylation of the conserved threonine at C-terminal of AMT1.1 and AMT1.2 (STRAUB; LUDEWIG; NEUHÄUSER, 2017).

In *A. thaliana*, the allosteric regulation is mediated by a cytosolic C-terminal trans-activation domain (Figure 3), which carries a conserved Thr (T460) in a critical position in the hinge region of the C-terminal (LANQUAR et al., 2009). Phosphorylation is triggered by ammonium depending on its concentration and time of exposure. The

T460 phosphorylation acts as a plant defense mechanism against ammonium toxicity, since it reduces the ammonium uptake in roots (LANQUAR et al., 2009; LANQUAR; FROMMER, 2010). Within the homo- or heterotrimeric complexes of AMT1s, the C-terminal domain interacts with a cytosolic loop of itself or with the adjacent monomer (KHADEMI et al., 2004; VAN DEN BERG et al., 2016). Although AtAMT1.1 monomers have independent pathways for ammonium transport, it is necessary that all trimer subunits are activated (or deactivated) for transport (LOQUÉ et al., 2007; NEUHÄUSER et al., 2007; LANQUAR et al., 2009). In addition, another mechanism for transport regulation can occur with the conformational coupling between monomers, which increases the dynamic range of ammonium detection and sensitivity (LOQUÉ et al., 2007).

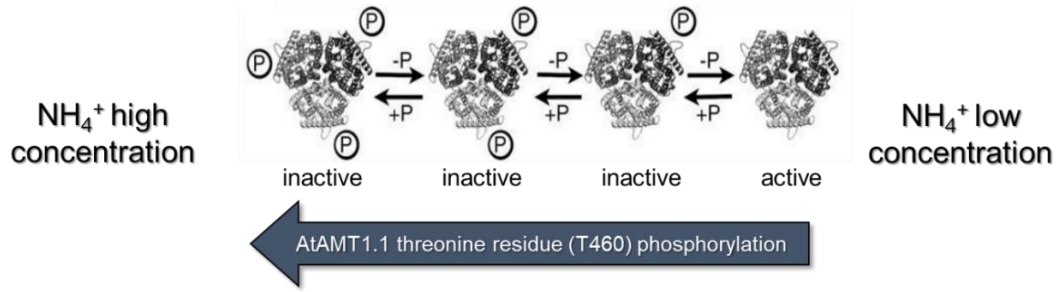


Figure 3 - Trans-activation mechanism for ammonium uptake regulation (LOQUÉ et al., 2007; NEUHÄUSER et al., 2007; LANQUAR et al., 2009).

Other AMT phosphorylation sites have been described in arabidopsis and rice involved in ammonium/nitrate uptake regulation, triggered by an ammonium signal, in AtAMT1.1 (S475, S488, S490, S492, T496, T497, and T499) (LANQUAR et al., 2009; REILAND et al., 2009); AtAMT1.2 (T472) (STRAUB; LUDEWIG; NEUHÄUSER, 2017); AtAMT1.3 (T464, S480, S487, and T494) (ENGELSBERGER; SCHULZE, 2012; YUAN et al., 2013; MENZ et al., 2016); and rice OsAMT1.2 (T453) (STRAUB; LUDEWIG; NEUHÄUSER, 2017; BEIER et al., 2018). Notably, different phosphorylation patterns are observed in response to distinct conditions of nutritional availability and the presence of different N forms. However, the way these phosphorylation sites are associated with the activity of AMT1 proteins and how they influence differential N supply response has not yet been elucidated (WU et al., 2019).

2.9 Natural genetic variation for N uptake in higher plants

In the model plant *A. thaliana*, natural genetic variation has been identified in plants grown under N limiting conditions, with two genotypes ('Bur-0' and 'Tsu-0') showing more nitrate uptake and biomass production, which resulted in better NUE (CHARDON et al., 2010), demonstrating the importance of the uptake and transport process for NUE (LEA; AZEVEDO, 2006; XU; FAN; MILLER, 2012). In two *Oryza* species, *O. sativa* and *O. rufipogon*, the variation in the ammonium uptake was associated with the presence of nucleotide polymorphism in the promoter sequence of *OsAMT1.1* (DING et al., 2011). The expression of the *O. rufipogon* allele was significantly higher compared to that of *O. sativa* (DING et al., 2011). This variation, analyzed among 216 rice accessions, suggests that during domestication there was a strong selection around the *OsAMT1.1* gene region, reducing diversity. Identification of allelic variation for nitrate-*NRT1.1B* transporter in *O. sativa* spp. *indica* proved to be advantageous for higher nitrate use efficiency when compared to *O. sativa* spp. *japonica* (HU et al., 2015a).

Near-isogenic lines (NILs) and transgenic lines from spp. *japonica* expressing the *NRT1.1B-indica* allele showed higher NUE due to the induction of nitrate uptake and increased N root-shoot transport, leading to higher grain yield. The presence of single nucleotide polymorphism (SNP) in *japonica* resulted in an amino acid substitution at the cytoplasmic loop region of NRT1.1B, essential for the nitrate transport (HU et al., 2015a). Therefore, the natural genetic variation of wild germplasm can be a source of beneficial alleles for breeding, and understanding the biology of plant membrane transporters can be a key factor in improving NUE (XU; FAN; MILLER, 2012), relevant to the rational use of N with less environmental impact from crop cultivation (GUTIÉRREZ, 2012; SCHROEDER et al., 2013).

2.10 Natural genetic variation studies of NUE in tomato

The occurrence of several signaling networks and regulation pathways for ammonium transporters hampers the process of obtaining plants with improved efficiency in N uptake and, consequently, better NUE (MASCLAUX-DAUBRESSE et al., 2010; GUTIÉRREZ, 2012). An alternative to overcome this problem is the

identification of natural genetic variation for N uptake in species within the same genus (HIREL et al., 2007; RISTOVA; BUSCH, 2014).

Adaptation to extreme environments among *Solanum* species seems to have caused a wide change in transcription networks, together with the positive selection of genes related to environmental adaptation (KOENIG et al., 2013). Analysis of sequence divergence and gene expression between cultivated *S. lycopersicum* and related wild species indicated that domestication may have affected a small number of changes in the DNA sequence and in the transcriptional regulation (KOENIG et al., 2013). However, many proteins and changes in gene expression are potentially harmful and have been associated to domestication. Even though the consequences of these changes are still unknown, it is likely that at least some of them may be associated with a decrease in the vigor of current cultivars (KOENIG et al., 2013).

As a result, wild species may have characteristics of interest that could potentially be valuable for the improvement of cultivated *S. lycopersicum*, as well as the identification of variation in characteristics that contribute to NUE, which could be transferred to elite cultivars by further improvement via conventional breeding or genome editing (XU; FAN; MILLER, 2012). Plants under low N stress conditions show remarkable mechanisms associated with stress responsiveness, *e.g.* increase in chlorophyll synthesis (BI et al., 2005), lignin content in the cell wall (PENG et al., 2008), changes in root architecture (ZHANG; FORDE, 1998), increased N assimilation (IMAMURA et al., 2009), and changes in the level of certain metabolites, such as sugars and sugar phosphates in the shoots (WATANABE et al., 2010). Distinctive characteristics of the cultivated *S. lycopersicum* and wild tomato species involved in N stress tolerance might be associated with of specific gene sets (BOLGER et al., 2014); however, to our knowledge, quantitative modulation of N responsive genes due to domestication or breeding has not yet been reported in tomato.

3 MATERIAL AND METHODS

3.1 Plant material

The tomato accessions *S. habrochaites* LA1718, *S. chmielewskii* LA2695, *S. pimpinellifolium* LA1584, and *S. lycopersicum* cv. M82 were used in ^{15}N-labeled ammonium influx experiments and RT-qPCR gene expression analysis. Seeds were kindly provided by Prof. Lázaro Eustáquio Pereira Peres from the Hormonal Control and Plant Development Laboratory at ESALQ/USP, Piracicaba, Sao Paulo, Brazil.

3.2 *In silico* material

The *in silico* analysis was based on 287 *Solanum* section *Lycopersicon* genomic sequences (Supplementary Table 1), obtained from the SolGenomics database (https://solgenomics.net/), including cultivated tomato (*S. lycopersicum*) and 13 wild relatives: *S. arcanum*, *S. chmielewskii*, *S. neorickii*, *S. pennellii*, *S. habrochaites*, *S. chilense*, *S. huaylasense*, *S. peruvianum*, *S. corneliomulleri*, *S. cheesmaniae*, *S. galapagense*, *S. pimpinellifolium,* and *S. lycopersicum* var. *cerasiforme*.

Genome Wide Association Study (GWAS) was performed based on transcriptomes of 31 *Solanum* section *Lycopersicon* accessions (Supplementary Table 2), including 12 cultivars (*S. lycopersicum*) accessions and 19 accessions of wild genotypes (*S. lycopersicum* var. *cerasiforme*, *S. arcanum, S. galapagense, S. habrochaites*, *S. huaylasense*, *S. neorickii, S. pimpinellifolium*, and *S. pennellii*).

Sequences of tomato genes and proteins were obtained from the SolGenomics database (https://solgenomics.net/). Sequences of *A. thaliana* genes and proteins were obtained from the TAIR database (https://www.arabidopsis.org/). AMT/MEP protein sequences from *Brassica napus, Capsicum annuum, Lotus japonica, Nicotiana tabacum, Oryza sativa, Physcomitrella patens, Populus trichocarpa, Solanum tuberosum, Sorghum bicolor, Triticum aestivum, Zea mays, Chlamydomonas reinhardtii, Escherichia coli, Galdieria sulphuraria* and *Saccharomyces cerevisiae* were obtained from the UniProt database (https://www.uniprot.org/) (Supplementary Table 3).

3.3 Sequence diversity and phylogenetic relationships

To determine conserved domains among protein families represented by multiple sequence alignments and hidden Markov model (HMMs), we used the Pfam database (https://pfam.xfam.org/) (MISTRY et al., 2021). Chromosomal mapping for spatial location of genes was performed using mapping data available at the SolGenomics database (https://solgenomics.net/cview/map.pl?map_version_id=151). To determine gene structure and exons and introns organization of the evaluated sequences, Gene Structure Display Server (http://gsds.gao-lab.org/) was employed (HU et al., 2015b).

Analysis of synonymous and non-synonymous variability and dN/dS in tomato sequences was performed using the SNAP Software (version 2.1.1) (https://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html) (KORBER, 2000). Graphical representations of an amino acid or nucleic acid multiple sequence alignment were performed using WebLogo (https://weblogo.berkeley.edu/) (CROOKS et al., 2004). Analysis of trans-membrane proteins topology were performed using Protter (http://wlab.ethz.ch/protter/) (OMASITS et al., 2014). For sequence alignments, NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and CLUSTAL OMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/) were used. Phylogenetic and molecular evolutionary analysis were conducted in MEGA version 7 (KUMAR; STECHER; TAMURA, 2016) using Maximum Likelihood statistical method with 1000 bootstraps replications.

3.4 RNAseq analysis

Public transcriptome files (FASTQ files) were obtained from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra).

For gene expression analysis in tomato tissues, *e.g.* roots, leaves, flowers and fruits, we used data from a high-quality genome sequence and transcriptome analysis of domesticated tomato *S. lycopersicum* cv. Heinz LA1706 (Bioproject SRA049915, SATO et al., 2012), where relative expression of all tomato genes was determined by three times replicated strand-specific Illumina RNA-Seq. For expression data between *Solanum* section *Lycopersicon* accessions, we have used to define

expression divergence between cultivated tomato *S. lycopersicum* cv. M82 LA3475 and four related wild species (*S. habrochaites* LA1777, *S. chmielewskii* LA1840, *S. pimpinellifolium* LA1589 and *S. pennellii* LA0716) from Bioproject PRJNA192978 (KOENIG et al., 2013) sequenced in 12 replicates runs of the Illumina GA II RNAseq. For analysis of expression in tomato fruit tissues (epidermis, collenchyma, parenchyma, vascular tissue, pericarp, septum, locular tissue, placenta, columella and seeds), we used the data from an high-resolution spatiotemporal transcriptome mapping of tomato fruit development and ripening (SHINOZAKI et al., 2018) available at the Tomato Expression Atlas (https://tea.solgenomics.net/). Lastly, for expression analysis used in the Genome Wide Association Study (GWAS), we used 22 accessions long-read sequencing data from a representative population-scale tomato panel (Bioproject PRJNA557253, ALONGE et al., 2020) and the whole transcriptomes from nine accessions in the ecologically and reproductively diverse wild tomato clade (Bioproject PRJNA305880, PEASE et al., 2016) (Supplementary Table 2).

FASTQ files were quality-checked using FastQC (version 0.11.9) (ANDREWS, 2019). When necessary, the files were filtered/trimmed using trimmomatic (version 0.39) (BOLGER; LOHSE; USADEL, 2014). The Salmon software package (version 0.12.0) (PATRO et al., 2017) was used in *quasi*-mapping-mode for transcript quantification, while transcript abundance was quantified in transcripts per kilobase million (TPM). The tximport (version 1.14.2) package (SONESON; LOVE; ROBINSON, 2015) was used to import into R (version 4.0.5) and summarize the TPM estimates at gene level obtained from Salmon. Heatmaps were generated using the R package pheatmap (version 1.0.12) (KOLDE, 2019).

3.5 Cultivation conditions

Tomato seeds were germinated in pots containing soaked vermiculite. Fourteen days after sowing, seedlings were transferred to a hydroponic system with nutrient solution containing 2 mM NH_4NO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 250 µM K_2SO_4, 250 µM $CaCl_2$, 100 µM Na-Fe-EDTA, 50 µM KCl, 50 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $ZnSO_4$, 1 µM $CuSO_4$, and 1 µM $NaMoO_4$, with pH adjusted to 5.8 with KOH, according to Loqué et al. (2006). The nutrient solution was aerated by a compressor at 30 min h^{-1} frequency and renewed weekly. Solution pH was monitored daily, and adjusted to 5.8 with KOH, whenever necessary. Plants were

grown hydroponically under non-sterile conditions for 30 d in a growth chamber under the following conditions: 12h/12h light/dark; light intensity 250 µmol m^{-2} s^{-1}; temperature 24ºC and 80% humidity.

3.6 Kinetics of N-ammonium uptake by tomato roots according to N availability

To investigate the kinetics of N-ammonium uptake by roots of tomato genotypes according to the N availability, plants were grown in nutrient solution under N sufficiency (2 mM NH_4NO_3) for 30 d, when two N availability conditions were imposed for 72 h, with a limiting concentration (-N, no N added) or a sufficient concentration (+N, 2 mM NH_4NO_3). After this period, roots were submitted to the short-term ^{15}N-labeled ammonium uptake analysis (LOQUÉ et al., 2006). The tomato roots were rinsed in 1 mM $CaSO_4$ solution for 1 min to eliminate apoplastic N, followed by an incubation of 10 min in nutrient solution containing 0.1 mM of $(^{15}NH_4)_2SO_4$ (60 atom% ^{15}N) as the sole N source, and 1 mM MES [2-(N-Morpholino)ethanesulfonic acid monohydrate], pH 5.8, followed by a final wash in 1 mM $CaSO_4$ solution for 1 min. All nutrient solutions were adjusted to pH 5.8. Roots were then harvested and separated for RNA extraction (stored at -80ºC) or dried at 50°C until constant weight for further analysis of ^{15}N determination by Stable Isotope Mass Spectrometry (HIDRA 20-20 ANCA-GLS Sercon, England).

3.7 Kinetics of N-ammonium uptake by tomato roots after ammonium resupply

Plants from the various genotypes were grown in nutrient solution under N sufficiency (2 mM NH_4NO_3) for 30 d, followed by N deficiency for 72 h. After N-deficiency, 4 mM NH_4Cl was used for ammonium resupply for 1 h, followed by the ^{15}N short-term uptake (LOQUÉ et al., 2006) analysis as described above. Roots were then harvested and separated for RNA extraction (stored at -80ºC) or dried at 50°C until constant weight for further analysis of ^{15}N determination by Stable Isotope Mass Spectrometry (HIDRA 20-20 ANCA-GLS Sercon, England).

3.8 RNA extraction and cDNA synthesis

Total RNA from root samples were extracted using TRIzol (Thermo Fisher Scientific, Carlsbad, California, USA). Samples were ground in liquid nitrogen and transferred to a 2 mL microtube, where 1 mL of TRIzol reagent was added for each 100 mg of sample. Tubes were vortexed and after 5 min incubation at room temperature, 200 µL of chloroform was added. Tubes were incubated for more 3 min at room temperature, centrifuged at 10,000 *g* for 15 min at 4ºC, and the upper aqueous phase was transferred to a new tube. Then, 500 µL of isopropanol was added per sample and after 10 min incubation at room temperature, tubes were centrifuged at 10,000 *g* for 10 min at 4ºC and the supernatant was discarded. Pellets were washed with 1 mL of 75% ethanol (inactive 0.01% DEPC H_2O) and centrifuged at 10,000 *g* for 5 min at 4ºC. Supernatant was removed and pellet was washed with 1 mL of 75% ethanol (inactive 0.01% DEPC H_2O) two more times. Finally, the pellet dried at room temperature and resuspended in 20 µL of inactive 0.01% DEPC H_2O. Total RNA was quantified in NanoDrop (Thermo Scientific) and the integrity confirmed via electrophoresis.

After quantification, 2 µg of RNA from each sample was treated using 1 U of DNAse I in an appropriate buffer, 2 U of RiboLock (Thermo Scientific) in a reaction incubated at 37ºC for 30 min. The reaction was stopped with the addition of 5 mM EDTA, and incubated at 65ºC for 10 min. For cDNA synthesis, samples of 1 µg of total RNA treated with DNase and the oligo dT (0.5 µg μL^{-1}) were denatured at 65°C for 10 min, incubated at 4°C for 2 min, followed by addition of 5x RT enzyme buffer, 1 mM dNTP, 20 U of RiboLock (Fermentas) and 200 U of SuperScript III Reverse Transcriptase enzyme (Thermo Scientific) in a final volume of 10 µL. The reaction was then incubated at 55ºC for 1 h, followed by 70ºC for 10 min and at 4ºC for 2 min.

3.9 Primer design for reverse transcription quantitative PCR (RT-qPCR)

Primers were designed based on *SlAMT1.1* orthologous genes obtained from tomato sequences (Table 2) extracted in the SolGenomics Network database (http://solgenomics.net/). Primers were designed using Primer3 (https://primer3.ut.ee/), defining the melting temperature (Tm) between 59 and 61ºC, and size of amplicons between 85 and 145 base pairs (Table 2). For each gene,

primers were tested using NetPrimer (http://www.premierbiosoft.com/netprimer/) for stability, Tm, GC content (%), and interactions between primers. Ubiquitin (*SlUBQ3*) and actin (*SlACT7*) were used as gene reference to perform RT-qPCR.

Table 2 - Sequence of primers used in RT-qPCR.

Gene	SolGenomics ID	Primer Sequence	Amplicon length
SlAMT1.1	Solyc09g090730	F- CCGGGTTATTCGCTAAAGGGG R- GATTATATGCGCCCCGAGTAGTTT	113 bp
SlUBQ3	Solyc01g056940	F- ACCAAGCCAAAGAAGATCAAGCACAA R- TGAACCTTTCCAGTGTCATCAACCTT	108 bp
SlACT7	Solyc03g078400	F- TGAATGCACGGTAGCAAACAACAGATT R- AATGCATCAGGCACCTCTCAAGTAT	99 bp

3.10 Reverse transcription quantitative PCR (RT-qPCR)

Reactions contained 5 µL of SYBR Green qPCR Master Mix (Invitrogen; Carlsbad, CA, USA), 0.2 µM of each primer, 1 µL of the 1:10 (v/v) cDNA and sterile Milli-Q water to a final volume of 14 µL. The RT-qPCR analysis was performed on RotorGene-6000 (Qiagen; Hilden, Germany), and included negative control (without cDNA). The amplification thermal profile started with two initial temperatures: 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of three steps: 95°C for 20 s, 60º C for 30 s and 72°C for 30 s. After amplification, the dissociation curve between 72 and 95°C was determined. In all experiments, C_Q (quantification cycle) values were used to determine the relative difference in gene expression. Relative expression was standardized using *SlACT7* and *SlUBQ3* as gene reference. Relative expression was calculated according to Pffaffl (2001).

3.11 Genome Wide Association (GWA) Mapping and Sequence Mining

SlAMT1.1 (Solyc09g090730) expression levels were calculated in transcripts per kilobase million (TPM) for each of the 31 accessions by Salmon (PATRO et al., 2017). The average expression of *SlAMT1.1* was then estimated. The accessions were then divided into two groups: (1) those that express *SlAMT1.1* above average;

and (2) those that express *SlAMT1.1* below the average. The difference in expression of *SlAMT1.1* for the two formed groups was used as phenotypic response in GWAS.

The *S. lycopersicum* cv. Heinz reference genome (version ITAG 2.4) was obtained from the SolGenomics database. Bowtie2 (version 2.3.5.1) (LANGMEAD; SALZBERG, 2012) was used to generate the reference genome index for mapping transcriptome reads using Tophat2 (version 2.1.1) (KIM et al., 2013). Subsequently, Samtools (version 1.10) (LI et al., 2009) was used to generate the VCF files of the hits obtained by Tophat2. The whole genome association analysis was performed by Plink (version 1.07) (PURCELL et al., 2007). The SNPs (single nucleotide polymorphism) associated with the expression of *SlAMT1.1* resulting from the GWA analysis was presented in a Manhattan plot using the qqman package (version 0.1.4) (TURNER, 2018) in software R (version 4.0.5).

3.12 Statistical analysis

Gene expression levels and ^{15}N-$(NH_4)_2SO_4$ influx data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test at $p<0.05$. Pairwise comparisons were carried out using the Welch *t* test. All statistical analysis was performed in GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, California USA).

4 RESULTS

4.1 Genome survey for *AMT*s gene family members in *S. lycopersicum*

In higher plants, ammonium transporters are categorized into two subfamilies, named *AMT1* and *AMT2* (NEUHÄUSER; DYNOWSKI; LUDEWIG, 2009). The number of *AMT* genes present in the genome of plant species varies considerably. In *A. thaliana,* six genes have been identified as members of the *AMT* gene family (GAZZARRINI et al., 1999). In the rice genome, 10 *AMT*s were found (LOQUÉ; VON WIRÉN, 2004), whereas 16 were found in soybean (KOBAE et al., 2010), and 16 in poplar (WU et al., 2015). To date, only four *AMT*s genes (*SlAMT1.1, SlAMT1.2, SlAMT1.3* and *SlAMT2.1)* have been described in *S. lycopersicum* (D'APUZZO et al., 2004), which might indicate that only part of the *AMT* genes have been identified in the *S. lycopersicum* genome.

Here, we have used public databases, that currently contain vast amounts of DNA sequences of the tomato pan-genome that deepen and complement the reference genome sequence (GAO et al., 2019), to survey for *AMT*-gene family members in *S. lycopersicum.* Based on BLAST (https://solgenomics.net/tools/blast/) (Genome version ITAG 2.4, SATO et al., 2012) and hidden Markov models (HMMs) (https://pfam.xfam.org/) profile searches, and subsequent AMT domain analysis, eight putative *AMT* gene members were identified (Table 3), four of which have not previously been described (D'APUZZO et al., 2004).

We then classified the eight *S. lycopersicum AMT* genes according to the AMT/MEP superfamily. A phylogenetic analysis was conducted using full length protein sequences from *S. lycopersicum* together with 12 representative members of plant AMT family, including those from *Arabidopsis thaliana*, *Brassica napus, Capsicum annuum*, *Lotus japonica*, *Nicotiana tabacum*, *Oryza sativa*, *Physcomitrella patens, Populus trichocarpa*, *Solanum tuberosum, Sorghum bicolor*, *Triticum aestivum* and *Zea mays*, plus three representative members of non-plant AMT family (*Chlamydomonas reinhardtii*, *Escherichia coli*, and *Galdieria sulphuraria*) and one representative of MEP family (*Saccharomyces cerevisiae*) (Figure 4; Supplementary Table 3). Based on the pattern of the tomato *AMT* members clustering, the new identified genes were denominated *SlAMT3.1, SlAMT3.2*, *SlAMT3.3,* and *SlAMT3.4*.

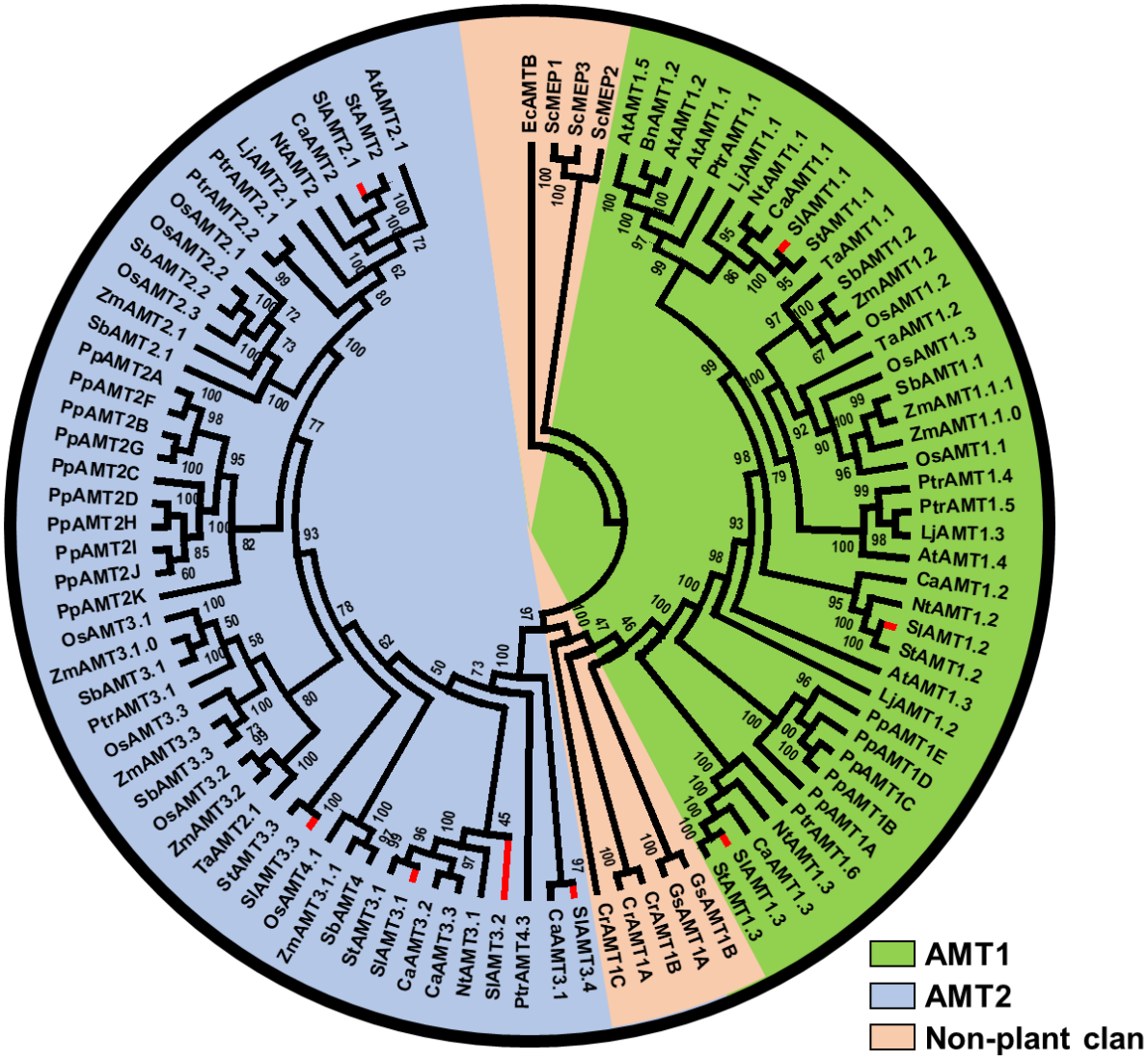


Figure 4 - Phylogenetic analysis of 17 AMT/MEP superfamily members. Protein sequences were aligned by ClustalW and the tree was constructed by MEGA7 based on maximum-likelihood with 1,000 bootstrap replicates. The eight tomato AMT proteins (SlAMT1.1, SlAMT1.2, SlAMT1.3, SlAMT2.1, SlAMT3.1, SlAMT3.2, SlAMT3.3, and SlAMT3.4) are highlighted with red branches.

