Mutations Found in the Asc1 Gene That Confer Susceptibility to the AAL-Toxin in Ancestral Tomatoes from Peru and Mexico

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Abstract: Tomato susceptibility/resistance to stem canker disease caused by Alternaria alternata f. sp. lycopersici and its pathogenic factor AAL-toxin is determined by the presence of the Asc1 gene. Several cultivars of commercial tomato (Solanum lycopersicum var. lycopersicum, SLL) are reported to have a mutation in Asc1, resulting in their susceptibility to AAL-toxin. We evaluated 119 ancestral tomato accessions including S. pimpinellifolium, resulting in their susceptibility to AAL-toxin. We evaluated 119 ancestral tomato accessions including S. pimpinellifolium (SP), S. lycopersicum var. cerasiforme (SLC) and S. lycopersicum var. lycopersicum "jitomate criollo" (SLJ) for AAL-toxin susceptibility. Three accessions, SP PER018805, SLC PER018894, and SLJ M5-3, were susceptible to AAL-toxin. SLC PER018894 and SLJ M5-3 had a two-nucleotide deletion (nt 854_855del) in Asc1 identical to that found in SLL cv. Aichi-first. Another mutation (nt 931_932insT) that may confer AAL-toxin susceptibility was identified in SP PER018805. In the phylogenetic tree based on the 18 COSII sequences, a clade (S3) is composed of SP, including the AAL-toxin susceptible PER018805, and SLC. AAL-toxin susceptible SLC PER018894 and SLJ M5-3 were in Clade S2 with SLL cultivars. As SLC is thought to be the ancestor of SLL and SLJ is an intermediate tomato between SLC and SLL, Asc1s with/without the mutation seem to have been inherited throughout the history of tomato domestication and breeding.

Keywords: Solanum pimpinellifolium; Solanum lycopersicum var. cerasiforme; alternaria alternata tomato pathotype; AAL-toxin; Peru
1. Introduction

Agricultural plant evolution has been driven by a complex process involving human activities and natural environment. Humans have selected individual wild plants displaying preferable traits, for example suitable for eating, resulting in domestication of plants [1]. Modern plant breeding has enhanced the selection of genes determining favorable phenotypes within a diverse gene pool, which has led to a reduction in genetic diversity among agricultural plants.

Tomato (Solanum lycopersicum L., formerly Lycopersicon esculentum Mill; SLL) is the most abundantly produced vegetable in the world. The total production of tomatoes was ca. 0.2 billion tons from ca. 5 million ha of fields in 2019 [2]. SLL originated from S. pimpinellifolium L. (SP) in the Andean region of South America, now occupied by Peru, Chile, Ecuador, and Bolivia [3–6]. The history of tomato domestication began about 2000 years ago, possibly in Mexico; subsequently, tomato was brought to Europe around 500 years ago [6–8].

The Andes region continues to sustain wild tomato species, including not only (Alternaria stem canker resistance protein 1) gene encodes an enzyme involved in ceramide (S. chmielewskii (cv.) Earlypak 7 in California, USA [13], followed by a 1977 report of the pathogen infecting alternaria species group (or, A. alternata tomato pathotype; Aal) Purified AAL-toxin produced by Aal, a host-specific toxin, is toxic only to those cultivars susceptible to AAL-toxin [17].

In 1975, the disease was reported for the first time in the SLL cultivar cv. Aichi-first in Japan [14]. Most of the other SLL cultivars are resistant to the disease [13,15]. Purified AAL-toxin produced by Aal, a host-specific toxin, is toxic only to those cultivars susceptible to Aal and causes necrotic lesions but not in the cultivars resistant to Aal [16]. Among wild tomatoes, SC and SG from the Galápagos Islands are known to be susceptible to the AAL-toxin [17].

AAL-toxin is the leading cause of symptom development in stem canker disease [18]. AAL-toxin induces apoptotic cell death in SLL tissues; however, cultivars resistant to AAL-toxin produce ceramide that protects the tissues from cell death [18]. The Asc1 (Alternaria stem canker resistance protein 1) gene encodes an enzyme involved in ceramide biosynthesis in SLL [18]. The SLL cv. Aichi-first, which is susceptible to AAL-toxin, has a two-nucleotide deletion in the Asc1 ORF, and SC and SG have ca. a 400 nucleotide-deletion that includes the 5′-UTR and a part of the 3′ ORF of Asc1 [17].

We hypothesized that the mutations found in Asc1 in the AAL-toxin susceptible cultivars and SC and SG originated from the gene pool of Asc1 in SP and SLC, the possible wild ancestors of SC and SG, and that we could find variations of Asc1 mutations in SP and SLC. To test this hypothesis, we established a collection of SP in Peru and Ecuador; SLC in Peru, Ecuador and Mexico; and SLJ, the archetypes of SLL, in Mexico. We investigated their...
susceptibilities to AAL-toxin and determined the nucleotide sequences of the respective Asc1 genes.

2. Results
2.1. UNALM-TUAT Collection of Peruvian Tomatoes

From 2016 to 2019, we collected wild tomatoes throughout several field trips in Peru and created the UNALM-TUAT Collection of Peruvian tomatoes composed of 41 SLC and 19 SP accessions (Table 1). In order to construct a diverse collection of wild tomatoes, we collected throughout a large area of Peru that encompassed the northwestern coast area including Tumbes, Piura, Lambayeque and La Libertad Regions, the northern highland and semi-jungle area, including the Cajamarca and Huánuco Regions, the Amazon rainforest area including the Ucayali Region, the south-central highland area including the Junin, Cusco and Ayacucho Regions, and the Pacific coastal area including the Lima and Ica Regions. Usually SP and SLC are found in coastal areas that are not over 800 m in elevation, but we also found SP and SLC in valleys in the Andean Mountains like Quillabamba City in the Cusco Region. SP and SLC were not distributed in the untouched natural environments but rather in fallow agriculture fields and near inhabited centers. Figure 1 schematically presents the sampling areas for SLC (squares) and SP (circles) in the UNALM-TUAT and INIA Collections used in this study.

2.2. Accessions Susceptible to AAL-Toxin

In bioassays using leaflets, one (M5-3 sampled in Querétaro, Mexico) among the two SLJ accessions, one (PER018894 from Huanuco, Peru) among the 62 SLC accessions, and one (PER018805 from Lambayeque, Peru) among the 51 SP accessions presented veinal necrosis and were determined to be susceptible to AAL-toxin (Table 1 and Figure 2). Other accessions presented no symptoms (Table 1 and Figure S2), suggesting that they are resistant to AAL-toxin. The references, SC (LA 0437 and 0521), SG (LA 0438 and 0528) and SLL cv. Aichi-first were susceptible to AAL-toxin, and SLL cv. Momotaro-8 was resistant to AAL-toxin.
Table 1. Tomato accessions and cultivars used in this study, their susceptibility to AAL-toxin and mutations found in Asc1.

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<tr>
<th>Species and Accessions</th>
<th>Sampling Site</th>
<th>AAL-Toxin Susceptibility</th>
<th>Gen Bank Accession No.</th>
<th>Mutations in Asc1 in Comparison to the Reference Sequence #AF198177 b</th>
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Note: * Denotes accessions with novel mutations.
Table 1. Cont.

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*S. lycopersicum var. cerasiforme (SLC)*

- **PER018795** Peru Lima S11°41'60" W75°52'11" 20150819 Resistant NT NT NT NT NT NT
- **PER018836** Peru Cajamarca S06°19'12" W78°41'90" 20111013 Resistant NT NT NT NT NT NT
- **PER018878** Peru Cusco S12°43'41" W72°32'44" 20111025 Resistant NT NT NT NT NT NT
- **PER018879** Peru Cusco S12°41'31" W72°31'07" 20111025 Resistant NT NT NT NT NT NT

- **PER018894** Peru Huanuco S09°50'08" W76°07'05" 2011109 Susceptible LC596580 No deletion 854_855del 911G>A 1065G>A, 1306T>G

- **PER018901** Peru Huanuco S09°48'06" W76°04'08" 20111110 Resistant NT NT NT NT NT NT
- **PER018902** Peru Huanuco S09°10'52" W75°57'36" 20111111 Resistant NT NT NT NT NT NT
- **PER018909** Peru Huanuco S09°22'55" W75°01'57" 20111113 Resistant NT NT NT NT NT NT

- **PER018923** Peru Ucayali S08°23'30" W75°07'41" 20111116 Resistant NT NT NT NT NT NT
- **PER018932** Peru Ayacucho S12°54'24" W74°17'05" 20111213 Resistant NT NT NT NT NT NT
- **PER018936** Peru Ayacucho S13°03'49" W73°57'27" 20111214 Resistant NT NT NT NT NT NT
- **PER018938** Peru Ayacucho S13°06'28" W73°54'36" 20111214 Resistant NT NT NT NT NT NT

*S. pimpinellifolium (SP)*

- **PER018780** Peru Lima S11°02'22" W77°37'37" 20110816 Resistant NT NT NT NT NT NT
- **PER018781** Peru Lima S11°02'22" W77°37'36" 20110816 Resistant NT NT NT NT NT NT
- **PER018782** Peru Lima S11°01'15" W77°37'20" 20110816 Resistant NT NT NT NT NT NT
- **PER018783** Peru Lima S10°59'37" W77°35'55" 20110816 Resistant NT NT NT NT NT NT
- **PER018785** Peru Lima S10°39'50" W77°45'66" 20110816 Resistant NT NT NT NT NT NT
- **PER018786** Peru Lima S10°39'82" W77°41'10" 20110817 Resistant NT NT NT NT NT NT
- **PER018788** Peru Lima S10°40'52" W77°44'07" 20150817 Resistant NT NT NT NT NT NT
- **PER018794** Peru Lima S11°29'46" W76°32'77" 20150817 Resistant NT NT NT NT NT NT
- **PER018796** Peru Lima S11°29'73" W77°15'61" 20150819 Resistant NT NT NT NT NT NT
- **PER018797** Peru Lima S11°29'74" W77°15'64" 20150819 Resistant NT NT NT NT NT NT
Table 1. Cont.