A new phylogenetic analysis was performed only with the eight proteins identified as tomato AMTs (Figure 5). The strict consensus tree indicates a grouping into two subfamilies: AMT1 including three members (SlAMT1.1, SlAMT1.2, and SlAMT1.3) and AMT2 including five members (SlAMT2.1, SlAMT3.1, SlAMT3.2, SlAMT3.3, and SlAMT3.4). The AMT2 subfamily can be further subdivided into two clades, the first one that includes only the previously described SlAMT2.1, and the second that contains the four new AMTs identified here (SlAMT3.1, SlAMT3.2, SlAMT3.3, and SlAMT3.4).

The *SlAMTs* genes structure analysis giving the organization of exons and introns was performed using the Gene Structure Display Server (http://gsds.gao-lab.org/), by comparing the coding and genomic sequences of the tomato *AMT*s genes (Figure 5). Among the members of the AMT1 subfamily, *SlAMT1.1* contains three exons and two introns, similar to *SlAMT1.2*, while *SlAMT1.3* has only one exon. Regarding the AMT2 subfamily, the three members that are phylogenetically closer (*SlAMT3.1, SlAMT3.2* and *SlAMT3.4*) also present a similar structure with three exons, while the other two members (*SlAMT2.1* and *SlAMT3.3*) have four exons and three introns.

We then determined the genomic position (locus), length of gene sequence, predicted length of amino acid sequence, full protein isoelectric point (http://www.isoelectric.org/), protein molecular weight (https://www.bioinformatics.org/sms2/protein_mw), and conceptually estimated the number of transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM/) of the identified AMT proteins (Table 3). The length of *AMT* genes ranged from 1383 (*SlAMT1.3*) to 3112 (*SlAMT3.3*) base pairs (bp), while AMT proteins ranged from 460 (SlAMT1.3) to 514 amino acids (SlAMT1.2). The isoelectric points of the AMT varied from 5.16 (SlAMT1.3) to 6.83 (SlAMT2.1), and the mass of these proteins were between 49.66 kDa (SlAMT1.3) and 55.38 kDa (SlAMT1.2). In general, the AMTs had 11 predicted transmembrane domains, with the exception of SlAMT1.3, which appeared to have 10 transmembrane domains.

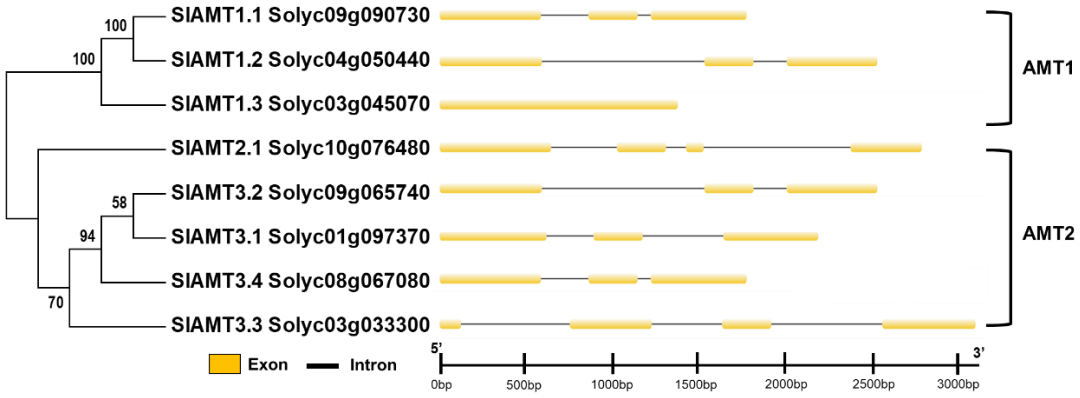


Figure 5 - Strict consensus tree based on AMTs protein sequences from *S. lycopersicum* cv. Heinz. Protein sequences were aligned by ClustalW and the tree was constructed by MEGA7 using maximum-likelihood method with 1,000 bootstrap replicates. The right side illustrates the exon-intron organization of *SlAMT*s genes, performed using Gene Structure Display Server (http://gsds.gao-lab.org/).

Table 3 - Characteristics of *SlAMT*s genes and the encoded AMT proteins identified in tomato, including the Gene ID in the SolGenomics ITAG 2.4 annotation database, location of the locus, size of the gene in base pairs, size of the protein in number of amino acids, estimated protein molecular weight, estimated isoelectric point, number of transmembrane domains and number of exons.

Gene	ID	Chromosome Location	Gene length (bp)	Protein length (aa)	Molecular Weight (kDa)	Isoelectric Point	Transmembrane domains	Number of exons
SlAMT1.1	Solyc09g090730	SL2.50**ch09**:70165532-70167004	1473	490	52.58	6.48	11	3
SlAMT1.2	Solyc04g050440	SL2.50**ch04**:47231399-47234372	2974	514	55.38	6.68	11	3
SlAMT1.3	Solyc03g045070	SL2.50c**h03**:11495587-11496969	1383	460	49.66	5.16	10	1
SlAMT2.1	Solyc10g076480	SL2.50**ch10**:59472160-9474959	2800	483	52.17	6.83	11	4
SlAMT3.1	Solyc01g097370	SL2.50**ch01**:88259856-88261638	1783	476	51.85	5.94	11	3
SlAMT3.2	Solyc09g065740	SL2.50**ch09**:64021974-64025085	3112	475	52.49	6.19	11	3
SlAMT3.3	Solyc03g033300	SL2.50**ch03**:4881297-4883838	2542	468	51.86	6.58	11	4
SlAMT3.4	Solyc08g067080	SL2.50**ch08**:55994765-55996963	2199	485	52.70	5.99	11	3

The location and distribution of *AMT*s genes in the *S. lycopersicum* genome were determined (Figure 6; Table 3), based in SolGenomics ITAG 2.3 annotation. Chromosomal location analysis showed that the eight tomato *AMT* genes are distributed in six of the 12 chromosomes: *SlAMT3.1* in chromosome 1, *SlAMT1.3* and *SlAMT3.3* in chromosome 3, *SlAMT1.2* in chromosome 4, *SlAMT3.4* in chromosome 8, *SlAMT1.1* and *SlAMT3.2* in chromosome 9, and *SlAMT2.1* in chromosome 10. No *AMT*s were identified on chromosomes 2, 5, 6, 7, 11, and 12, meaning that only half of the tomato chromosomes harbor *AMT* genes.

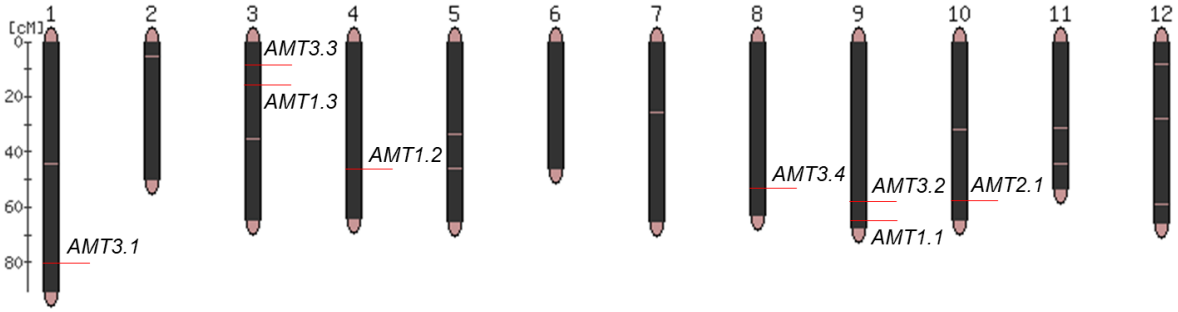


Figure 6 - Chromosomal location of the *AMT* gene family members in tomato. Chromosome number (ch01-ch12) is indicated at the top of each chromosome. Data obtained from SolGenomics ITAG 2.3 annotation (https://solgenomics.net/cview/map.pl?map_version_id=151).

4.2 *SlAMT*s differential expression in tomato

Orthologous members of the AMT family may differ in terms of their expression among plant tissues (LOQUÉ; VON WIRÉN, 2004). We characterized the expression of the *AMT* genes here identified in cv. Heinz in four tissues (leaves, roots, flowers and fruits) based on RNA-seq data publicly available to establish an inventory of expressed *AMT*-genes (Figure 7A). RNAseq sequencing data were obtained in duplicate and the results presented are the average gene expression in transcripts per kilobase million (TPM).

SlAMT1.1 showed significantly higher expression levels in tomato roots, followed by expression in flowers and leaves, with lower expression in fruits (Figure 7A). *SlAMT1.1* expression in roots was as much as three times higher than expression in flowers and nine times higher than in leaves. *SlAMT1.2* also presented higher expression in roots, followed by flowers and leaves with statistically similar values, but practically no expression in fruits (Figure 7A). *SlAMT1.3*, in turn, had significantly

higher expression levels in leaves than in flowers and had a minimal expression in roots and fruits (Figure 7A). *SlAMT1.3* expression in leaves is 4-fold higher than in flowers. Regarding the AMT2 genes, *SlAMT2.1* also showed higher expression values in roots, followed by an 18-fold lower expression in fruits, flower and leaves (Figure 7A). The new orthologous identified here (*SlAMT3.1*, *SlAMT3.2*, *SlAMT3.3*, and *SlAMT3.4*) displayed lower expression levels compared to the four previously described *AMT* genes (*SlAMT1.1, SlAMT1.2, SlAMT1.3,* and *SlAMT2.1*). While *SlAMT3.1* and *SlAMT3.2* were similarly expressed in leaf, root and fruit, *SlAMT3.3* transcripts were present only in flowers. In our expression analysis, no *SlAMT3.4* transcript accumulation was detected in any of the evaluated tissues in *S. lycopersicum* cv. Heinz (Figure 7A).

In addition, we also assessed the expression levels of the eight *AMT* genes within each tissue (Figure 7B). *SlAMT1.1* is the most expressed gene in tomato roots, followed by *SlAMT1.2* and *SlAMT2.1*. *SlAMT1.3*, *SlAMT3.1*, *SlAMT3.2,* and *SlAMT3.3* showed minimal expression in roots (Figure 7B). *SlAMT1.1* also displayed the highest level of expression in flowers and is the second gene more expressed in leaves. *SlAMT1.2* also showed expression in flowers and leaves, but 80% and 90% less than *SlAMT1.1* (Figure 7B). Although *SlAMT1.3* showed almost no expression in roots, it is the most expressed *AMT* gene in leaves and the second most expressed in flower. Moreover, *SlAMT2.1* is the *AMT* with the highest expression in fruits. The new genes identified (*SlAMT3.1* and *SlAMT3.3*) are grouped among those with lower expression in all tissues. Only *SlAMT3.2* stands out, which is the second most expressed *AMT* gene in fruits.

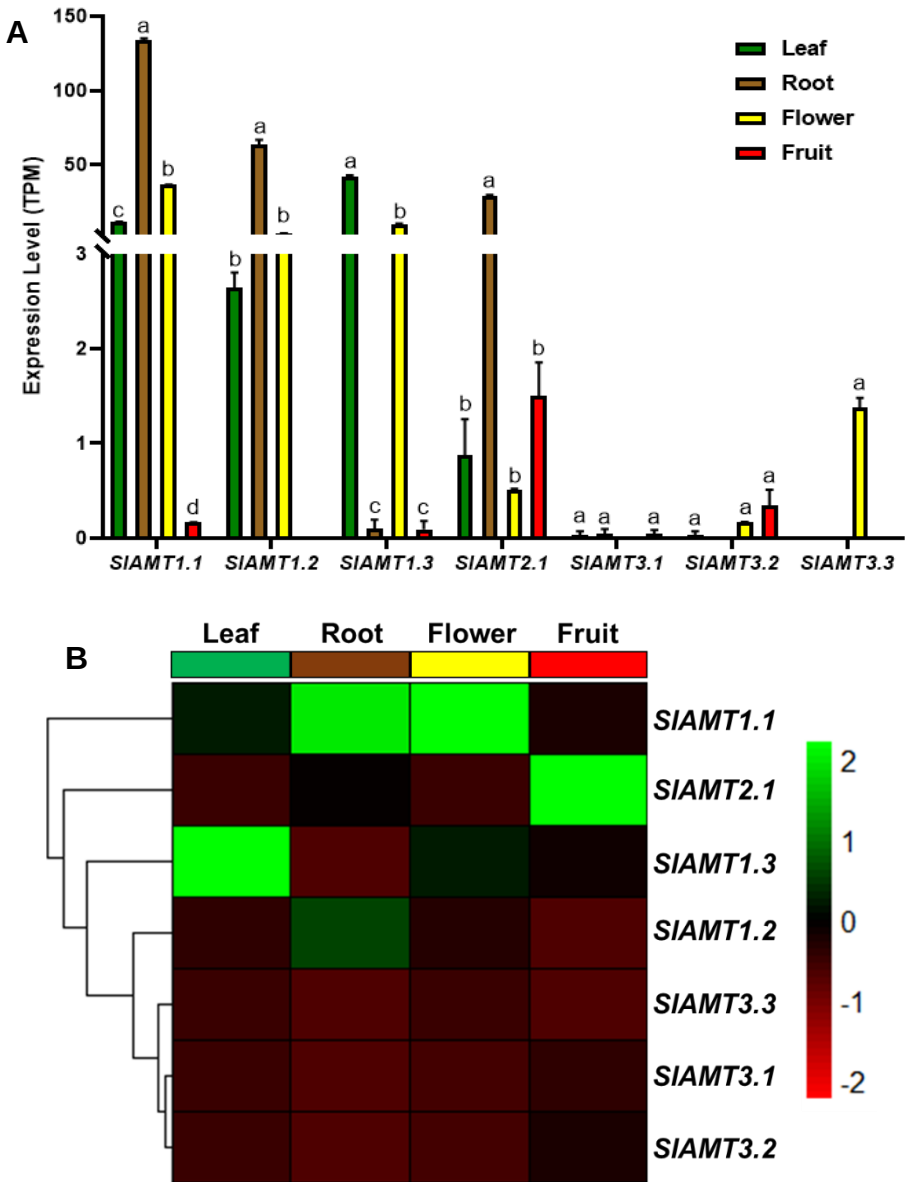


Figure 7 - *SlAMT* gene members expression in tomato tissues. (A) Absolute expression level in TPM (transcripts per kilobase million) of *SlAMT*s in roots, leaves, fully opened flowers and breaker fruits *of Solanum lycopersicum* cv. Heinz . Significant differences between tissues within genes at $p<0.05$ according to Tukey's test are indicated by different letters; (B) Heatmap for *AMT*s relative expression in roots, leaves, fully opened flowers and breaker fruits *in S. lycopersicum* cv. Heinz. Data is normalized by tissue (columns). Based on RNAseq data: (SATO et al., 2012).

Gene expression analysis revealed a substantial variation among wild and domesticated tomatoes, which might contribute to phenotypic diversity (ALONGE et al., 2020). We evaluated *AMT*s expression in seedling shoots of five species of the *Solanum* section *Lycopersicon* (KOENIG et al., 2013), including the closest wild relatives of domesticated tomato (*S. pimpinellifolium*), three green-fruited wild accessions from vastly distinct habitats (*S. habrochaites*, *S. chmielewskii*,

and *S. pennellii*) and one accession of domesticated tomato (*S. lycopersicum* cv. M82) using deeply sequenced transcriptomes (Figure 8).

SlAMT1.1 expression level was significantly higher in *S. pennellii*, followed by *S. chmielewskii*, *S. habrochaites* and the cultivar M82, and is less expressed in *S. pimpinellifolium*. Notably, *SlAMT1.2* and *SlAMT1.3* were statistically highly expressed in cv. M82 and *S. pennellii* (only for *SlAMT1.3*), showing intermediate levels in *S. chmielewskii* and *S. pimpinellifolium*, and reduced level of transcripts in *S. habrochaites*. These results indicate a significant variation on *AMT1* genes expression in wild and cultivated tomatoes.

For *AMT2* gene family members, there was relative lower level of transcripts compared to *AMT1* members. The substantial more expressed *SlAMT2.1*, displayed greater expression levels in *S. pennellii* and *S. chmielewskii* and significantly lower expression in cv. M82, *S. pimpinellifolium* and *S. habrochaites*, suggesting a broadly global changes in Sl*AMT2.1* expression in tomato wild species. Among the new identified *AMT2* genes, only *SlAMT3.2* and *SlAMT3.3* showed expression identified in this RNAseq. *SlAMT3.2* presented significantly higher expression levels in *S. pennellii* and cv. M82, while *SlAMT3.3* is more expressed in *S. habrochaites* and *S. pennellii*.

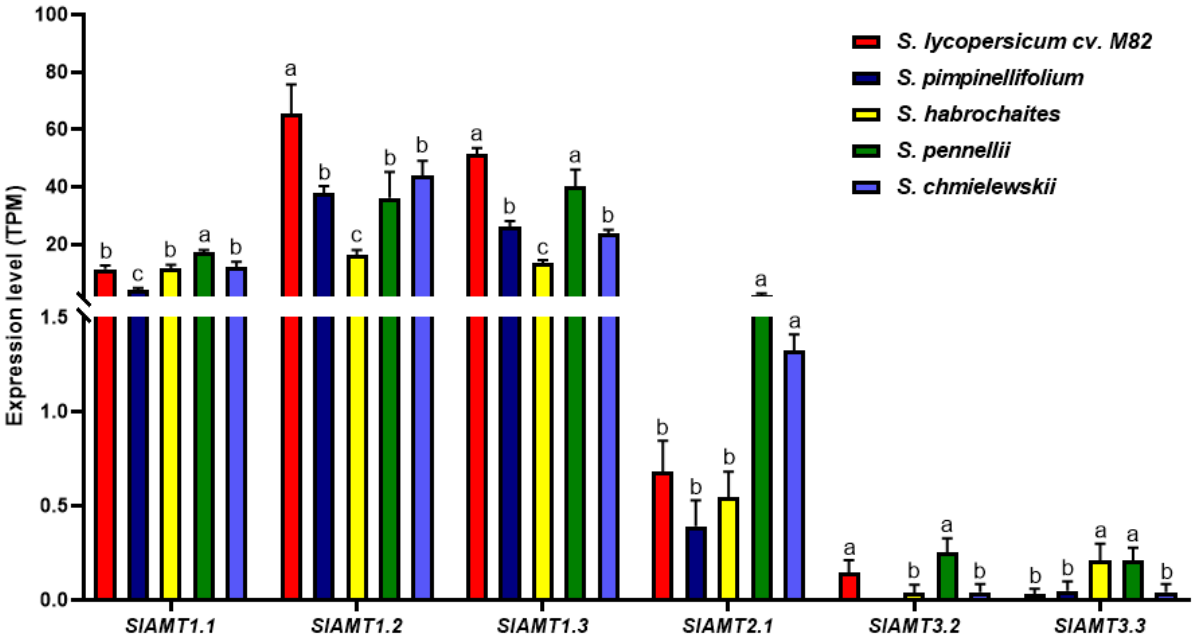


Figure 8 - *SlAMT*s expression in seedlings of tomato cultivar and wild relatives *S. pimpinellifolium*, *S. habrochaites*, *S. pennellii*, and *S. chmielewskii*. Absolute expression level of *AMT*s in tomato wild species and cultivar *S. lycopersicum* cv. M82 seedling shoots 10 days after sowing expressed in TPM (transcripts per kilobase million). Significant differences between genotypes within genes at $p<0.05$ according to the Tukey test are indicated by different letters. Based on RNAseq data: (KOENIG et al., 2013).

To identify *AMT* genes that may potentially have a role in tomato fruits (GAO et al., 2019), major sources of variance for absolute (Figure 9A) and relative (Figure 9B) expression level of *SlAMT*s in *S. lycopersicum* cv. M82 were analyzed in breaker fruit tissues. The RNAseq data (SHINOZAKI et al., 2018) were obtained from Tomato Expression Atlas (http://tea.solgenomics.net/expression_viewer/input). The RNAseq sequencing data were only available in mean values, without individual replicates, which did not allow statistical analysis for these results.

The clear mostly expressed *AMT* gene in fruits is *SlAMT2.1*, with consistent transcript accumulation in the epidermis, collenchyma, pericarp, and vascular tissues. Conversely, the pattern of *SlAMT1.1* expression is mainly present in the collenchyma, pericarp and seeds. While lower expression levels of *SlAMT1.2* occurs only in the tissues of the pericarp and in seeds, *SlAMT1.3* showed intermediate expression levels in tissues such as epidermis, collenchyma and pericarp.

Interestingly, the *AMT2* gene members identified here, may likely have a functional physiological function in tomato fruits. *SlAMT3.3* was the second most expressed gene in tomato fruits, with transcript accumulation mainly in the epidermis, collenchyma, parenchyma and vascular tissues. In addition, the relative expression of *SlAMT3.2* indicated clear accumulation in parenchyma, pericarp, septum, placenta, and seeds. The expression of *SlAMT3.1* was barely detectable in epidermis, collenchyma, parenchyma and vascular tissue, but *SlAMT3.4* showed the lowest expression levels among the *AMT2*-genes and mainly in seeds.

The overall expression of *AMT3* genes in tomato fruit tissues indicates a possible role of ammonium transport in outer tissues, such as epidermis, followed by the collenchyma and pericarp. By contrast, the inner tissues, septum, locular tissue and placenta showed lower transcripts accumulation of *AMT3* genes.

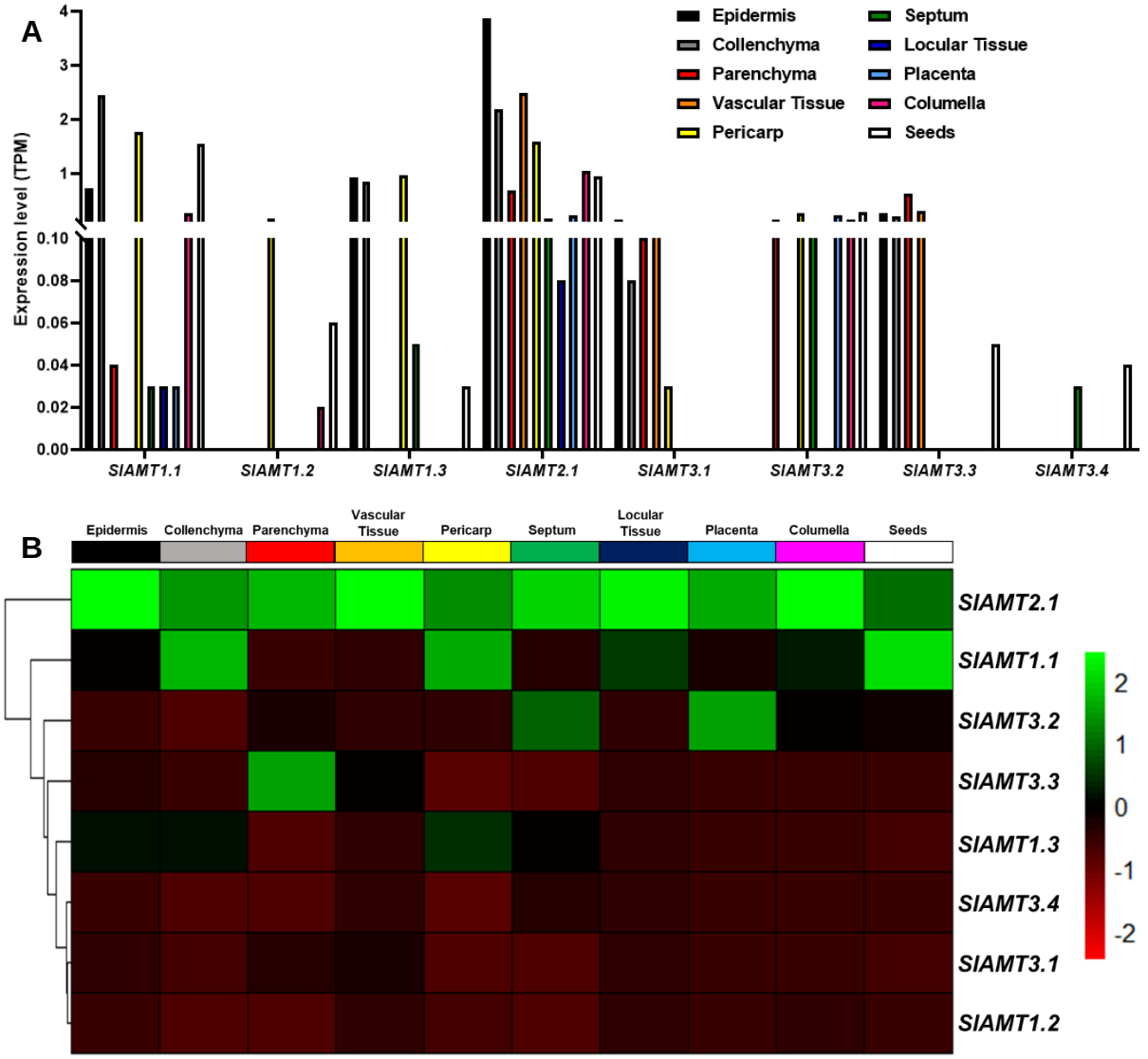


Figure 9 - *SlAMT*s expression in tomato fruit. (A) Absolute expression level of *SlAMT*s in *S. lycopersicum* cv. M82 breaker fruit tissues expressed in TPM (transcripts per kilobase million). Significant differences at $p<0.05$ according to Tukey test are indicated by different letters; (B) Heatmap for relative expression of *SlAMT*s in *S. lycopersicum* cv. M82 *AMT*s in breaker fruit tissues. Data is normalized by columns. Based on RNAseq data: (SHINOZAKI et al., 2018), Tomato Expression Atlas (http://tea.solgenomics.net/expression_viewer/input).

4.3 Genetic variability of the *SlAMT1.1* coding DNA sequence in *Solanum* section *Lycopersicon* clade

Ammonium influx studies on a T-DNA insertion line of *A. thaliana* knocking-out *AMT1.1* revealed that in N-deficient roots, *AtAMT1.1* confers approximately 30% of the total ammonium uptake capacity, that, together with *AtAMT1.3*, can significantly contribute to the overall ammonium influx in roots (KAISER et al., 2002; LOQUÉ et al., 2006). In *S. lycopersicum*, *SlAMT1.3* is not expressed in roots and therefore no

functional role in ammonium uptake in tomato root is expected, indicating that SlAMT1.1 may predominantly work in ammonium uptake in tomato roots (VON WIRÉN et al., 2000a).

To trace the evolutionary relationship among *Solanum* section *Lycopersicon* groups, we explored the diversity and phylogenetic relationships using the *SlAMT1.1* coding DNA sequence (CDS) among 107 wild accessions (83 from Lycopersicon, 16 from Eriopersicon, six from Arcanum, and two from Neolycopersicon) and 180 tomato cultivars. The resulting maximum-likelihood tree supports the clustering of the Lycopersicon group with the cultivar clade, indicating that the Lycopersicon group is evolutionarily closer to the cultivated species (Figure 10A). This close relationship is supported by the fact that the vast majority of tomato cultivars were obtained by breeding from the Lycopersicon group (AFLITOS et al., 2014). The Eriopersicon and Neolycopersicon groups are those phylogenetically more distant in relation to the cultivars. The Arcanum group belongs to an intermediate clade consistent with previous studies (PERALTA; SPOONER; KNAPP, 2008; AFLITOS et al., 2014).

We then evaluated the presence of CDS variability by aligning *SlAMT1.1* sequences from the 107 wild accessions and 180 cultivars to the tomato reference genomic sequence (*S. lycopersicum* cv. Heinz) to estimate the percent of synonymous (SNPs that do not change amino acid residues; Figure 10B) and non-synonymous SNPs (SNPs that change amino acid residues; Figure 10C). As expected, Eriopersicon, Neolycopersicon, and Arcanum groups presented a higher average percent of synonymous and non-synonymous SNPs in their CDS sequences, whereas the Lycopersicon group showed limited variability, and cultivars showed nearly no diversity in their *SlAMT1.1* sequences. These results corroborate the notion that cultivated tomato genomes have lost their variability during domestication and breeding (BAUCHET; CAUSSE, 2012; KOENIG et al., 2013; AFLITOS et al., 2014). Once the presence of SNPs, especially non-synonymous, may cause important changes in the function and/or structure of SlAMT1.1, the wild groups represent an important source for the search for advantageous variability associated with ammonium uptake.

The ratio of non-synonymous / synonymous SNPs (dN/dS) is a suitable statistic to measure the strength and direction of selection on protein-coding genes (JAFFARES et al., 2015). If this ratio is equal to one, the whole coding sequence selection is neutral; 0 < dN/dS < 1 represents evolution under constraint, and when > 1 a positive selection. Most genes exhibit a dN/dS less than 1 because non-synonymous SNPs, in general, are deleterious, while synonymous SNPs are neutral for protein function (JAFFARES et al., 2015). For all taxonomic groups investigated here, dN/dS was < 1 indicating that the *SlAMT1.1* underwent a negative selection. However, breeding through artificial selection can cause the maintenance of a small number of SNPs under positive selection (KOENIG et al., 2013; AFLITOS et al., 2014), which might reflect a slight increase in the cultivars and Lycopersicon dN/dS.

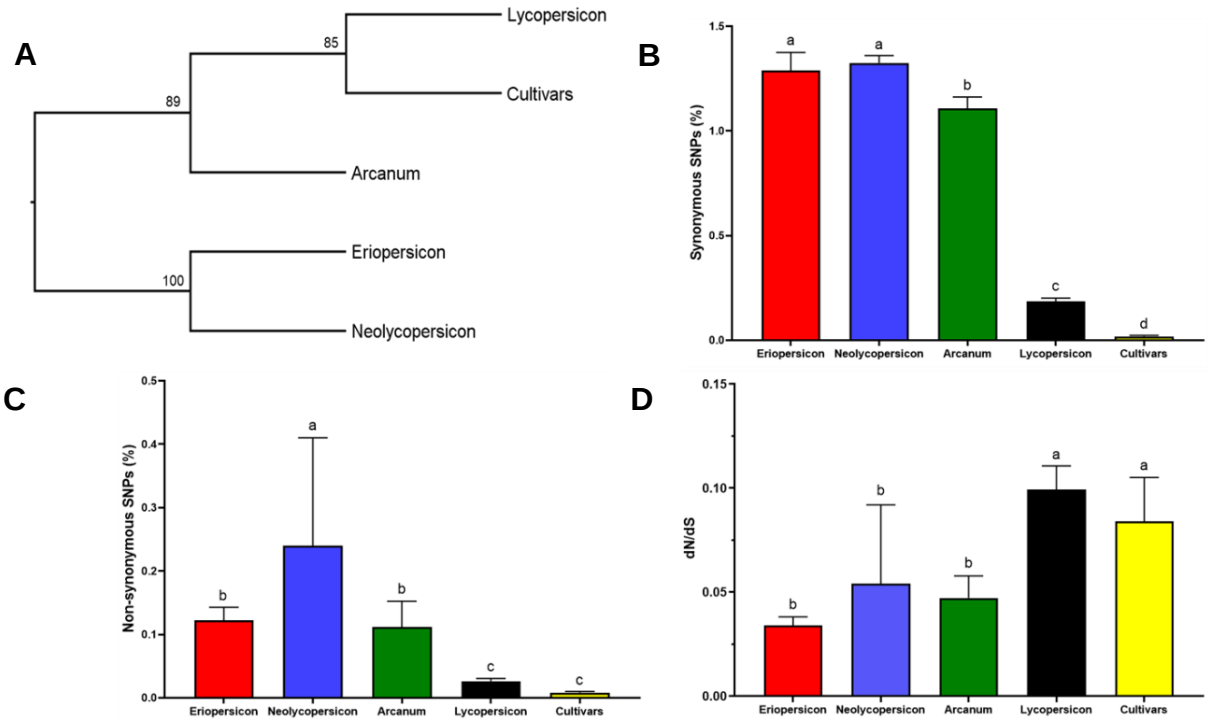


Figure 10 - Nucleotide variability for *SlAMT1.1* in *Solanum* sect. *Lycopersicon* clade. (A) Collapsed tree topology with 1,000 bootstraps of maximum-likelihood analysis based on *SlAMT1.1* CDS from 107 wild accessions and 180 tomato cultivars accessions; (B) Percent of synonymous SNPs in relation to *S. lycopersicum* cv. Heinz reference genome, (C) percent of non-synonymous SNPs in relation to *S. lycopersicum* cv. Heinz reference genome, and (D) dN/dS of *SlAMT1.1* in the various groups of *Solanum*. Bars indicates mean ± SE. Significant differences at $p<0.05$ according to the Tukey test are indicated by different letters.

Grouping the SNPs presence/absence matrix in *SlAMT1.1* sequence relative to the *S. lycopersicum* cv. Heinz reference genome revealed a structure that reflected the larger SNP-based tomato phylogeny, with accessions clustering within their known taxonomic groups (Figure 11). The SNP percent in *SlAMT1.1* for specific members of the Arcanum, Eriopersicon and Neolycopersicon groups are significantly higher than for members of Lycopersicon group and cultivars, which correlates with their more distant position in relation to the tomato clade (PERALTA; SPOONER; KNAPP, 2008). Notably, the sequence variation of the Eriopersicon and Neolycopersicon groups for synonymous and non-synonymous SNPs is larger than the Lycopersicon and cultivar groups. The Arcanum group also presents considerable *SlAMT1.1* variability, in particular, associated with synonymous SNPs (Figure 11). More natural variability found in some wild tomato groups offer an important potential for improving commercial cultivars, whether this variability could provide significant changes in the activity of the SlAMT1.1 protein. The extensive allelic polymorphism found to be enriched in *SlAMT1.1* CDS in wild tomato species needs to be investigated in SlAMT1.1 activity in ammonium uptake of tomato roots.

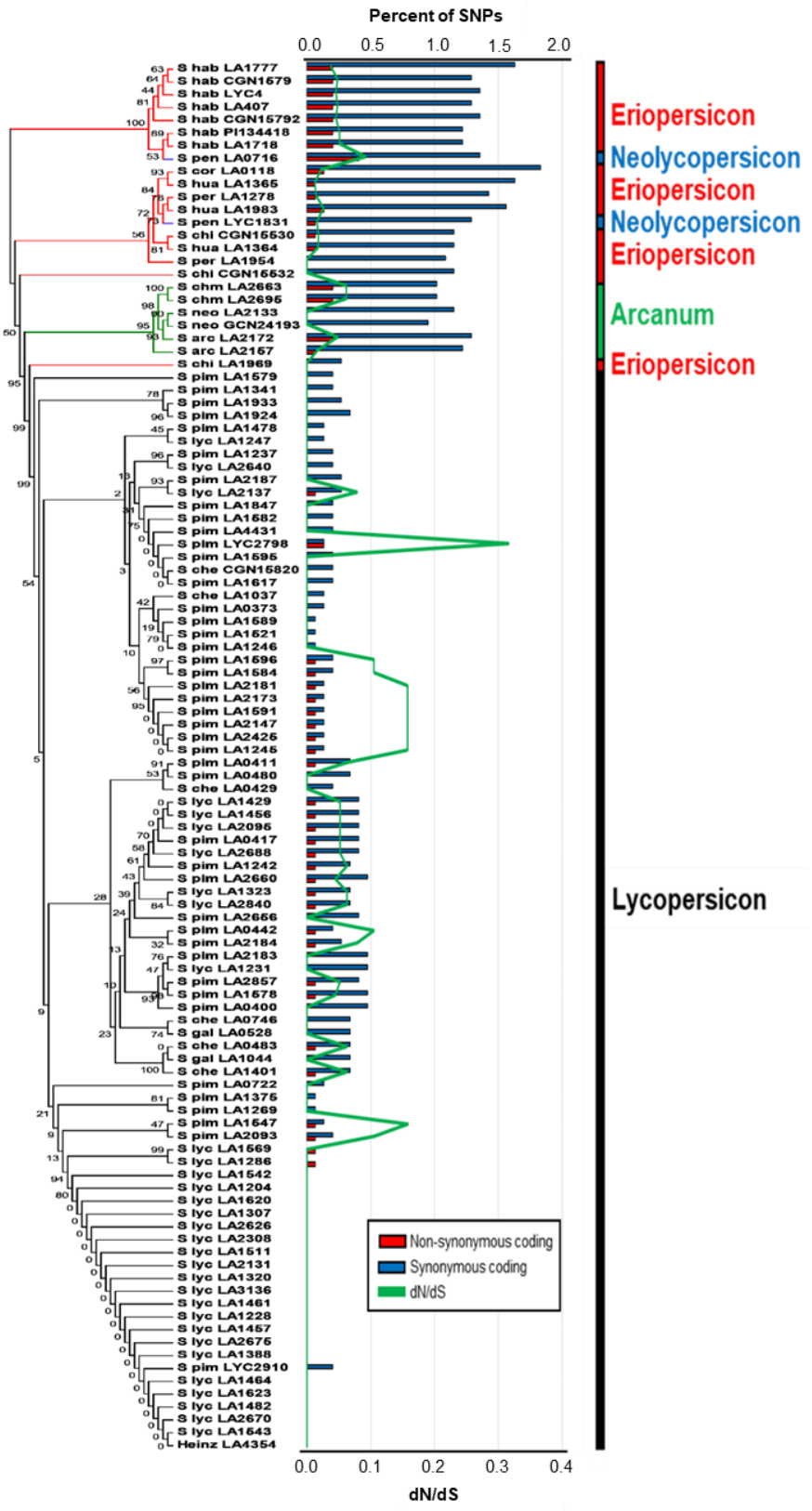


Figure 11 - Genetic diversity in percent of SNPs and dN/dS in accessions of tomato (*Solanum* sect. *Lycopersicon*) clade. Strict consensus tree based on *SlAMT1.1* CDS from 107 accessions with 1,000 bootstraps values obtained by maximum-likelihood analysis. Red bars show the percent of non-synonymous (dN) and blue bars shows the percent of synonymous (dS) SNPs in *SlAMT1.1* of tomato wild species against reference genome *S. lycopersicum* cv. Heinz. Green line shows the dN/dS ratio for *SlAMT1.1* of tomato wild species. Evolutionary analysis were conducted in MEGA7 (KUMAR; STECHER; TAMURA, 2016).

4.4 Identification of divergent SNPs associated with *SlAMT1.1* regulation in wild tomato species

To explore whether the genetic diversity in the *SlAMT1.1* coding sequence in the *Solanum* section *Lycopersicon* might have functional relevance in regulating AMT1s transporter activity, we quantified and classified SNPs for each tomato group. In general, there was a clear enrichment for variants of *SlAMT1.1* in wild tomato groups, but whether these variations in *SlAMT1.1* CDS play a role in the protein activity deserves further investigation. Comparative complete protein sequence alignment for group-, species- and accession-specific polymorphisms showed that more non-synonymous SNPs diversity in SlAMT1.1 exists in wild tomatoes relative to the domesticated species. Here, we performed a variability analysis for each codon that encodes the 490 amino acids of SlAMT1.1 between accessions of *Solanum* section *Lycopersicon* groups in comparation to the reference sequence of *S. lycopersicum* cv. Heinz (Figure 12A).

Non-synonymous SNPs cause amino acid changes throughout almost the entire length of SlAMT1.1. For the 6 assessed accessions of the Arcanum group, 33% had non-synonymous SNPs at codon 102 and 124, 17% had variability at codons 192, 330 and 478. However, the most significant variation occurs at position 236, where 50% of the sequences exhibit amino acid changes. The Neolycopersicon group showed 50% variability at codons 179, 187, 273, 418, and 480, while all sequences showed amino acid changes at position 477 in relation to the reference genome cv. Heinz. However, Neolycopersicon group has only 2 sequenced genotypes, which makes it difficult to observe patterns of variability. In turn, among the 16 accessions analyzed in the Eriopersicon group, 6% had amino acid changes at positions 179 and 348, 12% at position 418, 31% at position 476 and 44% at codon 273. However, the most significant variation for this group is present at position 477, where 81% of the sequences evaluated contained amino acids different from those found in cv. Heinz.

The larger SlAMT1.1 sequence variability in the wild groups Eriopersicon, Neolycopersicon and Arcanum is more evident than in the Lycopersicon and cultivars groups. Considering the 83 accessions evaluated in the Lycopersicon groups, only 18% showed variation in position 181, 12% in position 272, 4% in position 418 and 2% in position 294. For cultivars, only position 450 of the protein showed variability, where 10% of the 180 evaluated accessions presented amino acid change.

Observing all the variation sites presented in the sequences of the 287 analyzed accessions of the five groups of *Solanum* section *Lycopersicon*, nearly half of the non-synonymous coding SNPs are present at the C-terminal, which comprises positions 443 to 490 of the SlAMT1.1, suggesting the importance of this domain in the regulation of AMT activity.

The alignment of SlAMT1.1 from the 107 accessions of wild species and 180 tomato cultivars identified two majors highly variable sites caused by non-synonymous SNPs (Figure 12B). In this analysis, we assembled the consensus sequence for each of the five *Solanum* section *Lycopersicon* groups, aligning these sequences, and revealed the variation V236A substitution representative for the Arcanum group, S477L for the Eriopersicon group, and V477L for the Neolycopersicon group.

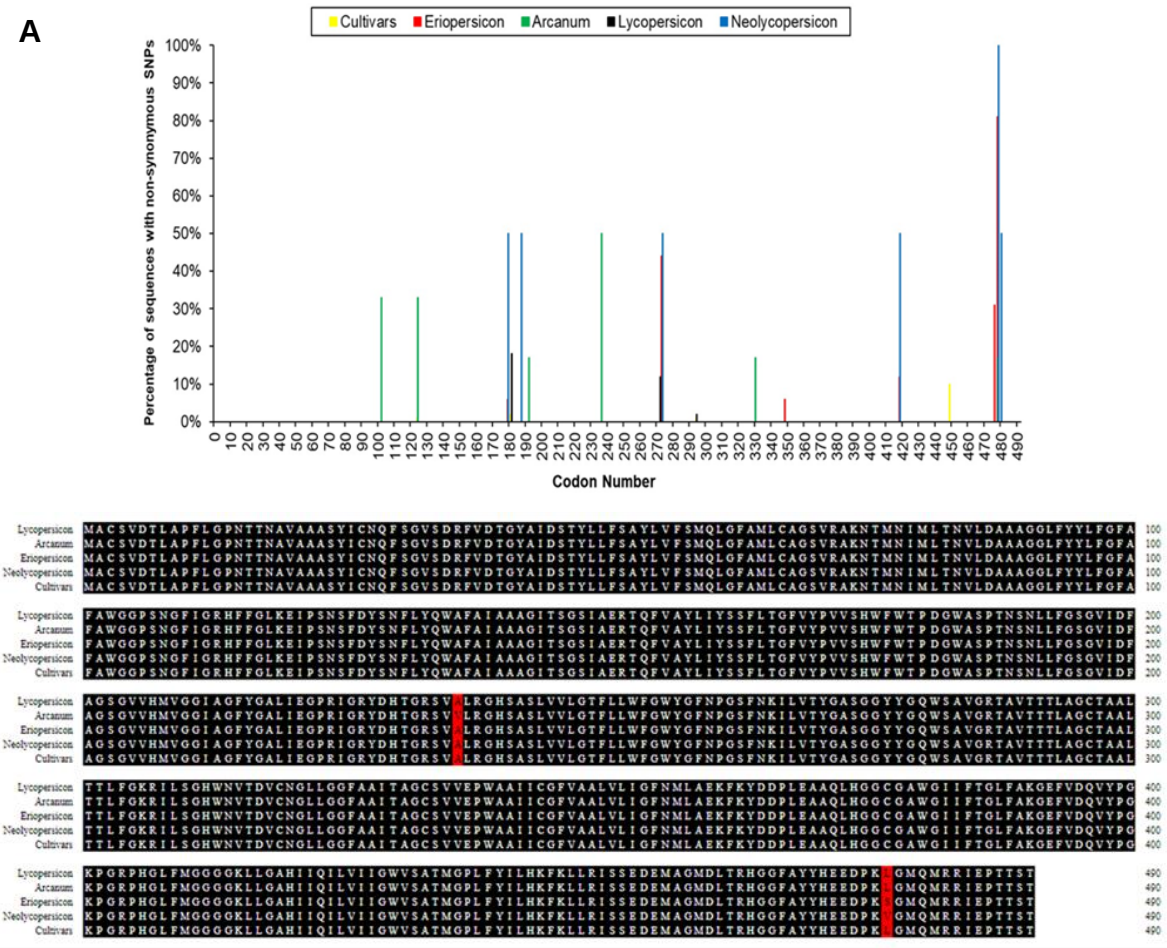


Figure 12 - Most divergent non-synonymous SNPs (causing amino acid changes) for SlAMT1.1 protein in *Solanum* section *Lycopersicon* groups. (A) Average non-synonymous SNPs behavior for each codon of SlAMT1.1 in cultivars and wild relatives; (B) Alignment of SlAMT1.1 consensus group sequence of 180 cultivars and 107 wild species accessions. V236A and S477L are highlighted in red.

The valine substitution of the Arcanum group is located at the SlAMT1.1 cytosolic loop number 6, which comprises the amino acids 221 to 240 (Figure 13A), a highly conserved region of the AMT1.1 protein (Figure 13B). However, the V236A substitution occurs in the Arcanum group members (Figure 13C), mainly in *S. chmielewskii*. By analyzing the variability at position 236 of SlAMT1.1 among 287 sequences of the five tomato groups (Figure 13D) confirmed that V236A variation is present only in representatives of the Arcanum group, with 50% among them showing V236, while another 50% hold alanine in that position.

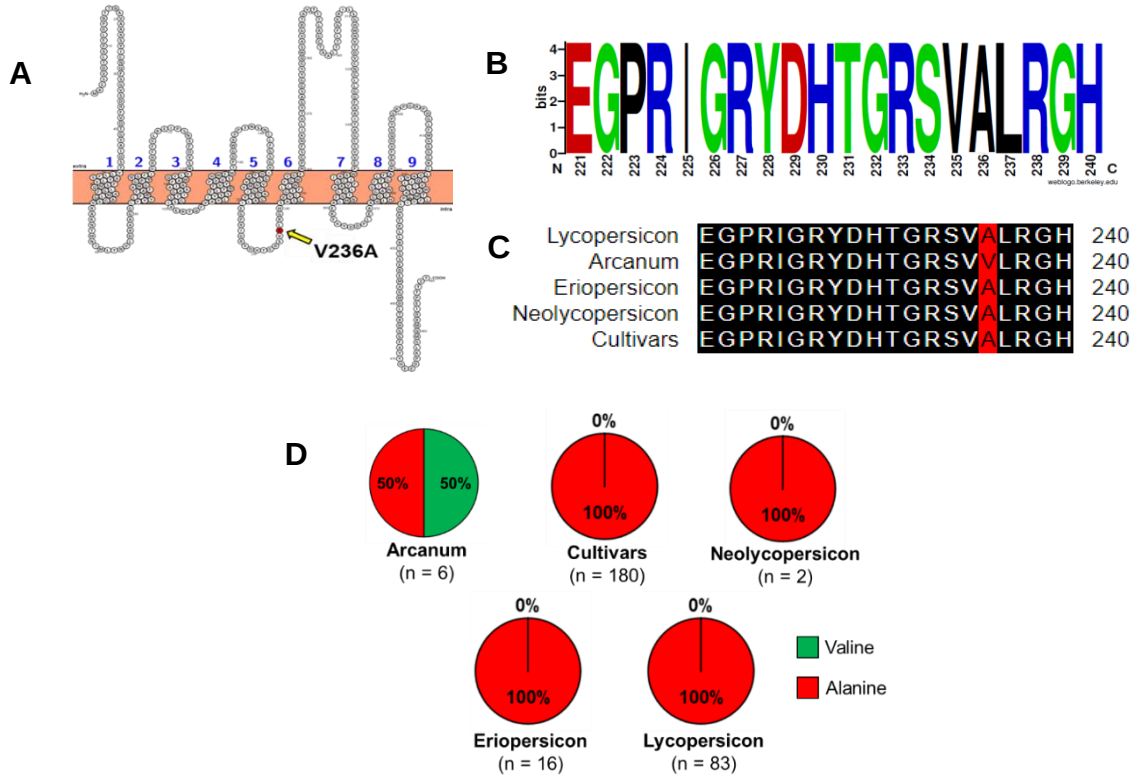


Figure 13 - Amino acid substitution in the SlAMT1.1 cytosolic loop number 6. (A) Topology of *Solanum lycopersicum* cv. Heinz SlAMT1.1 trans-membrane protein highlighting V236A; (B) WebLogo (https://weblogo.berkeley.edu/) representation for SlAMT1.1 cytosolic loop number 6 consensus sequences for tomato cultivars and wild species. (C) Alignment of SlAMT1.1 consensus sequences of tomato cultivars and wild species for the cytosolic loop number 6; (D) Amino acid variability frequency at position 236 of SlAMT1.1 protein in tomato cultivars and wild species.

AMT1 is post-translationally regulated by reversible phosphorylation triggered by an ammonium signal at the carboxy-terminal (LOQUÉ et al., 2007; WU et al., 2019). Under ammonium resupply, rapid phosphorylation of T460 and T472 at the C-terminal effectively inhibited the activity of AtAMT1.1 and AtAMT1.2, respectively, drastically reducing ammonium transport (LOQUÉ et al., 2007; NEUHÄUSER et al., 2007; LANQUAR et al., 2009). The SlAMT1.1 C-terminal region between amino acids

443 and 490 (Figure 14A) contains highly conserved amino acids sequence when considering 287 tomatoes accessions (Figure 14B), but with a consistent non-synonymous SNPs at position 477 present in Eriopersicon and Neolycopersicon (Figure 14C).

We evaluated the frequency of residues at position 477 of the AMT1.1 protein among wild and cultivars tomatoes (Figure 14D). Overall, 94% of the accessions present leucine, 4% serine and 2% valine at 477 position. In the Lycopersicon group and cultivars, leucine is present at position 477 100% of the cases, whereas Arcanum exhibited 83% leucine and 17% serine. In contrast, the Eriopersicon group showed more variability, with 3 amino acids at 477: 56% serine, 25% valine and only 19% leucine. The Neolycopersicon group showed a division with 50% serine and 50% valine (Figure 14D).

Comparative analysis among groups indicated that at position 477, leucine appears mainly in the cultivar groups (67.15%) and Lycopersicon (29.93%). On the other hand, serine is present mainly in the Eriopersicon sequences (81.82%) and valine is also present mainly in the Eriopersicon group sequences (80%). These results indicate that the SlAMT1.1 C-terminal sequence have relatively small genetic variation, with loss of variability during the tomato domestication/breeding.

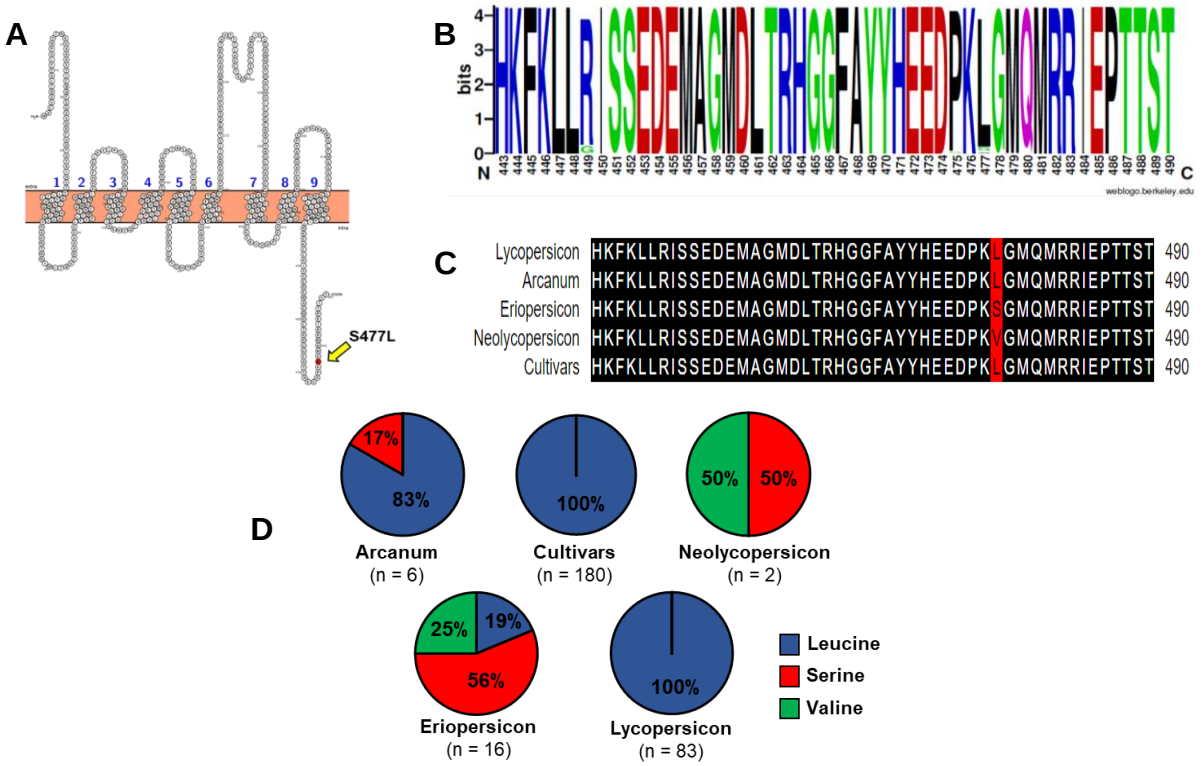


Figure 14 - Amino acid substitution at the SlAMT1.1 C-terminal sequences. (A) Predicted structure of *Solanum lycopersicum* cv. Heinz SlAMT1.1 trans-membrane protein, highlighting S477L; (B) WebLogo (https://weblogo.berkeley.edu/) representation for the AMT1.1 C-terminal consensus sequence for tomato cultivars and wild species; (C) Alignment of AMT1.1 C-terminal consensus sequences of tomato cultivars and wild species indicating in red the variable residue; (D) Amino acid variability frequency at position 477 of SlAMT1.1 protein in tomato cultivars and wild species.

Serine, as well as tyrosine and threonine, can undergo phosphorylation by kinases, usually altering protein structure or activity (WU et al., 2019). The presence of serine at the C-terminal of SlAMT1.1 of wild tomatoes could be associated with distinct phosphorylation regulation pattern at these multiple positions and could affect post-translational regulation of ammonium transport capacity.

Multiple phosphorylation sites at the carboxy-terminal of AMT have been previously described in the model plant *A. thaliana* (LOQUÉ et al., 2007; NEUHÄUSER et al., 2007; LANQUAR et al., 2009; WU et al., 2019). Therefore, we analyzed the sequences of SlAMT1.1 to predict possible phosphorylation sites in tomato by comparative alignment with three members of *A. thaliana* AMT1 family (AtAMT1.1, AtAMT1.2 and AtAMT1.3) (Table 4; Figure 15A).

For the analysis of potential phosphorylation sites at the C-terminal of SlAMT1.1, protein sequences of *S. lycopersicum* cv. M82 LA3475 were used as representative of the cultivar group, *S. pimpinellifolium* LA1584 from the Lycopersicon group, *S. habrochaites* LA1718 from the Eriopersicon group and *S. chmielewskii* LA2695 from Arcanum group. These genotypes were later used in plant assays for SlAMT1.1 activity for ammonium uptake.

The alignment of AtAMT1 and SlAMT1.1 showed that their C-terminal region (CTR) consisted of a conserved (CTR^{C}) and a non-conserved region (CTR^{NC}) with multiple putative phosphorylation sites. In *A. thaliana*, the C-terminal comprises amino acids 444-501 for AtAMT1.1, 456-514 for AtAMT1.2 and 448-498 for AtAMT1.3. The CTR^{C} is located between residues 444-467, 456-479 and 448-471 for AtAMT1.1, AtAMT1.2 and AtAMT1.3, respectively. The CTR^{NC} comprises amino acids 468-501, 480-514 and 472-498 for AtAMT1.1, AtAMT1.2 and AtAMT1.3. The *S. lycopersicum* C-terminal is the region between amino acids 443-490, presenting the CTR^{C} between amino acids 443-469 and the CTR^{NC} between amino acids 470-490.

Several phosphorylation sites experimentally confirmed in *A. thaliana* appear to be conserved in tomato (Table 4; Figure 15A). In addition to SlAMT1.1 T462 (equivalent to T460 in AtAMT1.1 and T464 in AtAMT1.3), T487 (T496 in AtAMT1.1), T488 (T497 in AtAMT1.1 and T494 in AtAMT1.3) and T490 (T499 in AtAMT1.1) are also present in the four evaluated tomato accessions *S. lycopersicum* cv. M82 LA3475, *S. habrochaites* LA1718, *S. pimpinellifolium* LA1584, and *S. chmielewskii* LA2695. However, among these accessions, only *S. habrochaites* (Eriopersicon) SlAMT1.1 presented S477. Position 477 in the tomato protein aligns with a conserved S475 in AtAMT1.1 and S480 in AtAMT1.3, both active phosphorylation sites in *A. thaliana* (WU et al., 2019) indicating a potential additional phosphorylation site in *S. habrochaites* LA1718.

Table 4 - Experimentally demonstrated phosphorylation sites at the C-terminal of AtAMT1s and predicted equivalency in SlAMT1.1.

Gene	Phosphorylation Site	*SlAMT1.1* equivalent	Species
AtAMT1.1	T460	T462	*S. lycopersicum* cv. M82 LA3475, *S. pimpinellifolium* LA1584, *S. habrochaites* LA1718 and *S. chmielewskii LA2695*
	S475	S477	*S. habrochaites* LA1718
		L477	*S. lycopersicum* cv. M82 LA3475, *S. pimpinellifolium* LA1584 and *S. chmielewskii LA2695*
	S488	-	*S. lycopersicum* cv. M82 LA3475, *S. pimpinellifolium* LA1584, *S. habrochaites* LA1718 and *S. chmielewskii LA2695*
	S490	-	
	S492	-	
	T496	T487	
	T497	T488	
	T499	T490	
AtAMT1.2	T472	T462	*S. lycopersicum* cv. M82 LA3475, *S. pimpinellifolium* LA1584, *S. habrochaites* LA1718 and *S. chmielewskii LA2695*
AtAMT1.3	T464	T462	*S. lycopersicum* cv. M82 LA3475, *S. pimpinellifolium* LA1584, *S. habrochaites* LA1718 and *S. chmielewskii LA2695*
	S480	S477	S. *habrochaites* LA1718
		L477	*S. lycopersicum* cv. M82 LA3475, *S. pimpinellifolium* LA1584 and *S. chmielewskii LA2695*
	S487	-	*S. lycopersicum* cv. M82 LA3475, *S. pimpinellifolium* LA1584, *S. habrochaites* LA1718 and *S. chmielewskii LA2695*
	T494	T488	

Data: (WU et al., 2019), adapted.

To further analyze the presence of putative phosphorylation sites at tomato SlAMT1.1 protein C-terminal, we performed an *in-silico* prediction (Figure 15B) using the DIPHOS1.3 software (http://www.dabi.temple.edu/disphos/) for estimating a probability that a phosphorylated amino acid is likely an active phosphorylation site. This analysis allows predicting potential phosphorylation sites present at the C-terminal of SlAMT1.1 through an approach other than alignment with *A. thaliana* AMT sequences. Amino acids with phosphorylation potential above the statistical threshold may suggest the presence of active phosphorylation sites at these positions.

Prediction analysis indicated that SlAMT1.1 of *S. lycopersicum* cv. M82, *S. pimpinellifolium* and *S. chmielewskii* potentially presented nine phosphorylation sites at the C-terminal sequences (S451, S452, T462, Y469, Y470, T487, T488, S489 and T490), whereas *S. habrochaites* LA1718 presented 10 sites, since the S477 also had the potential to be an active phosphorylation site (Figure 15B). In addition to the well characterized AtAMT1.1 T460 in the CTR^{C}, multiple phosphorylation sites are located at the CTR^{NC}, but the potential functional roles in the regulation of ammonium transporters are still unknown (WU et al., 2019). Thus, we hypothesized that the occurrence of S477 might function as putative phosphorylation target site, derived from the natural variability of non-synonymous SNPs present in the gene sequences in *S. habrochaites* wild tomatoes, which may be related to a possible differential activity of the SlAMT1.1.

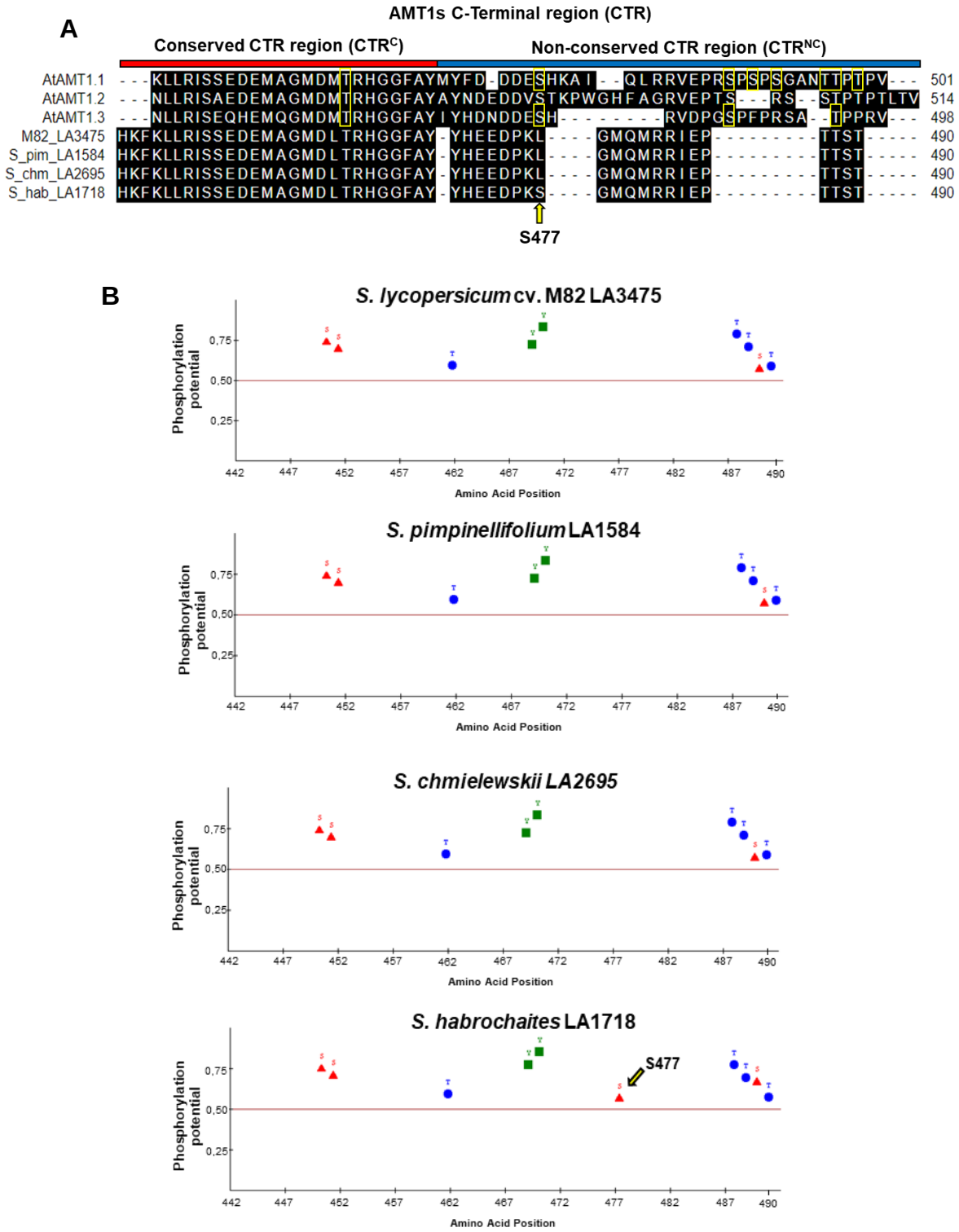


Figure 15 - Predicted phosphorylation sites at the SlAMT1.1 carboxy-terminal region (CTR). (A) Predicted phosphorylation sites at the CTR of AtAMT1 and AMT1.1 of *S. lycopersicum* cv. M82 LA3475, *S. pimpinellifolium* LA1584, *S. chmielewskii* LA2695 and *S. habrochaites* LA1718. The *Arabidopsis thaliana* phosphorylated Threonine/Serine residues confirmed by Wu et al. (2019) are marked with a yellow box. (B) Predicted phosphorylation sites on C-terminal of *S. lycopersicum* cv. M82 LA3475, *S. pimpinellifolium* LA1584, *S. chmielewskii* LA2695 and *S. habrochaites* LA1718 by DISPHOS 1.3 (http://www.dabi.temple.edu/disphos/).

4.5 Ammonium transport activity is affected by wild variation in SlAMT1.1 protein

We investigated whether resupply of ammonium can trigger contrasting changes in SlAMT1.1 transporter activity among representatives of the *Solanum* section *Lycopersicon* groups exhibiting differential amino acid substitution at the 236 and 477 SlAMT1.1 residues. We employed *S. lycopersicum* cv. M82 as representative for cultivars, *S. pimpinellifolium* LA1584 for Lycopersicon group, *S. chmielewskii* LA2695 (V236A substitution) for Arcanum group and *S. habrochaites* LA1718 (S477L substitution) for Eriopersicon group. We assessed the correlation between ^{15}N-labeled high-affinity ammonium influx (Figure 16A) and transcriptional abundance of *SlAMT1.1* (Figure 16B) to evaluate whether regulation was at the transcriptional or post-translational level under ammonium resupply. Tomato seedlings were cultivated upon N-sufficiency conditions (+N; 2 mM), N-deficiency for 72h (-N) or resupplied with 4 mM ammonium for 1 h after N-deficiency and analyzed using a short-term high-affinity ^{15}N-labeled ammonium influx assay.

Under N-sufficiency (+N) conditions, *S. lycopersicum* cv. M82, *S. pimpinellifolium* and *S. habrochaites* showed no significant difference in ^{15}N-ammonium influx. Conversely, *S. chmielewskii* showed an eight-fold greater ammonium influx rate than the other accessions. Upon N-deficiency, all accessions showed a significant increase in the ^{15}N-ammonium influx when compared to N-sufficiency (+N), indicating that N-starvation can de-repress the high-affinity ammonium uptake capacity in tomato roots. While M82, *S. pimpinellifolium* and *S. habrochaites* ammonium uptake increased by 8-12-fold, *S. chmielewskii* displayed a smaller ^{15}N-ammonium influx increase, 3-fold higher than N-sufficient condition. After ammonium resupply for 1 h, ammonium influx was moderately decreased in M82 roots (58%), but clearly reduced *S. pimpinellifolium* roots (80%), relative to N-deficiency (-N). However, in *S. habrochaites* and *S. chmielewskii*, the uptake decreased slightly, by 25% and 24%, respectively, in relation to the respective N-deficient (-N) roots.

In the analysis of the relative gene expression (Figure 16B) under N-sufficiency (+N), *S. chmielewskii* presented a statistically 3-fold higher expression of *SlAMT1.1* in relation to M82, *S. pimpinellifolium* and *S. habrochaites*. Under N-deficiency (-N), *S. chmielewskii* showed the highest relative expression values, followed by *S. habrochaites* and *S. pimpinellifolium*, while M82 displayed the lowest expression of *SlAMT1.1*. All species showed a significant increase in the *SlAMT1.1* expression levels

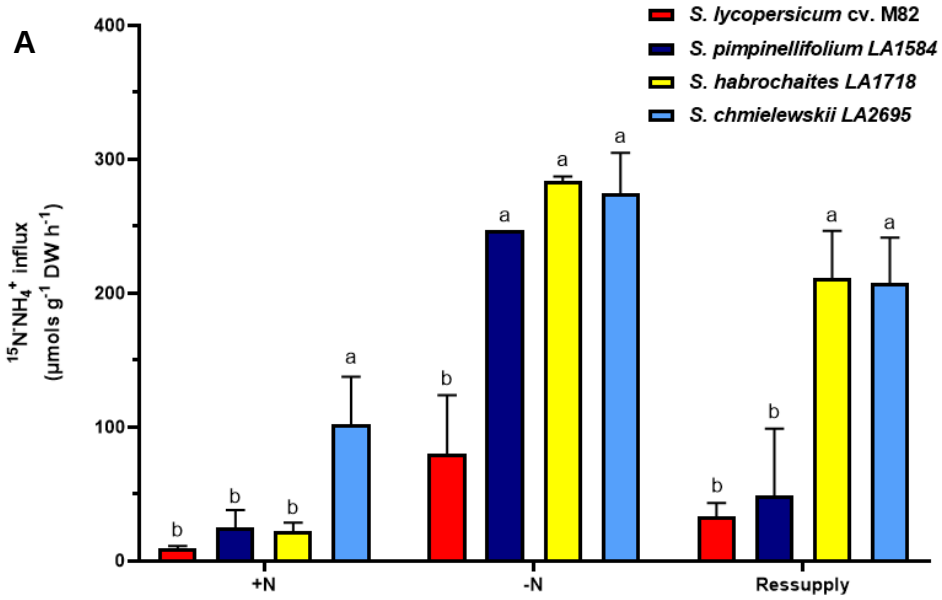
and ammonium influx under N-deficiency (-N). Compared to N-sufficiency (+N), M82 showed 2-fold higher expression of *SlAMT1.1*, while *S. pimpinellifolium* and *S. chmielewskii* presented 2.5 times higher expression, and *S. habrochaites* showed a three-fold increased expression. In the condition of 1 h ammonium resupply after 72 h of N-deficiency, *S. chmielewskii* also presented a significant highest *SlAMT1.1* expression, followed by *S. habrochaites*, while M82 and S*. pimpinellifolium* presented the lowest expression levels.

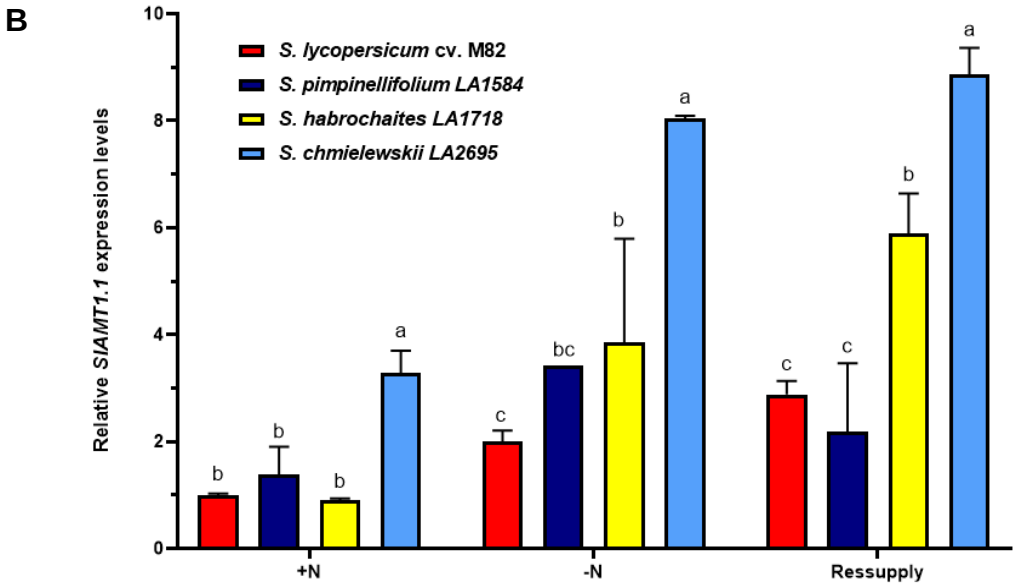
Despite the continuous decrease in ammonium uptake under ammonium resupply, the transcript abundance of *SlAMT1.1* was similar in N-starved roots and ammonium resupplied roots in genotypes (Figure 16B). A distinct pattern for ammonium uptake occurs in wild species and cultivated tomato roots. The regulation appeared to be at the post-translational level, because the transcript abundances of *SlAMT1.1* did not change accordingly. Compared with N-deficient roots, 1 h resupply of ammonium (Figure 16B) caused inhibition of root ammonium uptake by M82 and *S. pimpinellifolium* roots, whereas in roots of *S. habrochaites* and *S. chmielewskii* there was smaller repression relative to that of N-deficient plants. These observations corroborate the fact that these two wild species showed the greatest variability in the amino acid sequence of the SlAMT1.1 protein sequence and might contain a distinct post-translational regulation (Figure 16C).

Besides the S477L variation, *Solanum habrochaites* LA1718 also contains two other variations, T273A and A418G (Figure 16C). *Solanum chmielewskii*, in addition to the V236A substitution, also presented two serine substitutions at residues S102A and S124N, which might represent additional phosphorylation sites, associated with this differential response of SlAMT1.1 activity. *Solanum pimpinellifolium* LA1584 contains a serine substitution S272G but is unlikely this is ammonium-triggered phosphorylation site that affects SlAMT1.1 activity, since this species showed similar ammonium uptake to the cultivar M82 without such substitution, and therefore, S272G might not lead to SlAMT1.1 activity changes.

Our results suggest that S477L substitution at the C-terminal could be related to effectively inhibited SlAMT1.1-dependent ammonium uptake. Moreover, either phosphorylation of two serine at positions S102A and S124N or a V236A substitution in SlAMT1.1 from *S. chmielewskii* LA2695 might be involved with regulating ammonium uptake in response to ammonium resupply, and it deserves further functional analysis. Here, we disclosed a major genetic variation and three putative

causal variants for SlAMT1.1 in wild tomato accessions that could functionally affect ammonium transporter activity.





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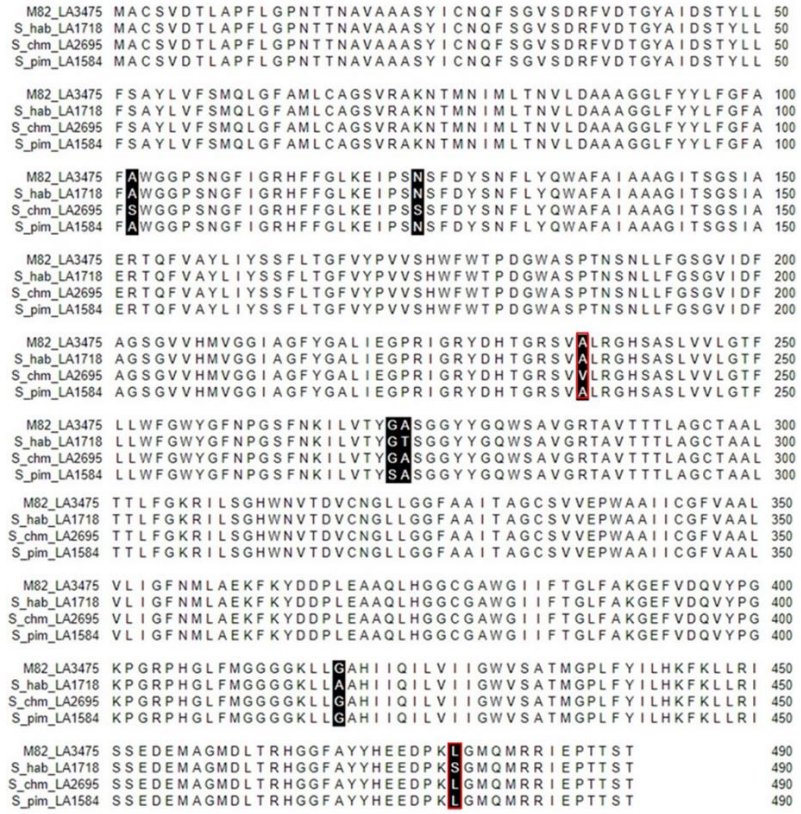


Figure 16 - (A) ^{15}N-$(NH_4)_2SO_4$ influx in tomato roots. Plants were grown for 30 days under sufficient N conditions (2 mM NH_4NO_3) and exposed to the treatments N sufficiency (+N, 2 mM NH_4NO_3); N deficient (-N) for 72 h; and 1 h ammonium (4 mM NH_4Cl) resupply after 72 h absence-deficiency. Influx assays were performed at the concentration of 0.1 mM $(^{15}NH_4)_2SO_4$ for 10 min in roots. Bars indicate mean ± SE, *n* = 5 plants. Significant differences at $p<0.05$ according to the Tukey test are indicated by different letters; (B) *SlAMT1.1* relative expression levels (RT-qPCR) in tomato or wild species accessions roots under contrasting ammonium provision. Bars indicate means ± SE, n = 3. The expression values are relative to the expression of *S. lycopersicum* cv. M82 LA3475 in the condition of N sufficiency (+N). Significant differences between genotypes in the same N-treatment at $p<0.05$ according to the Tukey test are indicated by different letters; (C) SlAMT1.1 protein sequence alignment for *S. lycopersicum* cv. M82*, S. pimpinellifolium* LA1584, *S. habrochaites* LA1718 and *S. chmielewskii* LA2695. Black boxes indicate the variations present between the protein sequences of the genotypes. Red boxes indicate variations V236A and S477L.

4.6 GWAS maps allelic variants that modulates *SlAMT1.1* expression

Differential *SlAMT1.1* expression between domesticated tomatoes (*S. lycopersicum)* and related wild species might indicate allelic variation (Figure 8). In order to identify causal genetic loci for the variation of *SlAMT1.1* expression between tomato cultivars and wild species, we performed a Genome-Wide Association Study (GWAS, Figure 17) using 12 tomato cultivars and 19 wild *Solanum* accessions with variable *SlAMT1.1* expression levels (Supplementary Table 2). The transcriptome files from each of the accessions were aligned with the reference genome *S. lycopersicum* cv. Heinz (ITAG 2.3 version) for the creation of a SNPs database

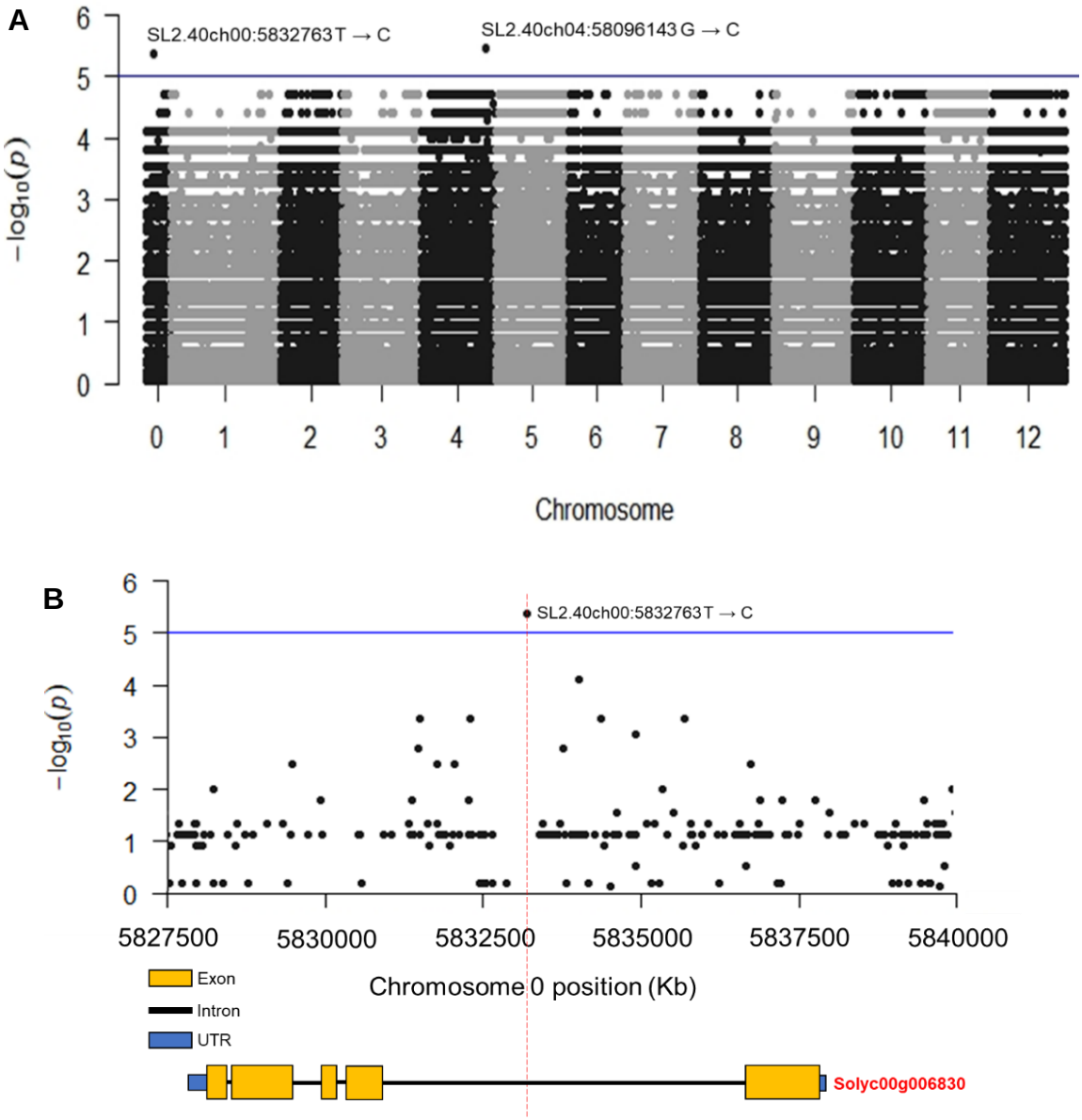
between these accessions. The genotypes were then separated into two groups: genotypes that express *SlAMT1.1* above the average and genotypes that express below the average among accessions. Then, we performed an association analysis of each of these SNPs in response to differences in *SlAMT1.1* expression among the studied genotypes.

It was not possible to identify any SNP with significant effect at 5% using the Bonferroni correction statistics. However, complex traits, such as efficiency in ammonium uptake by plants, are usually associated with the control of several genes, each with a small or moderate effect (KOOKE et al., 2016). Thus, using such a conservative threshold can negatively affect the search for associations (JIA; GIEHL; VON WIRÉN, 2020). We then used a less stringent arbitrary threshold of significance for $-\log_{10}$ (*P*-value) = 5 for the data.

Two significant SNPs were identified associated with the expression of the *SlAMT1.1* gene in cultivated tomato and *Solanum* wild species, one on chromosome 0 (at 5,732,763 bp) and the other on chromosome 4 (at 58,096,143 bp) (Figure 17A). While the SNP present on chromosome 0 was identified in the intronic region of the *NON-EXINE FORMATION-1* gene (Solyc00g006830) (Figure 17B), the SNP present on chromosome 4 was located at position 58,096,143 bp in the *14-3-3 GENERAL REGULATOR FACTOR* (Solyc04g074510), specifically at the 3′ untranslated region (3′ UTR) (Figure 17C). SNP SL2.40ch04:58096143 presented a *P*-value of 3.52×10^{-3}, while SL2.40ch00:5732763 presented a *P*-value of 4.17×10^{-3}. Among these two identified SNPs, the SNP of chromosome 4 showed a higher association value $-\log_{10}$ (*P*-value).

Noteworthy, the strongest association signal resided at 185 bp downstream of the stop codon of *14-3-3 GENERAL REGULATOR FACTOR* (Solyc04g074510), whose function is associated with signaling pathways regulating proteins involved in response to biotic as well as abiotic stress, *e.g.* nutrient metabolism in plants, such as potassium, nitrogen, phosphate and sulfur (ROBERTS, 2000; SHIN et al., 2011; YASHVARDHINI et al., 2018). The major regulatory role of 14-3-3 proteins is to physically interact with phosphorylated motifs of target proteins and lead to post-translational modifications, including alterations in conformation, subcellular localization, protein stability and protein-protein interaction (WILSON; SWATEK; THELEN, 2016; LEE et al., 2020a). Among Eukaryotes, plants have the largest number of 14-3-3 genes. Thirteen genes can be found in arabidopsis (DELILLE; SEHNKE;

FERL, 2001), five in barley (SCHOONHEIM et al., 2007), six in cotton (ZHANG et al., 2010), 17 in tobacco (KONAGAYA et al., 2004) and eight in rice (YAO et al., 2007). 14-3-3 proteins in plants comprise multiple isoforms with differential subcellular localization, suggesting that their isoforms specifically function and are involved in multiple protein–protein interactions (SHIN et al., 2011; YASHVARDHINI et al., 2018).



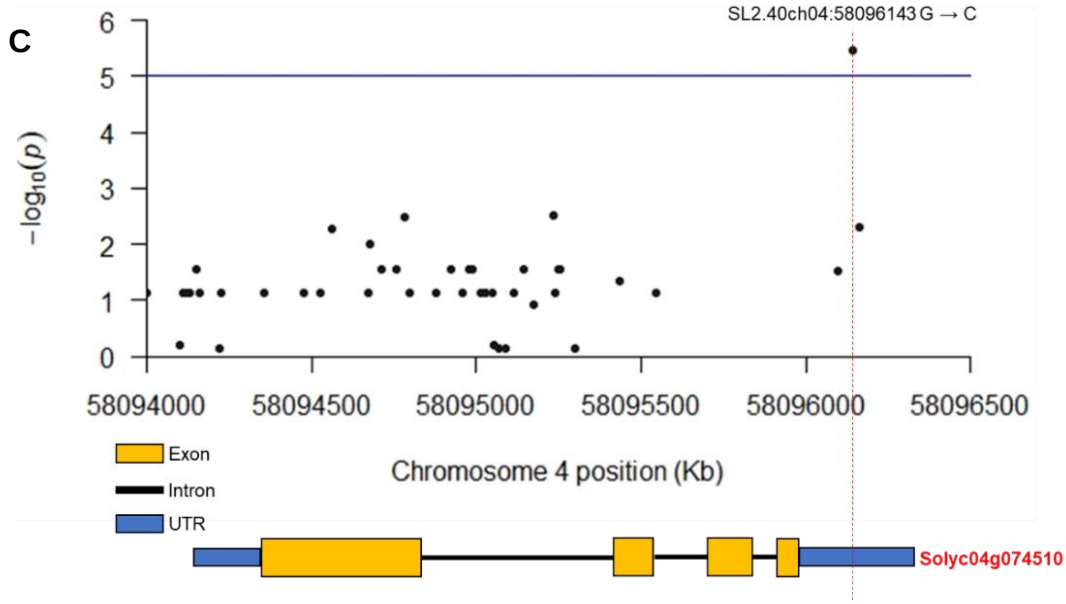


Figure 17 - GWAS maps natural variation of *AMT1.1* expression in 31 tomato cultivars and wild species. (A) Manhattan plot performed with the qqman package (TURNER, 2018) in R to determine SNP associations with *AMT1.1* expression levels in tomato cultivars and wild species. Negative $\log_{10}$-transformed *P*-values from a genome-wide scan were plotted against positions on each tomato chromosome. The blue line corresponds to the arbitrary threshold of $-\log_{10}$ (*P*-value) = 5; (B) The genomic region associated with the *NEF1* (Solyc00g006830) locus presenting significant SNP SL2.40ch00:5832763 T → C in the intronic region. (C) The genomic region associated with the *14-3-3* (Solyc04g074510) locus presenting significant SNP SL2.40ch04:58096143 G → C in the 3' UTR region.

To identify the 14-3-3 isoforms in *S. lycopersicum*, BLAST (https://solgenomics.net/tools/blast/) and hidden Markov models (HMMs) from Pfam tools (https://pfam.xfam.org/) were used, based on *A. thaliana* GRF/14-3-3 gene family sequences. Twelve isoforms of protein 14-3-3 were identified in the tomato genome (Table 5), classified as *TFT* gene family. The Solyc04g074510 highlighted in GWAS is homologue to the GRF4 protein of *A. thaliana* and it was classified as TFT3 in *S. lycopersicum* (Table 5).

We analyzed each *TFT* genomic position (Figure 18A), gene length, as well as length of amino acid sequence (Table 5). The *TFT* genes are well distributed throughout the tomato genome, being present in seven of the 12 chromosomes. Chromosome 4, where Solyc04g074510 (*TFT3*) is found, also includes three additional *TFT* isoforms, *TFT5*, *TFT7* and *TFT10*. In addition, the *TFT* genes differ widely in the size of the gene. The smallest gene is in the one from *TFT6*, presenting only 1193 bp, while the largest *TFT4* has 6880 bp. On the other hand, the protein length did not show such substantial variations, ranging from 250 to 286 amino acids.

To evaluate the evolutionary relationships among the 12 TFT proteins, we performed a phylogenetic analysis (Figure 18B) based on their full-length amino acid sequences, as well an exon-intron organization analysis of the corresponding *TFT* genes obtained by Gene Structure Display Server (http://gsds.gao-lab.org/). In the phylogenetic analysis, it is possible to notice that the tomato 14-3-3 members are divided into two main branches, which can be classified exactly as in *A. thaliana,* as Epsilon members and Non-Epsilon members (DELILLE; SEHNKE; FERL, 2001).

In *A. thaliana*, the Epsilon group is divided between the isoforms mu, epsilon, pi, iota, and omicron, while the non-Epsilon group has kappa, lambda, phi, chi, omega, psi, nu, and upsilon isoforms (DELILLE; SEHNKE; FERL, 2001). The Epsilon group is divided into two subbranches, with epsilon and pi on one subbranch and omicron, iota, and mu in the second subbranch. The Non-Epsilon group is divided into three very distinct subbranches. Kappa and lambda in one subbranch; phi, chi, and omega in a second subbranch; and psi, nu, and upsilon in the third subbranch (DELILLE; SEHNKE; FERL, 2001). Analyzing the tomato *TFT* genes that best align with each isoform of *A. thaliana* in the BLAST analyses (Table 5), it can be observed that the distribution of these genes among the branches happens in exactly the same way as in *A. thaliana*. Interestingly, this division between the Epsilon and Non-Epsilon groups is strongly associated with the intron/exon organization structure of the *TFT* genes (Figure 18B). While all members of the Non-Epsilon group have a structure containing 4 exons and 3 introns, members of the Epsilon group have sequences ranging from 6-7 exons and 5-6 introns.

Table 5 - Tomato *14-3-3* homologs identified through BLAST using *A. thaliana* sequences as probes.

Arabidopsis Gene	TAIR ID	Best BLAST hit	Tomato Gene	Chromosome location	Gene Length (bp)	Protein Length (aa)
GRF1 (14-3-3 chi)	AT4G09000	Solyc12g057110	*TFT2*	SL2.50**ch12**:63191706-63194481	2776	255
GRF2 (14-3-3 omega)	AT1G78300	Solyc04g012120	*TFT5*	SL2.50**ch04**:4428571-4430128	1558	256
GRF3 (14-3-3 psi)	AT5G38480	Solyc03g034180	*TFT11*	SL2.50**ch03**:5872975-5875083	2109	259
GRF4 (14-3-3 phi)	AT1G35160	Solyc04g074510	*TFT3*	SL2.50**ch04**:60500769-60502930	2162	269
GRF5 (14-3-3 upsilon)	AT5G16050	Solyc02g063070	*TFT4*	SL2.50**ch02**:35114568-35121447	6880	261
GRF6 (14-3-3 lambda)	AT5G10450	Solyc11g010470	*TFT1*	SL2.50**ch11**:3533525-3537417	3893	250
GRF7 (14-3-3 nu)	AT3G02520	Solyc03g034180	*TFT11*	SL2.50**ch04**:61027437-61032231	4795	253
GRF8 (14-3-3 kappa)	AT5G65430	Solyc04g076060	*TFT10*	SL2.50**ch07**:61735445-61739996	4552	262
GRF9 (14-3-3 mu)	AT2G42590	Solyc07g053260	*TFT9*	SL2.50**ch04**:60219831-60223658	3828	267
GRF10 (14-3-3 epsilon)	AT1G22300	Solyc04g074230	*TFT7*	SL2.50**ch05**:5666263-5669472	3210	286
GRF11 (14-3-3 omicron)	AT1G34760	Solyc04g074230	*TFT7*	SL2.50**ch04**:60219831-60223658	3828	267
GRF12 (14-3-3 iota)	AT1G26480	Solyc05g012420	*TFT12*	SL2.50**ch05**:5666263-5669472	3210	286
GRF13 (14-3-3 pi)	AT1G78220	Solyc05g012420	*TFT12*	SL2.50**ch05**:5666263-5669472	3210	286
-	-	Solyc11g010200	*TFT6*	SL2.50**ch11**:3274709-3275901	1193	270
-	-	Solyc12g010860	*TFT8*	SL2.50**ch12**:3781557-3784182	2626	262

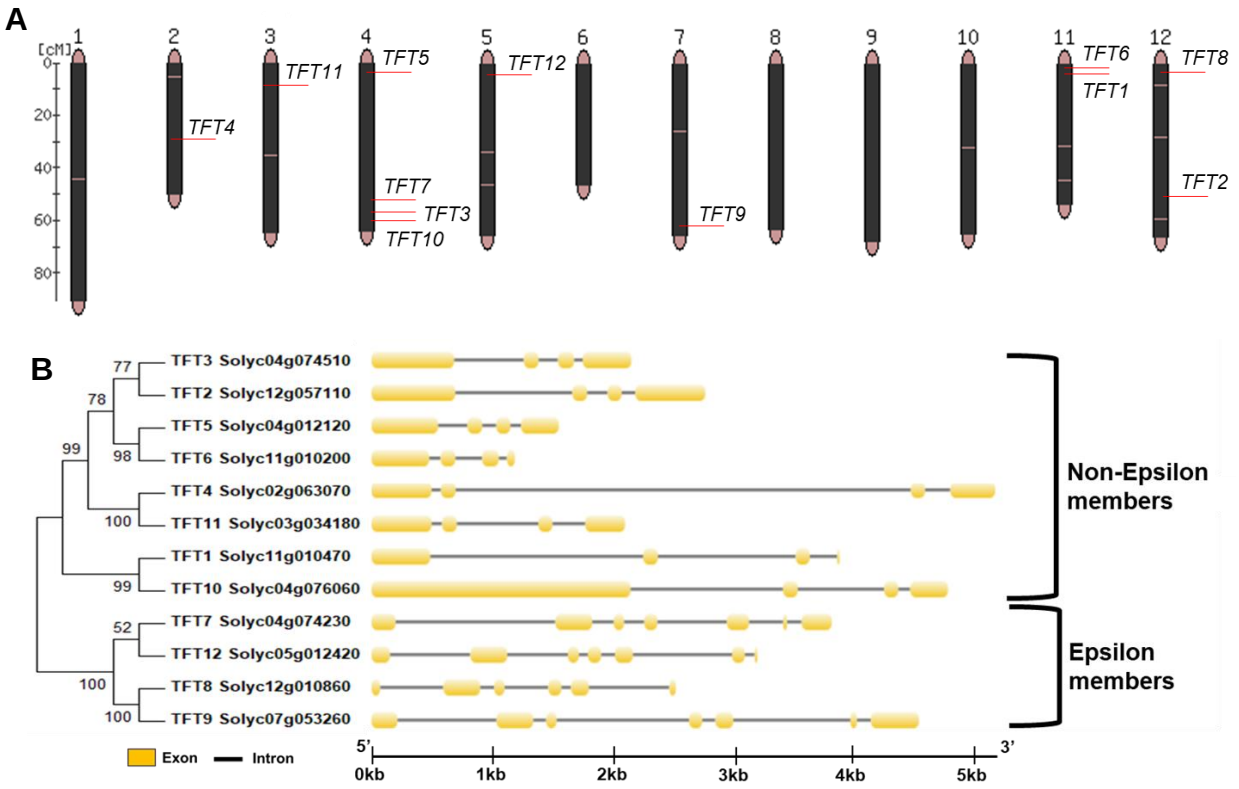


Figure 18 - Chromosomal distribuition, phylogenetic relationships and gene structure analyses of *14-3-3* genes from tomato. (A) Chromosomal distribuition analysis of the *14-3-3* gene family in *S. lycopersicum*. Chromosome number (ch01-ch12) is indicated at the top of each chromosome. Data obtained from SolGenomics ITAG 2.3 annotation. (B) Strict consensus tree based on 14-3-3 protein sequences from *S. lycopersicum* cv. Heinz with 1,000 bootstraps values obtained by maximum-likelihood analysis. The right side illustrates the exon-intron organization of the *14-3-3* genes, performed using Gene Structure Display Server (http://gsds.gao-lab.org/). Evolutionary analyses were conducted in MEGA7 (KUMAR; STECHER; TAMURA, 2016).

Since the different isoforms of 14-3-3 proteins can perform functional role in different organs/tissues (SHIN et al., 2011; YASHVARDHINI et al., 2018), we characterized the expression of the *TFT* genes identified in *S. lycopersicum* cv. Heinz in four tissues (leaves, roots, flowers and breaker fruits) based on public available data (Figure 19).

The expression of the *TFT* genes is well distributed among tomato tissues. In leaves, *TFT1* is highly expressed followed by *TFT9* and *TFT5*, whereas *TFT10* presented lower expression levels and *TFT12* was not detected in this organ. In roots, *TFT5* and *TFT1* transcripts were highly expressed, followed by *TFT3*, while *TFT11* has the least expression and *TFT12* is not expressed. In flowers, the highest expression levels occur for *TFT1*, followed by *TFT5, TFT7* and *TFT9* with similar levels of expression. Interestingly, *TFT12* was expressed only in flowers even though the transcript abundance is relative lower than the other family members. In tomato fruits,

TFT7 displayed the highest expression levels than the other genes, whereas *TFT2* has the lowest absolute expression value.

Interestingly, *TFT3* and *SlAMT1.1* showed more transcript abundance in roots (Figures 7 and 19). Since 14-3-3 has protein-protein interactions as the main mechanism of action, the root cell-type localization could directly affect its targets (DELILLE; SEHNKE; FERL, 2001).

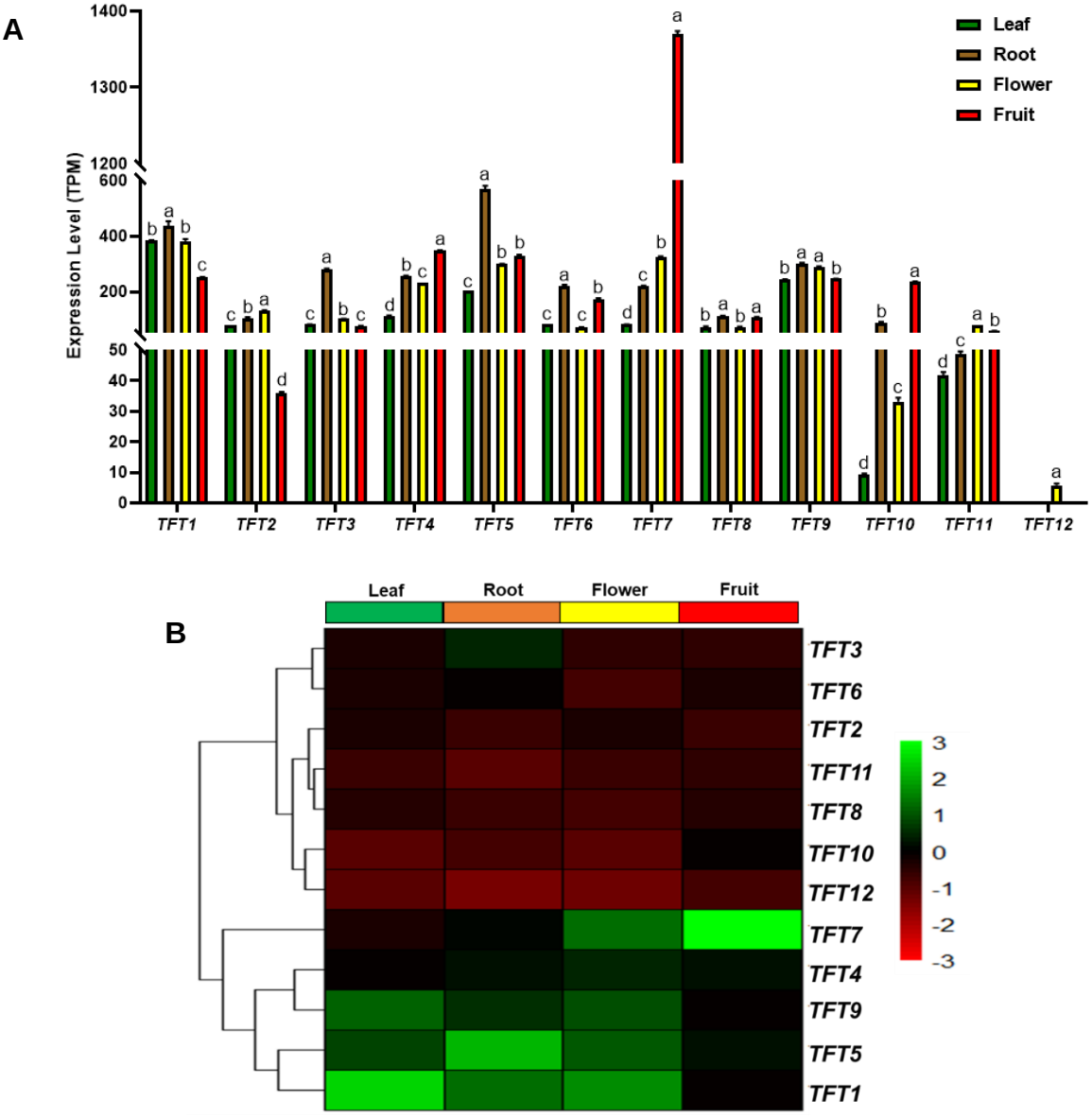


Figure 19 - *14-3-3* genes expression in tomato tissues. (A) Absolute expression level of *14-3-3*s in roots, leaves, fully opened flowers and breaker fruits of *S. lycopersicum* cv. Heinz expressed in TPM (transcripts per kilobase million). Significant differences between tissues within genes at $p<0.05$ according to Tukey's test are indicated by different letters; (B) Heatmap for *14-3-3*s relative expression in roots, leaves, fully opened flowers and breaker fruits in *S. lycopersicum* cv. Heinz. Data is normalized by columns. Based on RNAseq data: (SATO et al., 2012).

According to the GWAS analysis, a SNP variation caused the mutation of a G- allele to a C-allele in the *TFT3* gene at position 58,096,143 bp on chromosome 4. The distribution of alleles among 180 tomato cultivars and 107 wild species, whose genome resequencing is available in the SolGenomics database, indicates that the C-allele is present only in *TFT3* gene sequences from accessions of the Lycopersicon group and in tomato cultivars (Figure 20A). In the Lycopersicon group, 36% had C-allele, while the other 64% had G-allele. In turn, cultivars had 79% C-allele while only 21% presented G-allele. Within Lycopersicon group (Figure 20B), 70% accessions of *S. lycopersicum* var. cerasiforme, which is considered the probable ancestor of big-fruit tomato cultivars (LIN et al., 2014), presented C-allele. Besides, among 50 evaluated accessions of *S. pimpinellifolium*, 6% presented *TFT3* C-variant, while 17% of the six accessions of *S. cheesmaniae* presented C-allelic variation. Conversely, the two sequences of *S. galapagense* showed allele G.

These results suggest that is possible that during domestication there was an introgression of C-allele from the wild species *S. lycopersicum* var. cerasiforme from Lycopersicon group and it may have undergone a genetic drift, by which the genotypic distribution of the C-allele increased in frequency in cultivated species, likely derived from the wild species *S. lycopersicum* var. cerasiforme, *S. pimpinellifolium* or *S. cheesmaniae*. Thus, these results allowed us to identify underexplored germplasm that harbors derived “domestication alleles” of cultivated tomato.

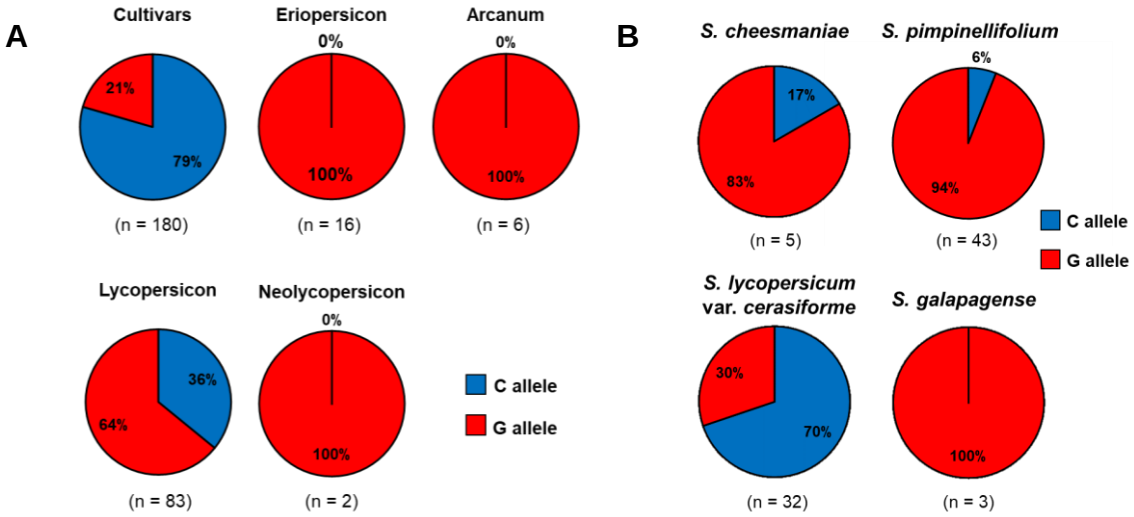
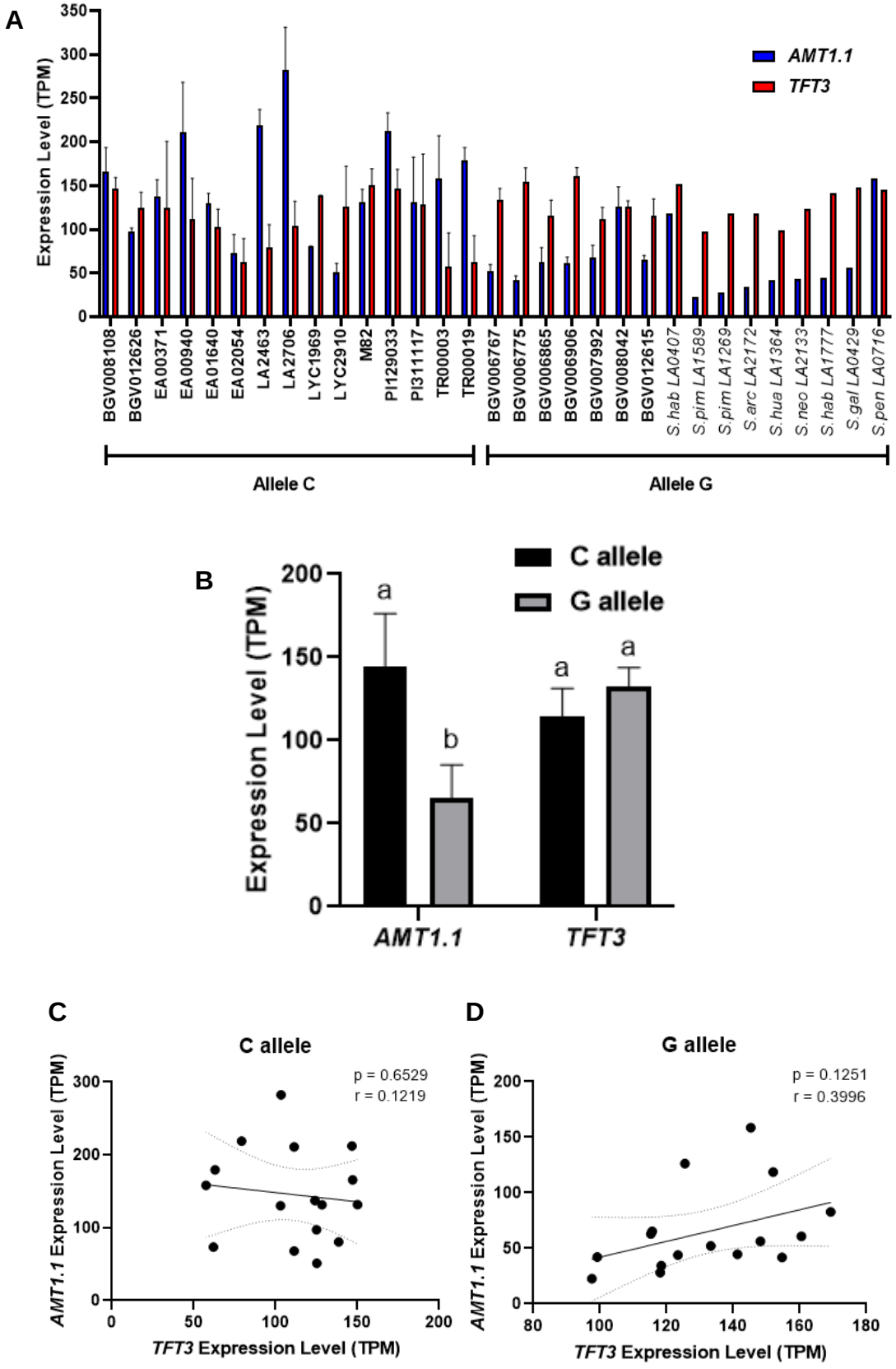


Figure 20 - *TFT3/14-3-3* allelic frequency in *Solanum* sect. *Lycopersicon.* (A) Distribution of *TFT3/14-3-3* C and G alleles among the *Solanum* sect. *Lycopersicon* groups; (B) Distribution of *TFT3/14-3-3* C and G alleles among species of Lycopersicon group.

To assess the effect of the *TFT3* G- and C-alleles on *SlAMT1.1* expression levels, we correlated this allelic variation with the expression levels of *TFT3* and *SlAMT1.1* in all 22 accessions of tomato cultivars and nine relative wild species previously considered in GWAS analysis. Tomato accessions with *TFT3* C-allele showed a higher expression of *SlAMT1.1* compared to those with the *TFT3* G-allele (Figure 21A and B). However, no significant difference in the expression was observed for *TFT3* between these allele groups (Figure 21B). Further, the correlation between the expressions of *TFT3* and *SlAMT1.1* in accessions with C-allele (Figure 20C) and with G-allele (Figure 21D) was not significant for either two alleles (*p*-value > 0.05).

We also estimate the CDS/UTR coverage (a.k.a. total read counts) data for both allelic variants as an important parameter to understand whether a 3' UTR-dependent regulatory mechanisms control *TFT3* gene expression (Figure 21E). In alternative polyadenylation, a mRNA transcript is processed to produce multiple isoforms that generally differ in their 3' UTR length (HELLENS et al., 2016). A typical polyadenylation process consists of a cleavage of pre-mRNA at the polyadenylation site (or poly(A) site) as a key contributor of gene expression regulation (TIAN; MANLEY, 2013). While such distal poly(A) site produces long 3' UTR, proximal poly(A) site instead can produces short 3' UTR (GUO et al., 2016; SRIVASTAVA et al., 2018). The average score of read coverage displayed a similar value for both allelic variants, suggesting that C>G mutation is unlikely to produce mRNA variants that differ in 3′ UTR length, and differently spliced isoforms for *TFT3* (Figure 21E). Hence, there is no evidence of a mechanistic link that connect 3' UTR length for *TFT3* with mRNA stability and functionality.

The analysis performed cannot confirm that *TFT3* allelic variant is causally associated with the natural variation to modulate its transcript stability. Nevertheless, several studies have shown that regulation by 3' UTR can be involved in transport, translation efficiency, and functioning and subcellular localization of the translated proteins (TIAN; MANLEY, 2013; GUO et al., 2016; SRIVASTAVA et al., 2018; BERNARDES; MENOSSI, 2020; TIAN et al., 2020).


A
Expression Level (TPM)
AMT1.1
TFT3
BGV008108
BGV012626
EA00371
EA00940
EA01640
EA02054
LA2463
LA2706
LYC1969
LYC2910
M82
PI129033
PI311117
TR00003
TR00019
BGV006767
BGV006775
BGV006865
BGV006906
BGV007992
BGV008042
BGV012615
S.hab LA0407
S.pim LA1589
S.pim LA1269
S.arc LA2172
S.hua LA1364
S.neo LA2133
S.hab LA1777
S.gal LA0429
S.pen LA0716
Allele C
Allele G
B
C allele
G allele
Expression Level (TPM)
AMT1.1
TFT3
C
C allele
p = 0.6529
r = 0.1219
AMT1.1 Expression Level (TPM)
TFT3 Expression Level (TPM)
D
G allele
p = 0.1251
r = 0.3996
AMT1.1 Expression Level (TPM)
TFT3 Expression Level (TPM)

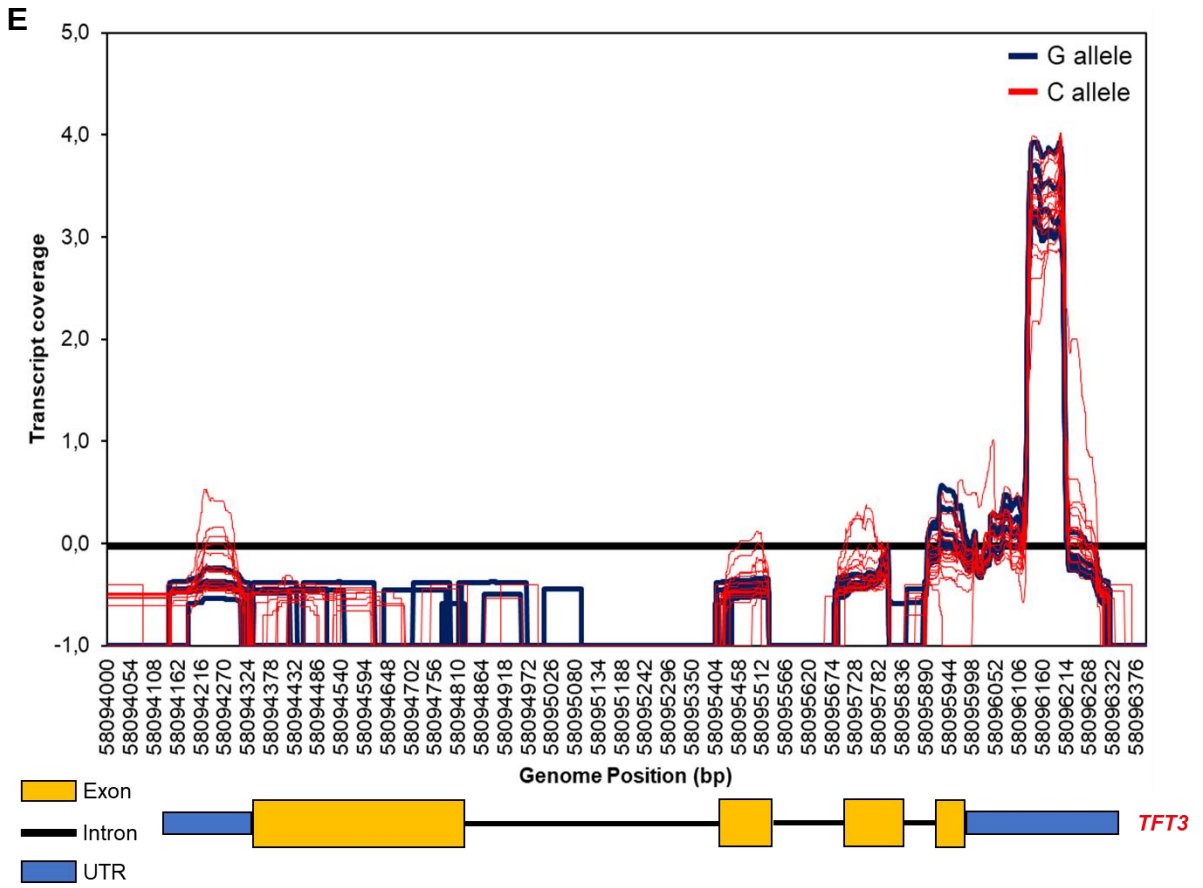


Figure 21 - *TFT3* alleles and *SlAMT1.1* expression levels. RNAseq data: (PEASE et al., 2016; ALONGE et al., 2020). (A) *TFT3* and *SlAMT1.1* expression levels (TPM) for 12 tomato cultivars and 19 wild relatives used in the GWAS analysis; (B) *TFT3* and *SlAMT1.1* expression (TPM) means into the C and G *TFT3* alleles. Bars indicate means ± SE. Significant mean differences at $P<0.05$ according to the Tukey test are indicated by different letters; (C) Analysis of correlation between *TFT3* and *SlAMT1.1* expression in samples containing the *TFT3* C-allele; (D) Analysis of correlation between *TFT3* and *SlAMT1.1* expressions in samples containing the *TFT3* G-allele; (E) Transcript coverage analysis z-score normalized in the *TFT3* gene in 12 tomato cultivars and 19 wild relatives accessions. Red lines indicate the total reads counts for accessions containing the *TFT3* C-allele. Blue lines indicate the total reads counts for accessions containing the *TFT3* G-allele. The x axis indicates the genomic position of the *TFT3* gene (SL2.40ch04:58094138-58096300).

14-3-3 proteins interact with large numbers of proteins in a sequence-specific and phosphorylation-dependent manner (SEHNKE; DELILLE; FERL, 2002). A conserved regulatory function of 14-3-3 was demonstrated in a brassinosteroid signaling pathway in dicots and monocots (BAI et al., 2007; GAMPALA et al., 2007; DENISON et al., 2011). This pathway (Figure 22) includes two cell-surface kinase receptors BRASSINOSTEROID INSENSITIVE-1 (BRI1) and BRI1-associated receptor kinase 1 (BAK1), a BRI1-KINASE INHIBITOR 1 (BKI1), kinases BRASSINOSTEROID-SIGNALING KINASES (BSKs), CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) and BRASSINOSTEROID INSENSITIVE-2 (BIN2), two phosphatases BRI1 SUPPRESSOR 1 (BSU1) and PROTEIN PHOSPHATASE 2A (PP2A), and two

homologous transcription factors (BZR1 and BZR2/BES1) (BAI et al., 2007; GAMPALA et al., 2007; TANG; HAN; CHAI, 2016).

Brassinosteroids bind to the BRI1 domains, activating BAK1 and intracellular kinase activity (LI et al., 2002; LI; NAM, 2002), inducing BKI1 phosphorylation, relieving BKI1 inhibition of BRI1 and fully activating BRI1 through mutual phosphorylation between BRI1 and BAK1 (TANG; HAN; CHAI, 2016). BRI1 activation phosphorylates the BSKs and CDG1 kinases, which in turn activate BSU1 and PP2A (MORA-GARCÍA et al., 2004; TANG; HAN; CHAI, 2016; REN et al., 2019). The BSU1 activation, dephosphorylate and inactivate BIN2, a component that negatively regulates BR signaling, allowing BR response transcription factors BZR1 and BES1 to accumulate in the nucleus and bind to DNA. The reactivation of phosphorylated BZR1 and BES1 is mediated by dephosphorylation through PP2A phosphate (TANG et al., 2011).

When BR signaling is not activated, BZR1 and BES1 are phosphorylated and trapped by 14-3-3 proteins and remain outside the nucleus (LI; NAM, 2002; GAMPALA et al., 2007; TANG; HAN; CHAI, 2016). BZR1 and BES1 in active forms allow their roles as regulator of genes downstream to the signaling pathway of brassinosteroids, such as DWF4, CPD and SAUR-AC1 (HE et al., 2005; YIN et al., 2005).

In *A. thaliana*, BIN2-catalyzed phosphorylation of BZR1 and BZR2/BES1 not only inhibits DNA binding, but also promotes binding to the 14-3-3 proteins (GAMPALA et al., 2007). Mutants of a BIN2 binding site in BZR1 showed no 14-3-3 protein binding, leading to a greater BZR1 nuclear localization and increased BR response, suggesting that 14-3-3 is needed to potentiate the inhibition of BR transcription factors, mainly due to retention in the cytoplasm (GAMPALA et al., 2007). In rice, a mutation at a putative 14-3-3 binding site in BZR1 showed increased nuclear localization compared to the wild type, also suggesting an inhibition of BZR1 function by 14-3-3, reducing the BZR1 nuclear localization (BAI et al., 2007). In addition, BRs signaling was shown to modulate root elongation and foraging (changes in spatial root system allocation in response to a nutrient or water gradient) under low N conditions (JIA et al., 2019; JIA; GIEHL; VON WIRÉN, 2020).

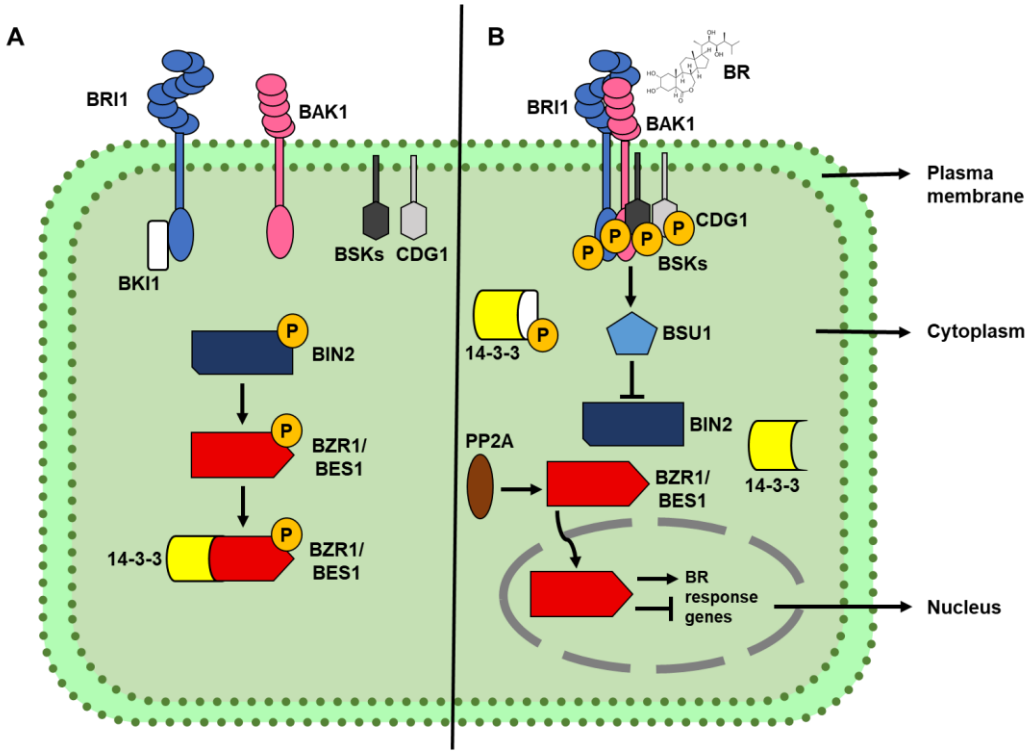


Figure 22 - The brassinosteroid signaling pathway in the absence and presence of brassinosteroids (BR) in *A. thaliana*. In the absence of BR (A), BKI1 interacts with an inactive form of BRI1 to prevent BAK1 from binding to BRI1. BIN2 phosphorylates BZR1 and BES1 which are retained by 14-3-3 proteins in the cytoplasm. In the presence of BR (B), BR binds to BRI1, inducing phosphorylation of BKI1 and BAK1. Phosphorylated BKI1 dissociates from BRI1 and is retained by 14-3-3 proteins. BAK1 and BRI1 form heterodimers and phosphorylation transmit the signal via CDG1 and BSKs. Phosphorylated CDG1 and BSK1s activate BSU1. Activated BSU1 inhibits BIN2. Freed from BIN2 inhibition, BZR1 and BES1 transcriptionally regulate their target genes. BZR1 and BES1 can either induce or inhibit their target genes. Arrows and short lines represent promotion and inhibition, respectively. The circles containing the letter 'P' indicate phosphorylation. Based on: (CHUNG; CHOE, 2013; TANG; HAN; CHAI, 2016).

As *SlAMT1.1* expression appears to be regulated by allelic variation in *14-3-3/TFT3* gene in tomato (Figure 20B), we hypothesized that G>C polymorphism in the 3' UTR of *TFT3* might enter BR signaling downstream of *BZR/BES* determining quantitative *SlAMT1.1* expression. Therefore, we assessed whether allelic differences in *TFT3/14-3-3* gene modulates BR signaling between the *TFT3* C-allele and *TFT3* G-allele in tomato accessions. To address this question, we performed genome-wide identification for orthologous of *BZR/BES1* genes in *S. lycopersicum* reference genome (cv. Heinz) using BLASTP with all genes from *A. thaliana* as query. In total, nine orthologous of *BZR1/BES1* were identified in the tomato genome based on sequence similarity (E-value < $1e^{-10}$) with *A. thaliana* proteins (Table 6).

Table 6 - BZR/BES orthologous identified in *S. lycopersicum* cv. Heinz genome.

Gene	ID	Chromosome location	Gene length (bp)	Protein length (aa)
BES1.1	Solyc01g094580	SL2.50**ch01**: 85997496-86006359	8864	695
BES1.2	Solyc02g063010	SL2.50**ch02**: 35030416-35032639	2224	319
BES1.3	Solyc02g071990	SL2.50**ch02**: 41313401-41318179	4779	324
BES1.4	Solyc03g005990	SL2.50**ch03**: 667344-672399	5056	323
BES1.5	Solyc04g079980	SL2.50**ch04**: 64289859-64291884	2026	328
BES1.6	Solyc07g062260	SL2.50**ch07**: 65038606-65041740	3135	315
BES1.7	Solyc08g005780	SL2.50**ch08**: 604998-612717	7720	666
BES1.8	Solyc10g076390	SL2.50**ch10**: 59363764-59364788	1025	180
BES1.9	Solyc12g089040	SL2.50**ch12**: 64193208-64195373	2166	333

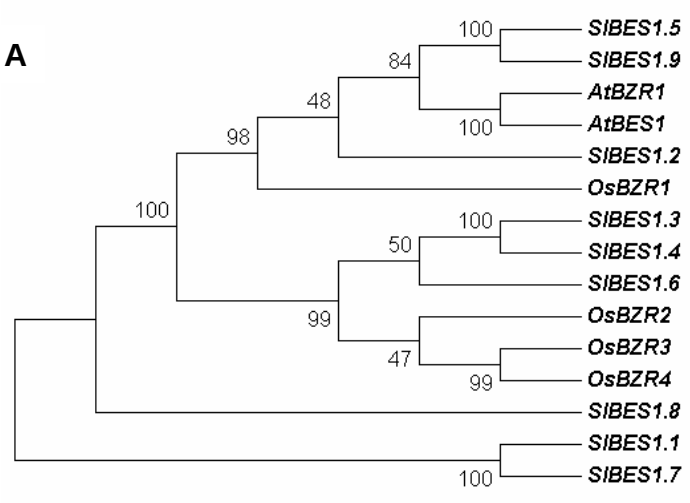
Among the nine identified proteins, BES1.9 shows the highest amino acid sequence identity with AtBZR1 (68.47%) and AtBZR2/BES1 (67.41%), followed by BES1.5 (66.98% and 65.29%), and BES1.2 (56.13% and 57.01%). We also performed an analysis of phylogenetic relationship (Figure 23A) between the homologous BZR/BES identified in tomato, BZR1 and BZR2/BES1 of *A. thaliana* and the four BZRs identified in *O. sativa* (BAI et al., 2007). The three robust main clusters with 100% bootstrap were identified: the first comprising SlBES1.2, SlBES1.5, SlBES1.9, AtBZR1, AtBES1 and OsBZR1; the second containing SlBES1.3, SlBES1.4, SlBES1.6, and three OsBZRs (OsBZR2, OsBZR3 and OsBZR4), and the third group contains only tomato BZRs (SlBES1.8, SlBES1.1 and SlBES1.7). Highly conserved sequences between OsBZR1 and BZR1 or BZR2/BES1 include potential 14-3-3 binding site RXXXpSXP (where X is any amino acid, R is arginine, pS is phosphoserine, and P is proline) in the PEST domain (BAI et al., 2007). Only three tomato proteins (SlBES1.2, SlBES1.5 and SlBES1.9) presented this 14-3-3 predicted binding site (Figure 23B), indicating that these SlBES1 proteins are likely targets for degradation by 14-3-3

binding. Interestingly, these three proteins showed the greatest sequence identity and phylogenetic relationship relative to BZR1 and BES1 proteins from *A. thaliana*.

To assess whether allelic differences in *14-3-3*/*TFT3* gene could also be affecting brassinosteroid signaling among the 15 genotypes with *TFT3* C-allele and the 16 genotypes with *TFT3* G-allele, we evaluated the expression of the *BZR1/BES1* genes in tomato (Figure 23C). The group with *TFT3* G-allele displayed more expression of *BES1.1*, *BES1.4, BES1.5, BES1.6*, *BES1.7 BES1.8* and *BES1.9* relative to *TFT3* C-allele. In contrast, *BES1.2* and *BES1.3* did not show variation in expression between the two group of accession contrasting for the two alleles of *TFT3*.

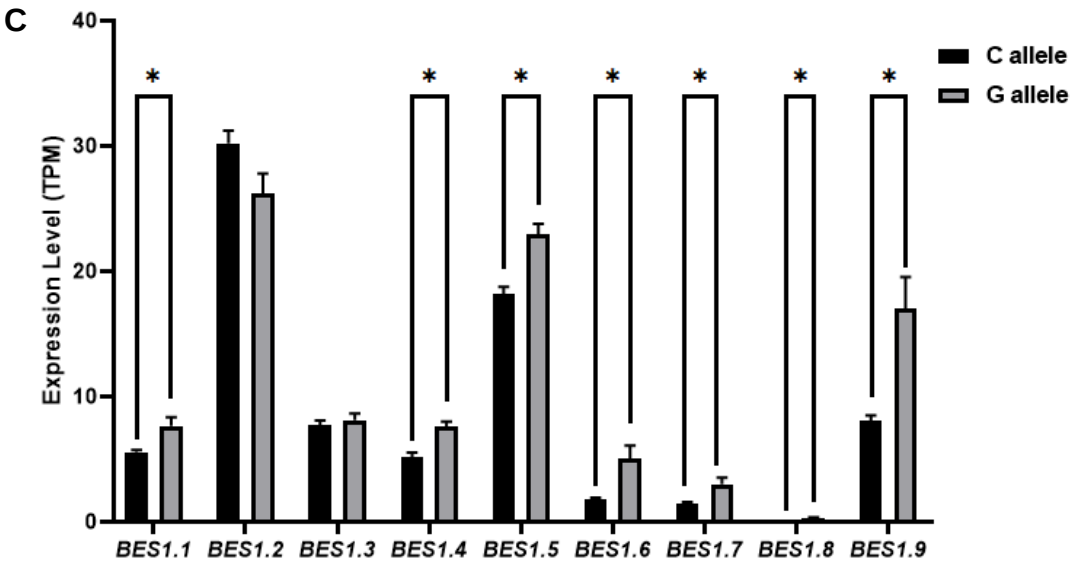
These results suggest that *TFT3* G-allele might specifically upregulates transcript levels of seven transcription factors *BZR1*/*BES1* that are key effector of BRs action. BZR1 and BZR2/BES1 have been shown to directly bind to promoters of BR-responsive genes. In *A. thaliana*, the BZR1 protein binds to specific promoter sequences to mediate feedback inhibition of the BR biosynthetic genes such as *DWARF3/CONSTITUTIVE PHOTOMORPHOGENIC DWARF (DWF3/CPD)* and *DWARF4* (*DWF4)* (HE et al., 2005). We further evaluate whether *TFT3* alleles modulate the expression level of the BR biosynthesis genes *DWF3/CPD* and *DWF4* orthologous in tomato. Relative to *TFT3* C-allele, *TFT3* G-allele is associated to upregulation of transcript levels of *DWF3/CPD* and *DWF4* (Figure 23D).

To further test whether the C to G substitution in *TFT3* gene affects BR signaling, we assessed the expression of *BZR2/BES1*-target gene. BES1 binds to promoters of BR-target gene *SAUR-AC1* for transcriptional activation (YIN et al., 2005). Notably, *SAUR-AC1* transcript levels were found more expressed in accessions containing the allelic G-variant of *TFT3* compared to the C-allele (Figure 23D), suggesting BES1-dependent signaling is enhanced in tomato accessions with *TFT3* G-allelic polymorphism. Taken together, the data suggests that the *TFT3* G-allele variant increases the responsiveness to BRs, whereas *TFT3* C-allele variant reduces BRs response.



B

AtBZR1	SLPSLRISN---SCPVTPPVSS	165
AtBES1	SLPPLRISN---SAPVTPPVSS	182
OsBZR1	NLPPLRVSS---SAPVTPPLSS	202
SlBES1.1	QVVDIHTKLQERDFAGTPYIPV	260
SlBES1.2	TLPPLRISN---SAPVTPPLSS	171
SlBES1.3	FPHHLCIPGGSISAPVTPPLSS	170
SlBES1.4	FPHHLYIPGSSISAPVTPPLSS	172
SlBES1.5	SLPPLRISN---SAPVTPPLSS	188
SlBES1.6	KLPNFHIHTGSISAPVTPPFSS	170
SlBES1.7	LMQELHCGEHGGSFSETQYVPV	236
SlBES1.8	GRPEGSLTSSAFDVNLTLSLSS	180
SlBES1.9	SLPPLRISN---SAPVTPPLSS	196



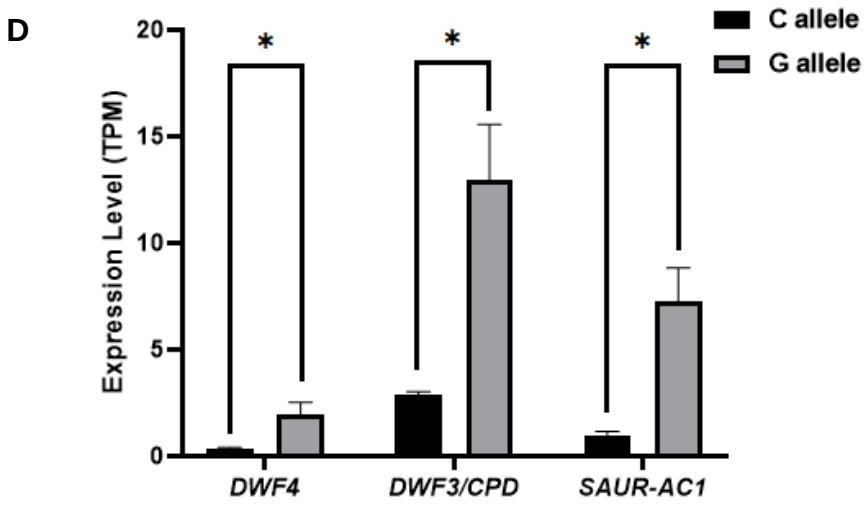


Figure 23 - *TFT3* alleles modulates expression for BR signaling and biosynthesis genes. (A) Strict consensus tree based on BZR/BES protein sequences from *S. lycopersicum*, *A. thaliana* and *O. sativa* with 1,000 bootstraps values obtained by maximum-likelihood analysis; (B) Alignment of the BZR/BES protein portion of *S. lycopersicum*, *A. thaliana* and *O. sativa* containing 14-3-3 predicted binding site; (C) Expression levels for tomato *BZR1/BES1* isoforms between accessions with G and C *TFT3* alleles; (D) Expression levels for tomato *DWF4*, *CPD* and *SAUR-AC1* between accessions with G and C *TFT3* alleles. Bars indicate mean ± SE. Asterisk indicates significant statistical differences in the expression of the indicated gene, comparing the two alleles G and C of *TFT3*. Pairwise statistics were performed using Welch's t test. *DWARF4* (*DWF4* - Solyc02g085360), *DWARF3/CONSTITUTIVE PHOTOMORPHOGENIC DWARF* (*DWF3/CPD* - Solyc06g051750), *SMALL AUXIN UPREGULATED 15* (*SAUR-AC1* - Solyc10g052580).

To understand whether *BES1* directly regulates the expression of *SlAMT1.1* in tomato, we search for *BES1* transcription factor binding sites in the 5000 bp upstream promoter sequence of *SlAMT1.1* gene through the Motif-based analysis tool MEME-suite (https://meme-suite.org/meme/). However, no binding site of the BES family was found, suggesting that the regulation of *SlAMT1.1* expression is not directly regulated by BES1 in tomato. In *A. thaliana*, *AtAMT1* genes expression was inhibited by exogenously supplied BR in gain-of-function *BES1* mutant roots. Although the mechanism is still unclear, there was a higher accumulation of ammonium and glutamine in this mutant compared to the wild type, which could influence the repression of *AtAMT1* expression, mediated by different GS/GOGAT expression patterns (ZHAO et al., 2016). These results indicate that there are regulatory mechanisms shared between brassinosteroids, ammonium uptake and *AMT*s expression, although additional studies are needed to further clarify these mechanisms.

5 DISCUSSION

5.1 Identification and characterization of new *AMT*s in tomato

By revisiting the reference genome sequence released of cultivated tomato (*S. lycopersicum*) version ITAG 2.4 (GAO et al., 2019), we identified four new homologous *AMT*s in tomato from the *AMT2* subfamily. The proposed nomenclature for the new *AMT*s identified in *S. lycopersicum* (*SlAMT3.1, SlAMT3.2, SlAMT3.3* and *SlAMT3.4*) was based on the grouping observed in the phylogenetic analysis with other AMT/MEP superfamily members. The new tomato *AMT* members clustered with *AMT3* members from other species, such as poplar, maize, rice, potato, tobacco and pepper. Further, the phylogenetic analysis containing only the tomato *AMT*s members showed an *AMT2* subdivision into two clades, the first contained only *SlAMT2.1*, while the second had the four newly identified genes (*SlAMT3.1, SlAMT3.2, SlAMT3.3* and *SlAMT3.4*). Previous phylogenetic analyses showed grouping of the *AMT2* subfamily into three different clusters (*AMT2, AMT3* and *AMT4*) (LOQUÉ; VON WIRÉN, 2004; KOEGEL et al., 2013; WU et al., 2015). In other species, such as poplar, the *AMT2* subfamily has been described as originating from whole-genome duplication events (WU et al., 2015). This evolutionary hypothesis may also have happened in tomato. Through our chromosomal localization analysis, it is possible to perceive a homogeneous distribution of *AMT*s in the tomato genome, which may not suggest the existence of segmental or tandem duplications.

The identified SlAMT3.1, SlAMT3.2, SlAMT3.3, and SlAMT3.4 share features similarity to previously described AMTs in other plant species, including protein size (468-485 amino acids), molecular weight (51-52 kDa), isoelectric point (5.9-6.5), and the 11 transmembrane domains, typical for AMTs transporters (LUDEWIG; VON WIREN; FROMMER, 2002; FILIZ; AKBUDAK, 2020). Gene structure analysis based on the CDS and genomic sequences of each tomato *AMT* gene indicated that the tomato subfamilies AMT1 and AMT2 have structural diversity. AMT1 members (*SlAMT1.1* and *SlAMT1.2*) contain three exons, with the exception of *SlAMT1.3*, which has only one exon. AMT2 members presented three (*SlAMT3.1* and *SlAMT3.4*) or four exons (*SlAMT3.2* and *SlAMT3.3*). Similar results were reported for poplar and *A. thaliana*, where AMT2 subfamily members had larger number of exons than members from the AMT1 subfamily (SOHLENKAMP et al., 2002; COUTURIER et al., 2007).

The newly identified tomato *AMT* genes had significantly lower expression levels than those previously described, which may have been the reason why these genes have not yet been described. The expression analysis of *AMT*s genes among tomato tissues demonstrated that the members of the AMT1 subfamily (*SlAMT1.1*, *SlAMT1.2* and *SlAMT1.3*) are more expressed in roots, leaves and flowers, while the AMT2 members (in particular *SlAMT2.1* and *SlAMT3.2*) showed more accumulation of transcripts in fruits at breaker stage, which may indicate a possible specific function in that organ or stage of development. To date, no functional study has described AMTs as playing a specific role in fruits. However, the expression of *AMT*s in reproductive tissues has already been identified in other species. Cc03_g06810, the *AtAMT1.1* orthologous from *Coffea canephora* presented high expression in perisperm, indicating that ammonium transport may have some impact in fruit development (SANTOS et al., 2017). In *Populus trichocarpa*, *PtrAMT1.1*, *PtrAMT1.6* and *PtrAMT4.5* presented expression in fruits (COUTURIER et al., 2007). In maize, *ZmAMT1.1A*, *ZmAMT2.1* and two putative low affinity transporters *AMMONIUM FACILITATOR 1* (*ZmAMF1.1* and *ZmAMF1.2*), are expressed in cobs (DECHORGNAT et al., 2019). Besides, *AtAMT1.4* mediates ammonium uptake across the plasma membrane in pollen in *A. thaliana* to contribute to N nutrition of pollen via ammonium uptake or retrieval (YUAN et al., 2009).

Annotating the underlying AMT2 genes is the first step towards a more complete characterization of tomato AMT2 function and potential of relevant traits. Here, our analysis revealed substantial gene expression diversity for the newly identified *SlAMT3.2* and *SlAMT3.3* in accessions of *Solanum* section *Lycopersicon*. In addition, gene expression analysis indicated higher expression levels of *SlAMT3.3,* but variable for *SlAMT3.2* in wild species compared to cv. M82. *SlAMT3.3* showed more expression in *S. pennellii* and *S. habrochaites* genotypes, while *SlAMT3.2* is more expressed in cv. M82 and *S. pennellii*. Both were less expressed in *S. pimpinellifolium* and *S. chmielewskii*. Given that the tomato RNA-Seq data used derived from samples of a single tissue at one developmental stage (seedlings shoots), these expression frequencies represent a conservative estimate. As the *SlAMT3.2* and *SlAMT3.3* genes show different expression patterns between wild and cultivar accessions, studies of gene sequence diversity, as well as functional studies seem to be relevant to search for interesting traits and for the introgression of possible beneficial wild alleles in cultivars. Moreover, the tissue-specific expression of *SlAMT3.1*, *SlAMT3.2*, *SlAMT3.3*, and *SlAMT3.4* is positively associated with big-fruited tomato development, suggesting

a presumed link with ammonium nutrition. Therefore, such natural variation in *SlAMT3* expression may have relevant phenotypic outcomes that could contribute to crop improvement. Functional studies of *SlAMT3.1*, *SlAMT3.2*, *SlAMT3.3*, and *SlAMT3.4* should enable the understanding of the physiological relevance in tomato fruit.

5.2 Wild tomato species exhibit a genetic diversity in the SlAMT1.1 protein that could be relevant for root ammonium influx

In *A. thaliana*, post-translational regulation of AMT1 proteins occurs mainly through the phosphorylation of a specific Thr residue (T460 in AtAMT1.1 and T464 in AtAMT1.3) at the carboxy-terminal under conditions of high external ammonium supply (LOQUÉ et al., 2007; LANQUAR et al., 2009; YUAN et al., 2013), leading to a trans-inactivation of the entire trimer of the AMT protein in a allosteric way, and thereby inhibiting ammonium uptake in roots (LOQUÉ et al., 2007). However, besides T460, there are multiple presumed phosphorylation sites at the C-terminal, but their potential functional roles in regulating AMTs are still unknown (WU et al., 2019).

Thus, we pursued to verify possible variations in the *SlAMT1.1* coding sequence and how these variations may be associated with different patterns of regulation of SlAMT1.1 activity. Our findings within the *Lycopersicon* section revealed nucleotide variation along *SlAMT1.1*, with a substantial higher diversity in *SlAMT1.1* CDS sequences of the wild groups Eriopersicon, Neolycopersicon and Arcanum, as compared with the Lycopersicon and cultivars groups. The Eriopersicon and Neolycopersicon groups, which are the most phylogenetically distant to cultivars, were those that presented a greater variability in the *SlAMT1.1* sequence. A remarkably *SlAMT1.1* sequence diversity was found in wild species, but a relatively low sequence diversity between genetically closer species within the groups of cultivars and Lycopersicon, displaying essentially conserved gene sequences. Notably, for all taxonomic groups dN/dS was <1 evidencing a negative selection for *SlAMT1.1* and differences in selection pressure between cultivars and wild species.

Several lines of evidence suggest a role of the genetic diversity within SlAMT1.1 in the tomato clade in the regulation of the ammonium transport activity. Firstly, *S. habrochaites* LA1718 from Eriopersicon showed the substitution S477L at the non-conserved region of the C-terminal (CTR^{NC}). SlAMT1.1 S477 site in *S. habrochaites* is equivalent to the S475 of AtAMT1.1 and S480 present in the CTR^{NC} of AtAMT1.3,

previously identified in phosphoproteomic studies (ENGELSBERGER; SCHULZE, 2012; WU et al., 2019). In *A. thaliana*, during the nitrate resupply, AtAMT1.3 S480 is phosphorylated in N-deficient roots. By contrast, mutants of AtAMT1.3 S480 did not cause a significant decrease in transport activity compared to the wild type roots. However, this response is not seen during ammonium resupply (WU et al., 2019). Our findings show that *S. habrochaites* LA1718 failed to shut off ammonium transport activity under ammonium resupply treatment. This differential activity of SlAMT1.1 appears to be associated with the predicted additional S477 phosphorylation site at the C-terminal region of SlAMT1.1.

Multisite phosphorylation is a sophisticated regulatory mechanism to modulate protein function by different kinases/phosphatases via different signaling pathways (COHEN, 2000; HARUTA; GRAY; SUSSMAN, 2015; STRAUB; LUDEWIG; NEUHÄUSER, 2017). Phosphorylation at various sites may lead to distinct conformational changes of the C-terminal. Upon ammonium resupply, T464 phosphorylation at the CTR^{C} acts as a prime switch preventing excessive uptake of ammonium, while Thr or Ser phosphorylation sites in the CTR^{NC} can finely adjust AtAMT1.3 transport activity (WU et al., 2019). Simultaneous phosphorylation of T464 and T494 were shown to effectively inhibit AtAMT1.3 transport activity (WU et al., 2019). Similar to AtAMT1.3, the SlAMT1.1 activity can be modulated by multisite phosphorylation under control of various N signals. High external ammonium availability triggers post-translational regulation in AMT1 proteins, mainly through the phosphorylation of specific Thr site (T462 in SlAMT1.1, T460 in AtAMT1.1, T464 in AtAMT1.3) at CTR^{C} (LOQUÉ et al., 2007; LANQUAR et al., 2009; YUAN et al., 2013).

Therefore, ammonium-triggered phosphorylation of conserved T460 residue at the CTR^{C} (T462 in SlAMT1.1, T464 in AtAMT1.3) for shutting off the transporter activity via the allosteric regulation, preventing excessive uptake of ammonium seems to be a conserved mechanism. T462 is present in cultivars and all related wild tomato species representative of the Lycopersicon, whereas Eriopersicon species have an additional S477 phosphorylation site at the CTR^{NC}, which may give distinct dynamic phosphorylation patterns. Putative phosphorylation sites in the CTR^{NC} of SlAMT1.1 (*e.g.* three Thr residues – T497, T498 and T490) are conserved among wild species and cultivars, indicating that it is unlikely that these phosphorylation sites have a major role in regulating distinct AMT1 transport activity crop accession and wild species. However, it is clear that among multiple phosphorylation sites found in SlAMT1.1,

the identification phosphosites modulated by ammonium needs further functional confirmation.

The second evidence that the genetic diversity within SlAMT1.1 in the tomato clade can regulate ammonium transport activity, the Arcanum group member *S. chmielewskii* LA2695 exhibit substitutions at S102A, S124N and V236A and presented a less repressed transport activity upon ammonium resupply. Although these variations are not present at the C-terminal, phosphorylation sites in the cytosolic loop helices (*e.g.* S102A) may serve as a general mechanism to modulate membrane proteins transport (ZOURELIDOU et al., 2014). These variants are restricted to the *S. chmielewskii* species and not found in *S. arcanum* or *S. neorickii*, suggesting a more diverse and likely a rare allelic variation for *SlAMT1.1* in *S. chmielewskii*. Moreover, phylogenetic relationship likely correlated with habitat, however, may indicate the occurrence of distinct geographical races within Arcanum group.

Third, either *S. lycopersicum* cv. M82 and *S. pimpinellifolium* LA1584, representative of the Lycopersicon without the substitution at S102A, S124N, V236A and S477L residues, can rapidly inactivate SlAMT1.1 transport activity and inhibit root ammonium uptake. In addition, the rate of ^{15}N-ammonium uptake during ammonium resupply for *S. chmielewskii* LA2695 and *S. habrochaites* LA1718 suggested a reduced allosteric regulation for ammonium transport activity when compared to *S. lycopersicum* cv. M82 LA3475 and *S. pimpinellifolium* LA1584. Despite the distinct dynamic for root influx of ^{15}N-ammonium among tomato groups, the transcript abundance of *SlAMT1.1* did not show any change after resupply of ammonium in N-deficient roots in either wild species or cultivar. This suggests that the regulation was at the post-translational level, because transcripts and protein activity were not synergistically affected. Thus, besides the substitution S477L in CTR^{NC} present in Eriopersicon group, modifications at S102A and V236A in the cytosolic loop within Arcanum group members can be involved in modulating AMT transporter activity in an allosteric manner.

Our finding of novel phosphorylation site prediction and *in planta* ammonium influx analysis extended our understanding of SlAMT1s presumed phospho-dependent regulation that evolved within Eriopersicon and Arcanum groups are functionally involved in regulating AMT1s transporter activity and how they differentially respond to variable N supply.

5.3 A putative role of *TFT3* alleles and brassinosteroids in the regulation of *SlAMT1.1* expression

Using genome-wide association mapping (GWAS), we found that patterns of gene expression in *SlAMT1.1* transcript level associates with two main loci: a SNP T>C identified in the intronic region of the *NON EXINE FORMATION-1* gene (*NEF1*, Solyc00g006830) and a SNP G>C present in the 3' UTR region of a *14-3-3* gene (*TFT3*, Solyc04g074510). The *TFT3* gene encodes to 14-3-3 protein family involved in the regulation of several metabolic processes in eukaryotes through the binding and modulation of phosphorylated proteins (WILSON; SWATEK; THELEN, 2016). In addition, 14-3-3 proteins are found as multiple isoforms in organisms, with plants having the highest number of isoforms. The presence of multiple 14-3-3 generates complexity in the mechanism of action of these proteins, in particular, due to the variation of the spatiotemporal expression and the diversity of subcellular location between the paralogues (WILSON; SWATEK; THELEN, 2016).

Several studies have revealed an impressive list of putative 14-3-3 targets in plants (JASPERT; THROM; OECKING, 2011). 14-3-3s play an important role in stress signaling pathways, such as nutrient deficiency, salt stress and drought, by increasing membrane transporter activity, such as Arabidopsis H^+-ATPase (AHA), V-type H^+-ATPase (VHA) and H^+-pyrophosphatase (H^+-PPase) (DENISON et al., 2011; JASPERT; THROM; OECKING, 2011). 14-3-3s also can display changes in the transcriptional and/or protein levels in response to pathogen perception or infection (LOZANO-DURÁN; ROBATZEK, 2015). For example, in tomato, 14-3-3 may act by stabilizing the MAPKKKα kinase to activate Mitogen-activated protein kinase (MAPK) downstream cascade, leading to programmed cell death in response to *Pseudomonas syringae* infection (OH; PEDLEY; MARTIN, 2010). Another plant environmental response in which 14-3-3s have a functional role is the response to light (DENISON et al., 2011). In *A. thaliana*, 14-3-3 insertional mutants show a delay in flowering of 3 to 5 days under long-day conditions (MAYFIELD et al., 2007). In addition, two 14-3-3 isoform mutants also presented hypocotyl growth under red light, suggesting a possible role in phytochrome B signaling pathway (MAYFIELD et al., 2007).

However, a number of 14-3-3 roles in physiological processes may be at least partially due to their effects on the regulation of hormone signaling pathways (DENISON et al., 2011). Studies demonstrated an essential role for 14-3-3 proteins in

brassinosteroid (BR) signal transduction (BAI et al. 2007; GAMPALA et al., 2007, DENISON et al. 2011). In the absence of BR, two transcription factors involved in BR signaling BZR1 and BES1 are phosphorylated by BIN2 kinase. 14-3-3 binds to phosphorylated BZR1/BES1, retaining them in the cytoplasm where they are degraded by the proteasome and reducing their nuclear localization. In the presence of BR, BIN2 kinase is deactivated, inhibiting the binding of 14-3-3 in BZR1/BES1, which remain in the nucleus and can interact with BR-responsive genes (BAI et al., 2007; GAMPALA et al. 2007).

Here, we found that distinct expression levels of the *14-3-3* genes occur in tomato among the plant tissues, and *TFT3/14-3-3* gene has a predominant expression in roots, which might suggest a functional role in root cells for regulating *SlAMT1.1* expression levels. We could not significantly associate *TFT3* transcript abundance with naturally occurring variants G and C alleles of *TFT3*, but we showed that the G-allelic variant for *TFT3* was associated with an increased expression of BR signaling component in tomato *BZR1/BES1* orthologous, contributing to higher expression levels of the BR biosynthetic genes, such as *DWF4*, *CPD* and *SAUR-AC1*. As accessions with the C-allele showed a reduced BR response than those carrying G-allele, we hypothesized that the G>C polymorphism in in the 3' UTR region of *TFT3* might be an associated quantitative trait nucleotide underlying *SlAMT1.1* transcripts variation in tomato groups. A group of tomato accessions carrying the *TFT3 C*-allele exhibited significantly more *SlAMT1.1* transcript level than those accessions with the *TFT3 G*-variant. Thus, our results provide some evidence that natural variation of the *TFT3/14-3-3* locus might be associated with BR response in tomato root to modulate *SlAMT1.1* transcriptional levels, by which reduced BR signaling in *TFT3 C*-variant enhanced *SlAMT1.1* expression than tomato accessions carrying *TFT3 G*-allelic variation. Although the analysis performed cannot confirm, we hypothesized that the G>C variation in the 3' UTR region may be related to the stability of the *TFT3* mRNA, and thus cause variations in the protein's activity.

The role of BR signaling in N uptake has been previously described in *A. thaliana.* BR synthesis contributes to the root foraging adaptative response DWARF1 (DWF1), a key gene involved in BR biosynthesis, as a further molecular determinant for root elongation in response to low N availability (JIA et al., 2020). In addition, allelic variation BSK3/BIN2 kinase in *A. thaliana* accessions was shown to be determinant to modulate BR sensitivity and root elongation at low N. Further,

low N specifically upregulates transcript levels of the BR co-receptor BAK1 to activate BR signaling and stimulate root elongation (JIA et al., 2019). Thus, BR signaling is clearly modulated by N signaling. This conclusion is further supported by functional studies in *A. thaliana* which have shown that *BES1* regulates *AMT1* expression in roots and BR treatment reduced the expression of AMT1 genes (ZHAO et al., 2016). Using ^{15}N-ammonium influx analysis, mutants with BES1 gain-of-function showed higher root ammonium content with the BR treatment, indicating that the activation of BR signaling increases the ammonium uptake, and consequently, repress the expression of the *AtAMT1* genes (ZHAO et al., 2016). However, BES1 does not appear to regulate *AMT1* directly, since BES1 failed to direct bind E-box elements present in the *AMT1* promoter region (ZHAO et al., 2016). BR signaling activation accumulated more cellular ammonium, and suppression of BR-mediated *AtATM1*-repression was inhibited in *BRI1* mutant, suggesting that BR-mediated *AtATM1*-repression required *BRI1* activity in *A. thaliana* roots (ZHAO et al., 2016). Further, the repression of the expression of *AMT1* genes is not mainly caused by ammonium itself, but by the perception of ammonium assimilation products, *i.e.* glutamine (RAWAT et al., 1999). This could suggest a potential regulatory role for BES1 in the genes associated with the assimilation of ammonium products, such as GS/GOGAT. Together, these previous studies indicate that BRs regulate several physiological and developmental processes in plants according to fluctuations in N availability.

As N deficiency was previously shown to activate BR signaling upstream of BSK3/BIN2 by upregulating BAK1 expression (JIA et al., 2019), thereby activating the BR signaling cascade, we hypothesized that downstream of BAK1 receptor, the amplitude of BR signaling can be modulated irrespective of N nutritional status as a result of allelic variation in the downstream gene/protein targets. In agreement with this hypothesis, our results demonstrated that the *TFT3 C*-allele relays a weaker BR signaling output than the *TFT3 G*-allele, associated with higher expression of *SlAMT1.1* in tomato accessions. In line with this possibility, it is possible to speculate that *TFT3 C*-variant is more efficient in directly inhibiting BZR/BES1 function and decreasing BR response, suggesting that 14-3-3 potentiate the inhibition of BR transcription factors, at least in part by reducing its nuclear localization. Thus, irrespective of N conditions in soils, natural allelic variation *TFT3-C* might have an increased SlAMT1.1-mediated uptake levels than *TFT3 G*-variants. The molecular regulatory mechanism underlying the potential role of 14-3-3 in *SlAMT1.1* expression

remains elusive. Future research will be necessary to test whether tomato accession with *TFT3* variants may have distinct root adaptive response to low N.

SNPs variants were shared within the members of Lycopersicon clade, but not in the Arcanum, Eriopersicon and Neolycopersicon groups that carries only the G-allelic variation in the *TFT3* locus. In addition, the distribution of *TFT3 G*-variant relative to C- variant was found for specific members of Lycopersicon. Indeed, among 50 evaluated accessions of *S. pimpinellifolium*, 6% presented *TFT3* C-variant, while 17% of the six accessions of *S. cheesmaniae* and 70% of the 53 accessions of *S. lycopersicum* var. cerasiforme presented the C allelic variation. Conversely, the two sequences of *S. galapagense* showed allele G. Thus, *TFT3-C* is clearly at minor allele frequency among wild species and allelic variation of *TFT3* arisen independently within the members of Lycopersicon clade. By contrast, *TFT3 G*-variant decreased in frequency among modern cultivars compared to *TFT3 C*-allele, suggesting that *TFT3-C* haplotype is likely a human-related selection during domestication associated with increased *SlAMT1.1* transcript abundance in roots. Accordingly, previous studies revealed that genomic variation in tomato domestication, from wild ancestors to contemporary breeding accessions, are associated with introgression of genes for favorable traits with evolutionary trajectory of alleles from Lycopersicon group, with the origin of modern cultivated tomato from convergent evolution of wild *S. pimpinellifolium* to *S. lycopersicum* var. cerasiforme in South America, and consistent subsequent selection gave rise to cultivated tomato in Mesoamerica (LIN et al., 2014; RAZIFARD et al., 2020). Supporting this notion, we demonstrated that natural acessions of *S. pimpinellifolium* carrying the *TFT3-C* are present at only 6%, whereas 94% are *TFT3-G* haplotype. Conversely, *TFT3 C*-variant are predominantly in the *S. lycopersicum* var. cerasiforme (70% *TFT3-C* and 30% *TFT3-G*).

Taken together, these results suggest that prevalence of the naturally rare *TFT3 C*-allele probably originated from wild ancestor *S. pimpinellifolium*, and subsequent indirect human selection for further increased *SlAMT1.1* expression levels in cultivated tomato roots, caused increase in *TFT3 C*-allele frequency. Therefore, here we discovered a major genomic signature for modern cultivated tomato, identifying *TFT3* variants that potentially confer increased ammonium nutrition uptake with conventional breeding.

6 CONCLUSION

- Four new *AMT*s genes were identified in the *S. lycopersicum* genome, and named *SlAMT3.1*, *SlAMT3.2*, *SlAMT3.3,* and *SlAMT3.4* based on phylogenetic relationships with *AMT*s from other species;
- *SlAMT3.1*, *SlAMT3.2*, *SlAMT3.3* and *SlAMT3.4* displayed distinct pattern of expression between cultivars and wild species of *Solanum*, and among tomato tissues, suggesting function specificities in tomato;
- Evolutionary studies and sequence analysis of *SlAMT1.1* among 287 accessions of the five groups of *Solanum* section *Lycopersicon* revealed synonymous and non-synonymous SNPs, which could be associated with differences in expression patterns among tomato accessions, and in protein activity by structural differences and/or post-translational regulation;
- ^{15}N-labeled high-affinity ammonium influx experiment using *S. lycopersicum* cv. M82, *S. pimpinellifolium* LA1584, *S. chmielewskii* LA2695 and *S. habrochaites* LA1718 under contrasting N availability showed that *S. chmielewskii* had a higher ammonium uptake compared to the other genotypes, which could be associated with more *SlAMT1.1* expression. Under N-deficiency, *S. pimpinellifolium*, *S. chmielewskii* and *S. habrochaites* showed similar levels of SlAMT1.1 protein activity;
- During ammonium resupply, *S. chmielewskii* and *S. habrochaites* showed less inhibition of the ammonium uptake compared to cv. M82 and *S. pimpinellifolium*. The lack of correlation between expression and uptake suggests a differential allosteric regulation of the SlAMT1.1 in these genotypes, that may be associated with amino acids variation in the protein sequence, especially S477L present in *S. habrochaites*;
- Genome wide association study identified a SNP G>C at the 3' UTR of a *14-3-3* gene associated with differences in the *SlAMT1.1* expression in tomato. The G-allele decreases expression of *SlAMT1.1* but does not alter the expression of the *14-3-3* itself, indicating that it is not a transcriptional regulation;
- The SNP at the *14-3-3* gene alters the expression of genes associated with signaling and synthesis of brassinosteroids (*BES1, DWF4, CPD* and *SAUR-AC1*) suggesting that *14-3-3* may be modulating the expression of *SlAMT1.1* through the brassinosteroid signaling pathway, but further studies should be carried out to better elucidate the mechanism.

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SUPPLEMENTARY DATA

Supplementary Table 1 - Information of the 287 accessions of *Solanum* sect. *Lycopersicon* used for *in silico* analysis

Group	Genotype	Code
Cultivar	M82	LA3475
Cultivar	Ailsa Craig	-
Cultivar	All Round	EA02617
Cultivar	Anto	EA01835
Cultivar	Belmonte	EA00892
Cultivar	Black Cherry	EA00027
Cultivar	Bloody Butcher	TR00019
Cultivar	Brandywine	EA01019
Cultivar	Cal J	EA02054
Cultivar	Chih-Mu-Tao-Se	EA04828
Cultivar	Cross Country	EA03701
Cultivar	Dana	EA01155
Cultivar	Dixy Golden Giant	TR00020
Cultivar	Dolmalik	EA04861
Cultivar	Galina	EA00325
Cultivar	Garderners Delight	EA06086
Cultivar	Giant Belgium	EA01037
Cultivar	Heinz	LA4345
Cultivar	Iidi	EA03362
Cultivar	Jersey Devil	EA00990
Cultivar	John Big Orange	EA00371
Cultivar	Katinka Cherry	EA00375
Cultivar	Kentucky Beefsteak	TR00021
Cultivar	Large Pink	EA01049
Cultivar	Large Red Cherry	TR00018
Cultivar	Marmande	TR00022
Cultivar	MicroTom	-
Cultivar	Momotaro	TR00003
Cultivar	Moneymaker	-
Cultivar	Polish Joe	EA00157
Cultivar	Ponderosa	EA00448
Cultivar	Porter	EA00940
Cultivar	Rote Beere	EA01965
Cultivar	Rutgers	EA00465
Cultivar	Sonato	EA02724
Cultivar	The Dutchman	EA05581
Cultivar	Thessaloniki	TR00023
Cultivar	Tiffen Mennonite	EA01088

(to be continued)

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Supplementary Table 1 - Information of the 287 accessions of *Solanum* sect. *Lycopersicon* used for *in silico* analysis

Group	Genotype	Code
Cultivar	Watermelon Beefsteak	EA01640
Cultivar	Wheatley Frost	EA04939
Cultivar	Winter Tipe	EA01854
Cultivar	*S. lycopersicum*	EA00240
Cultivar	*S. lycopersicum*	EA00304
Cultivar	*S. lycopersicum*	EA00369
Cultivar	*S. lycopersicum*	EA00389
Cultivar	*S. lycopersicum*	EA00422
Cultivar	*S. lycopersicum*	EA00526
Cultivar	*S. lycopersicum*	EA00840
Cultivar	*S. lycopersicum*	EA00915
Cultivar	*S. lycopersicum*	EA00951
Cultivar	*S. lycopersicum*	EA00983
Cultivar	*S. lycopersicum*	EA01002
Cultivar	*S. lycopersicum*	EA01020
Cultivar	*S. lycopersicum*	EA01185
Cultivar	*S. lycopersicum*	EA01198
Cultivar	*S. lycopersicum*	EA01230
Cultivar	*S. lycopersicum*	EA01237
Cultivar	*S. lycopersicum*	EA01270
Cultivar	*S. lycopersicum*	EA01371
Cultivar	*S. lycopersicum*	EA01756
Cultivar	*S. lycopersicum*	EA01802
Cultivar	*S. lycopersicum*	EA01804
Cultivar	*S. lycopersicum*	EA01915
Cultivar	*S. lycopersicum*	EA01953
Cultivar	*S. lycopersicum*	EA01960
Cultivar	*S. lycopersicum*	EA01982
Cultivar	*S. lycopersicum*	EA01989
Cultivar	*S. lycopersicum*	EA02304
Cultivar	*S. lycopersicum*	EA02435
Cultivar	*S. lycopersicum*	EA02586
Cultivar	*S. lycopersicum*	EA02655
Cultivar	*S. lycopersicum*	EA02660
Cultivar	*S. lycopersicum*	EA02669
Cultivar	*S. lycopersicum*	EA02728
Cultivar	*S. lycopersicum*	EA02732
Cultivar	*S. lycopersicum*	EA02761
Cultivar	*S. lycopersicum*	EA02764

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Supplementary Table 1 - Information of the 287 accessions of *Solanum* sect. *Lycopersicon* used for *in silico* analysis

Group	Genotype	Code
Cultivar	*S. lycopersicum*	EA02895
Cultivar	*S. lycopersicum*	EA02898
Cultivar	*S. lycopersicum*	EA02960
Cultivar	*S. lycopersicum*	EA03002
Cultivar	*S. lycopersicum*	EA03028
Cultivar	*S. lycopersicum*	EA03083
Cultivar	*S. lycopersicum*	EA03107
Cultivar	*S. lycopersicum*	EA03126
Cultivar	*S. lycopersicum*	EA03221
Cultivar	*S. lycopersicum*	EA03222
Cultivar	*S. lycopersicum*	EA03274
Cultivar	*S. lycopersicum*	EA03426
Cultivar	*S. lycopersicum*	EA03439
Cultivar	*S. lycopersicum*	EA03456
Cultivar	*S. lycopersicum*	EA03463
Cultivar	*S. lycopersicum*	EA03525
Cultivar	*S. lycopersicum*	EA03533
Cultivar	*S. lycopersicum*	EA03577
Cultivar	*S. lycopersicum*	EA03586
Cultivar	*S. lycopersicum*	EA03611
Cultivar	*S. lycopersicum*	EA03613
Cultivar	*S. lycopersicum*	EA03648
Cultivar	*S. lycopersicum*	EA03650
Cultivar	*S. lycopersicum*	EA03673
Cultivar	*S. lycopersicum*	EA04236
Cultivar	*S. lycopersicum*	EA04243
Cultivar	*S. lycopersicum*	EA04710
Cultivar	*S. lycopersicum*	EA05170
Cultivar	*S. lycopersicum*	EA05480
Cultivar	*S. lycopersicum*	EA05578
Cultivar	*S. lycopersicum*	EA05612
Cultivar	*S. lycopersicum*	EA05701
Cultivar	*S. lycopersicum*	EA05747
Cultivar	*S. lycopersicum*	EA05808
Cultivar	*S. lycopersicum*	EA05891
Cultivar	*S. lycopersicum*	EA06485
Cultivar	*S. lycopersicum*	EA06902
Cultivar	*S. lycopersicum*	LA0012
Cultivar	*S. lycopersicum*	LA0025

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Supplementary Table 1 - Information of the 287 accessions of *Solanum* sect. *Lycopersicon* used for *in silico* analysis

Group	Genotype	Code
Cultivar	*S. lycopersicum*	LA0089
Cultivar	*S. lycopersicum*	LA0113
Cultivar	*S. lycopersicum*	LA0126
Cultivar	*S. lycopersicum*	LA0134C
Cultivar	*S. lycopersicum*	LA0146
Cultivar	*S. lycopersicum*	LA0147
Cultivar	*S. lycopersicum*	LA0172
Cultivar	*S. lycopersicum*	LA0292
Cultivar	*S. lycopersicum*	LA0395
Cultivar	*S. lycopersicum*	LA0404
Cultivar	*S. lycopersicum*	LA0410
Cultivar	*S. lycopersicum*	LA0466
Cultivar	*S. lycopersicum*	LA0490
Cultivar	*S. lycopersicum*	LA0502
Cultivar	*S. lycopersicum*	LA0516
Cultivar	*S. lycopersicum*	LA0517
Cultivar	*S. lycopersicum*	LA0533
Cultivar	*S. lycopersicum*	LA1021
Cultivar	*S. lycopersicum*	LA1090
Cultivar	*S. lycopersicum*	LA1162
Cultivar	*S. lycopersicum*	LA1210
Cultivar	*S. lycopersicum*	LA1218
Cultivar	*S. lycopersicum*	LA1263
Cultivar	*S. lycopersicum*	LA1421
Cultivar	*S. lycopersicum*	LA1425
Cultivar	*S. lycopersicum*	LA1459
Cultivar	*S. lycopersicum*	LA1462
Cultivar	*S. lycopersicum*	LA1479
Cultivar	*S. lycopersicum*	LA1504
Cultivar	*S. lycopersicum*	LA1506
Cultivar	*S. lycopersicum*	LA1544
Cultivar	*S. lycopersicum*	LA1701
Cultivar	*S. lycopersicum*	LA1706
Cultivar	*S. lycopersicum*	LA2009
Cultivar	*S. lycopersicum*	LA2260
Cultivar	*S. lycopersicum*	LA2285
Cultivar	*S. lycopersicum*	LA2307
Cultivar	*S. lycopersicum*	LA2399
Cultivar	*S. lycopersicum*	LA2402

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Supplementary Table 1 - Information of the 287 accessions of *Solanum* sect. *Lycopersicon* used for *in silico* analysis

Group	Genotype	Code
Cultivar	*S. lycopersicum*	LA2413
Cultivar	*S. lycopersicum*	LA2706
Cultivar	*S. lycopersicum*	LA2711
Cultivar	*S. lycopersicum*	LA2838A
Cultivar	*S. lycopersicum*	LA3008
Cultivar	*S. lycopersicum*	LA3144
Cultivar	*S. lycopersicum*	LA3214
Cultivar	*S. lycopersicum*	LA3242
Cultivar	*S. lycopersicum*	LA3243
Cultivar	*S. lycopersicum*	LA3320
Cultivar	*S. lycopersicum*	LA3343
Cultivar	*S. lycopersicum*	LA3528
Cultivar	*S. lycopersicum*	LA3625
Cultivar	*S. lycopersicum*	LA3840
Cultivar	*S. lycopersicum*	LA3845
Cultivar	*S. lycopersicum*	LA3846
Cultivar	*S. lycopersicum*	LA3856
Cultivar	*S. lycopersicum*	LA3903
Cultivar	*S. lycopersicum*	LA3911
Cultivar	*S. lycopersicum*	LA4024
Cultivar	*S. lycopersicum*	LA4025
Cultivar	*S. lycopersicum*	LA4133
Cultivar	*S. lycopersicum*	LA4347
Cultivar	*S. lycopersicum*	LA4354
Cultivar	*S. lycopersicum*	LA4355
Arcanum	*S. arcanum*	LA2157
Arcanum	*S. arcanum*	LA2172
Arcanum	*S. chmielewskii*	LA2663
Arcanum	*S. chmielewskii*	LA2695
Arcanum	*S. neorickii*	LA2133
Arcanum	*S. neorickii*	GCN24193
Eriopersicon	*S. chilense*	LA1969
Eriopersicon	*S. chilense*	CGN15532
Eriopersicon	*S. chilense*	CGN15530
Eriopersicon	*S. corneliomulleri*	LA0118
Eriopersicon	*S. habrochaites*	CGN15791
Eriopersicon	*S. habrochaites*	PI134418
Eriopersicon	*S. habrochaites*	CGN15792
Eriopersicon	*S. habrochaites*	LA1718

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Supplementary Table 1 - Information of the 287 accessions of *Solanum* sect. *Lycopersicon* used for *in silico* analysis

Group	Genotype	Code
Eriopersicon	*S. habrochaites*	LA1777
Eriopersicon	*S. habrochaites*	LA407
Eriopersicon	*S. habrochaites*	LYC4
Eriopersicon	*S. huaylasense*	LA1983
Eriopersicon	*S. huaylasense*	LA1365
Eriopersicon	*S. huaylasense*	LA1364
Eriopersicon	*S. peruvianum*	LA1278
Eriopersicon	*S. peruvianum*	LA1954
Lycopersicon	*S. cheesmaniae*	CGN15820
Lycopersicon	*S. cheesmaniae*	LA1401
Lycopersicon	*S. cheesmaniae*	LA0746
Lycopersicon	*S. cheesmaniae*	LA1037
Lycopersicon	*S. cheesmaniae*	LA0483
Lycopersicon	*S. galapagense*	LA1044
Lycopersicon	*S. galapagense*	LA0528
Lycopersicon	*S. galapagense*	LA0429
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1542
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1429
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1323
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1623
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2626
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1457
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1569
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1620
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1228
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA3136
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1511
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1247
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2640
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1286
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2308
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2131
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1231
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2688
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1543
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1461
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1204
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1464
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2137

(to be continued)

(Continued)

Supplementary Table 1 - Information of the 287 accessions of *Solanum* sect. *Lycopersicon* used for *in silico* analysis

Group	Genotype	Code
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2675
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2840
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2095
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1320
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1307
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA2670
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1482
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1388
Lycopersicon	*S. lycopersicum var. cerasiforme*	LA1456
Lycopersicon	*S. pimpinellifolium*	LA1245
Lycopersicon	*S. pimpinellifolium*	LA0411
Lycopersicon	*S. pimpinellifolium*	LA1617
Lycopersicon	*S. pimpinellifolium*	LA1547
Lycopersicon	*S. pimpinellifolium*	LA2181
Lycopersicon	*S. pimpinellifolium*	LA2093
Lycopersicon	*S. pimpinellifolium*	LA1584
Lycopersicon	*S. pimpinellifolium*	LA1246
Lycopersicon	*S. pimpinellifolium*	LA0373
Lycopersicon	*S. pimpinellifolium*	LA2183
Lycopersicon	*S. pimpinellifolium*	LA1579
Lycopersicon	*S. pimpinellifolium*	LA1589
Lycopersicon	*S. pimpinellifolium*	LA0442
Lycopersicon	*S. pimpinellifolium*	LA1375
Lycopersicon	*S. pimpinellifolium*	LA1269
Lycopersicon	*S. pimpinellifolium*	LA1521
Lycopersicon	*S. pimpinellifolium*	LA0400
Lycopersicon	*S. pimpinellifolium*	LA1478
Lycopersicon	*S. pimpinellifolium*	LA2660
Lycopersicon	*S. pimpinellifolium*	LA0480
Lycopersicon	*S. pimpinellifolium*	LA0722
Lycopersicon	*S. pimpinellifolium*	LA1242
Lycopersicon	*S. pimpinellifolium*	LA1341
Lycopersicon	*S. pimpinellifolium*	LA1596
Lycopersicon	*S. pimpinellifolium*	LA1847
Lycopersicon	*S. pimpinellifolium*	LA1933
Lycopersicon	*S. pimpinellifolium*	LA2147
Lycopersicon	*S. pimpinellifolium*	LA2173
Lycopersicon	*S. pimpinellifolium*	LA2184
Lycopersicon	*S. pimpinellifolium*	LA2187

(to be continued)

(Continued)

Supplementary Table 1 - Information of the 287 accessions of *Solanum* sect. *Lycopersicon* used for *in silico* analysis

Group	Genotype	Code
Lycopersicon	*S. pimpinellifolium*	LA2425
Lycopersicon	*S. pimpinellifolium*	LA1591
Lycopersicon	*S. pimpinellifolium*	LA1595
Lycopersicon	*S. pimpinellifolium*	LA2656
Lycopersicon	*S. pimpinellifolium*	LA2857
Lycopersicon	*S. pimpinellifolium*	LA4431
Lycopersicon	*S. pimpinellifolium*	LA0417
Lycopersicon	*S. pimpinellifolium*	LA1237
Lycopersicon	*S. pimpinellifolium*	LA1924
Lycopersicon	*S. pimpinellifolium*	LA1582
Lycopersicon	*S. pimpinellifolium*	LYC2910
Lycopersicon	*S. pimpinellifolium*	LYC2798
Lycopersicon	*S. pimpinellifolium*	LA1578
Neolycopersicon	*S. pennellii*	LYC1831
Neolycopersicon	*S. pennellii*	LA0716

Supplementary Table 2 - Information of the 31 accessions used to perform GWAS analysis, including *Solanum* section *Lycopersicon* group, genotype, and accession code

Group	Genotype	Code
Cultivar	*S. lycopersicum*	EA00371
Cultivar	*S. lycopersicum*	EA00940
Cultivar	*S. lycopersicum*	EA01640
Cultivar	*S. lycopersicum*	EA02054
Cultivar	*S. lycopersicum*	LA2463
Cultivar	*S. lycopersicum*	LA2706
Cultivar	*S. lycopersicum*	LYC1969
Cultivar	*S. lycopersicum*	LYC2910
Cultivar	*S. lycopersicum*	LA3475
Cultivar	*S. lycopersicum*	PI311117
Cultivar	*S. lycopersicum*	TR00003
Cultivar	*S. lycopersicum*	TR00019
Arcanum	*S. arcanum*	LA2172
Arcanum	*S. neorickii*	LA2133
Eriopersicon	*S. habrochaites*	LA0407
Eriopersicon	*S. habrochaites*	LA1777
Eriopersicon	*S. huaylasense*	LA1364
Lycopersicon	*S. lycopersicum var. cerasiforme*	BGV006767
Lycopersicon	*S. lycopersicum var. cerasiforme*	BGV006865
Lycopersicon	*S. lycopersicum var. cerasiforme*	BGV006906
Lycopersicon	*S. lycopersicum var. cerasiforme*	BGV007992
Lycopersicon	*S. lycopersicum var. cerasiforme*	BGV008042
Lycopersicon	*S. lycopersicum var. cerasiforme*	BGV008108
Lycopersicon	*S. lycopersicum var. cerasiforme*	BGV012615
Lycopersicon	*S. lycopersicum var. cerasiforme*	BGV012626
Lycopersicon	*S. lycopersicum var. cerasiforme*	PI129033
Lycopersicon	*S. galapagense*	LA0429
Lycopersicon	*S. pimpinellifolium*	BGV006775
Lycopersicon	*S. pimpinellifolium*	LA1269
Lycopersicon	*S. pimpinellifolium*	LA1589
Neolycopersicon	*S. pennellii*	LA0716

Supplementary Table 3 - Proteins included in AMT/MEP phylogenetic analyses including name used, species of origin, categorization applied, and database accessions

Protein	Specie	Category	Accession
AtAMT1.1	*A. thaliana*	AMT1/Plant group	At4g13510
AtAMT1.2	*A. thaliana*	AMT1/Plant group	At1g64780
AtAMT1.3	*A. thaliana*	AMT1/Plant group	At3g24300
AtAMT1.4	*A. thaliana*	AMT1/Plant group	At4g28700
AtAMT1.5	*A. thaliana*	AMT1/Plant group	At3g24290
AtAMT2.1	*A. thaliana*	AMT2/Plant group	At2g38290
OsAMT1.1	*Oryza sativa*	AMT1/Plant group	Q7XQ12
OsAMT1.2	*Oryza sativa*	AMT1/Plant group	Q6K9G1
OsAMT1.3	*Oryza sativa*	AMT1/Plant group	Q6K9G3
OsAMT2.1	*Oryza sativa*	AMT2/Plant group	Q84KJ7
OsAMT2.2	*Oryza sativa*	AMT2/Plant group	Q8S230
OsAMT2.3	*Oryza sativa*	AMT2/Plant group	Q8S233
OsAMT3.1	*Oryza sativa*	AMT2/Plant group	Q84KJ6
OsAMT3.2	*Oryza sativa*	AMT2/Plant group	Q851M9
OsAMT3.3	*Oryza sativa*	AMT2/Plant group	Q69T29
OsAMT4.1	*Oryza sativa*	AMT2/Plant group	Q10CV4
StAMT1.1	*Solanum tuberosum*	AMT1/Plant group	XP006340957.1
StAMT1.2	*Solanum tuberosum*	AMT1/Plant group	XP006340472.1
StAMT1.3	*Solanum tuberosum*	AMT1/Plant group	XP006346855.1
StAMT2	*Solanum tuberosum*	AMT2/Plant group	XP006363622.1
StAMT3.1	*Solanum tuberosum*	AMT2/Plant group	XP006347752.1
StAMT3.3	*Solanum tuberosum*	AMT2/Plant group	XP006343424.1
BnAMT1.2	*Brassica napus*	AMT1/Plant group	Q9FUH7
LjAMT1.1	*Lotus japonica*	AMT1/Plant group	Q9FSH3
LjAMT1.2	*Lotus japonica*	AMT1/Plant group	Q7Y1B9
LjAMT1.3	*Lotus japonica*	AMT1/Plant group	Q70KK9
LjAMT2.1	*Lotus japonica*	AMT2/Plant group	Q93X02
ZmAMT1.1.0	*Zea mays*	AMT1/Plant group	A0A3L6G6C1
ZmAMT1.1.1	*Zea mays*	AMT1/Plant group	A0A3L6FVW9
ZmAMT1.2	*Zea mays*	AMT1/Plant group	A0A3L6ET75
ZmAMT2.1	*Zea mays*	AMT2/Plant group	A0A3L6EC55
ZmAMT3.1.0	*Zea mays*	AMT2/Plant group	A0A3L6DQA1
ZmAMT3.1.1	*Zea mays*	AMT2/Plant group	A0A3L6EZQ8
ZmAMT3.2	*Zea mays*	AMT2/Plant group	A0A317YJQ9
ZmAMT3.3	*Zea mays*	AMT2/Plant group	A0A3L6D701
CaAMT1.1	*Capsicum annuum*	AMT1/Plant group	KAF3665461.1
CaAMT1.2	*Capsicum annuum*	AMT1/Plant group	KAF3636911.1
CaAMT1.3	*Capsicum annuum*	AMT1/Plant group	XP016570093.1
CaAMT2	*Capsicum annuum*	AMT2/Plant group	XP016546003.1
CaAMT3.1	*Capsicum annuum*	AMT2/Plant group	PHT86816.1
CaAMT3.2	*Capsicum annuum*	AMT2/Plant group	PHT74908.1
CaAMT3.3	*Capsicum annuum*	AMT2/Plant group	KAF3647346.1
PtrAMT1.1	*Populus trichocarpa*	AMT1/Plant group	B9HSW3
PtrAMT1.4	*Populus trichocarpa*	AMT1/Plant group	B9GRB5
PtrAMT1.5	*Populus trichocarpa*	AMT1/Plant group	B9GRB4
PtrAMT1.6	*Populus trichocarpa*	AMT1/Plant group	B9HP47
PtrAMT2.1	*Populus trichocarpa*	AMT2/Plant group	B9HCZ0
PtrAMT2.2	*Populus trichocarpa*	AMT2/Plant group	B9IGE2
PtrAMT3.1	*Populus trichocarpa*	AMT2/Plant group	B9GHA5

(to be continued)

(Continued)

Supplementary Table 3 - Proteins included in AMT/MEP phylogenetic analyses including name used, species of origin, categorization applied, and database accessions

Protein	Specie	Category	Accession
PtrAMT4.3	*Populus trichocarpa*	AMT2/Plant group	B9H8E7
SbAMT1.1	*Sorghum bicolor*	AMT1/Plant group	Sb06g022230
SbAMT1.2	*Sorghum bicolor*	AMT1/Plant group	Sb04g026290
SbAMT2.1	*Sorghum bicolor*	AMT2/Plant group	Sb09g023030
SbAMT2.2	*Sorghum bicolor*	AMT2/Plant group	Sb03g038840
SbAMT3.1	*Sorghum bicolor*	AMT2/Plant group	Sb03g041140
SbAMT3.3	*Sorghum bicolor*	AMT2/Plant group	Sb04g022390
SbAMT4	*Sorghum bicolor*	AMT2/Plant group	Sb01g008060
NtAMT1.1	*Nicotiana tabacum*	AMT1/Plant group	XP016507020.1
NtAMT1.2	*Nicotiana tabacum*	AMT1/Plant group	XP016481446.1
NtAMT1.3	*Nicotiana tabacum*	AMT1/Plant group	XP016472765.1
NtAMT2	*Nicotiana tabacum*	AMT2/Plant group	XP016502016.1
NtAMT3.1	*Nicotiana tabacum*	AMT2/Plant group	XP016458328.1
SlAMT1.1	*Solanum lycopersicum*	AMT1/Plant group	Solyc09g090730
SlAMT1.2	*Solanum lycopersicum*	AMT1/Plant group	Solyc04g050440
SlAMT1.3	*Solanum lycopersicum*	AMT1/Plant group	Solyc03g045070
SlAMT2.1	*Solanum lycopersicum*	AMT2/Plant group	Solyc10g076480
SlAMT3.1	*Solanum lycopersicum*	AMT2/Plant group	Solyc01g097370
SlAMT3.2	*Solanum lycopersicum*	AMT2/Plant group	Solyc09g065740
SlAMT3.3	*Solanum lycopersicum*	AMT2/Plant group	Solyc03g033300
SlAMT3.4	*Solanum lycopersicum*	AMT2/Plant group	Solyc08g067080
TaAMT1.1	*Triticum aestivum*	AMT1/Plant group	Q6QU81
TaAMT1.2	*Triticum aestivum*	AMT1/Plant group	Q6QU80
TaAMT2.1	*Triticum aestivum*	AMT2/Plant group	Q6T8L6
CrAMT1A	*Chlamydomonas reinhardtii*	AMT1/Chlorophytes	Cre03.g159250.t1.1
CrAMT1B	*Chlamydomonas reinhardtii*	AMT1/Chlorophytes	Cre06.g293050.t1.1
CrAMT1C	*Chlamydomonas reinhardtii*	AMT1/Chlorophytes	Cre13.g569850.t1.1
EcAMTB	*Escherichia coli*	AMT1/Bacteria	P69681
GsAMT1A	*Galdieria sulphuraria*	AMT1/Rhodophytes	EME30100.1
GsAMT1B	*Galdieria sulphuraria*	AMT1/Rhodophytes	EME32138.1
PpAMT1A	*Physcomitrella patens*	AMT1/bryophytes/Plant group	Pp1s33_11V6.1
PpAMT1B	*Physcomitrella patens*	AMT1/bryophytes/Plant group	Pp1s33_380V6.1
PpAMT1C	*Physcomitrella patens*	AMT1/bryophytes/Plant group	Pp1s33_381V6.1
PpAMT1D	*Physcomitrella patens*	AMT1/bryophytes/Plant group	Pp1s33_385V6.1
PpAMT1E	*Physcomitrella patens*	AMT1/bryophytes/Plant group	Pp1s475_29V6.1
PpAMT2A	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s4_201V6.1
PpAMT2B	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s12_395V6.1
PpAMT2C	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s15_321V6.1
PpAMT2D	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s30_275V6.1
PpAMT2F	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s57_114V6.1
PpAMT2G	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s109_32V6.1
PpAMT2H	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s120_140V6.1
PpAMT2I	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s240_51V6.1
PpAMT2J	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s297_9V6.1
PpAMT2K	*Physcomitrella patens*	AMT2/bryophytes/Plant group	Pp1s352_2V6.1
ScMEP1	*Saccharomyces cerevisiae*	MEP/Yeast	P40260
ScMEP2	*Saccharomyces cerevisiae*	MEP/Yeast	P41948
ScMEP3	*Saccharomyces cerevisiae*	MEP/Yeast	P53390