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<th>Species and Accessions</th>
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TGRC Collection used as references

*S. lycopersicum var. cerasiforme (SLC)*

| S. lycopersicum var. cerasiforme (SLC) LA 1456 | Mexico | Veracruz | N19°10’00” | W96°08’00” | 1971 | Resistant | LC596520 | No deletion | 1306T>G |

*S. pimpinellifolium (SP)*

| S. pimpinellifolium (SP) LA 3123 | Ecuador | Santa Cruz Island | S00°37’30” | W90°22’59” | 19910516 | Resistant | LC596565 | No deletion | 836A>T |

*S. cheesmaniae (SC)*

| S. cheesmaniae (SC) LA 0437 | Ecuador | Isabela Island | S00°57’09” | W90°58’39” | 19561125 | Susceptible | LC596568 | 400 bp-deletion |  |
Table 1. Cont.

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Commercial cultivars used as references

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<td>* cv. Momotaro-8 (Takii &amp; Co, Kyoto, Japan)</td>
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* Date, yymmdd. b Blank, identical to #AF198177; NT, not tested. c An approximately 400 bp-deletion including the 5' UTR and a part of the 5' ORF of Asc1 as determined by PCR; * Accessions used in the phylogenetic analyses.

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<td>* cv. Momotaro-8 (Takii &amp; Co, Kyoto, Japan)</td>
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* Date, yymmdd. b Blank, identical to #AF198177; NT, not tested. c An approximately 400 bp-deletion including the 5' UTR and a part of the 5' ORF of Asc1 as determined by PCR; * Accessions used in the phylogenetic analyses.
Figure 1. Map of the collection sites of Peruvian tomato accessions. Squares shown within each province represent accessions of *Solanum lycopersicum* var. *cersiforme* (SLC) and circles represent *S. pimpinellifolium* (SP) from the UNALM-TUAT Collection and the INIA Collection (Table 1). Each black square and circle shows an AAL-toxin susceptible accession. Map from Aflo Co. [19] and modified.
Figure 2. Leaflet bioassay for AAL-toxin from a culture extract of Aal As-27. The abaxial side of each tomato leaflet was wounded and a small piece of filter paper containing either the culture extract or water was placed on the wound and incubated in a humidified chamber (25 °C for 3 days). The necrosis of the leaflet was evaluated, and a susceptible reaction or resistant reaction is indicated with an “S” or “R”, respectively, in the figure. SLL cv. Aichi-first is a representative cultivar with susceptibility to AAL-toxin. SLL cv. Momotaro-8 is a representative cultivar with resistance to AAL-toxin. M5-3 was susceptible to AAL-toxin among two accessions of SLJ. Among 60 accessions of SLC, one accession, PER018894, was susceptible to AAL-toxin, and the others were resistant to AAL-toxin. The reaction of BRC016 is representative of AAL-resistant SLC accessions. Among 37 accessions of SP one accession, PER018805, was susceptible to AAL-toxin, and the others were resistant to AAL-toxin. CPN032 is representative of AAL-resistant SP accessions.

2.3. Absence of ca. 400-bp Deletion in Asc1 in SP, SLC and SLJ

The susceptibility to AAL-toxin in SC and SG is determined by a ca. 400-bp deletion that includes the 5’-UTR and part of the 5’ ORF of Asc1 (Figure 3) [17]. PCR using a F10/R10 primer set reveals that all tested accessions, including the three AAL-toxin susceptible accessions (SLJ M5-3, SLC PER018894 and SP PER018805), did not have the ca. 400-bp deletion in the Asc1 region (Table 1 and Figure 4). The references SC (LA 0437 and LA 0521) and SG (LA 0438 and LA 0528) had the ca. 400-bp deletion as previously reported [15].

Figure 3. Schematic structure of Asc1 from SLL in the DDBJ/EMBL/GenBank databases identified as accession #AF198177. Asc1 is composed of 6 exons and encodes an ASC1 protein of 308 amino acids. Primers indicated by arrows are listed in Table 2. The primer set BASC87+R12 was used to amplify a ca. 1600-bp fragment containing Asc1 for cloning and sequencing. The primer set F10+R10 was used to detect the ca. 400-bp deletion including the 5’-UTR and part of the 5’ ORF of Asc1. White gaps shown in exon 2 represent a two-nucleotide deletion reported in SLL cv. Aichi-first and found in SLJ PER018894 and SLC M5-3 in this study and a nucleotide insertion found in SP PER018805, respectively. An approximately 400-bp deletion including the upstream region and part of the 5’ ORF region indicated by a gray bar has been reported in SC and SG [17].
Table 2. *Ascl* primers used in this study.

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<th>Position a</th>
<th>Tm °C</th>
<th>Thermal Conditions</th>
<th>Reference</th>
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<td>BASC87 GGAATCTGCAATTCATTTGAAACTACAAC</td>
<td>EcoRI recognition site + nt 424–447</td>
<td>70</td>
<td>98 °C, 2 min; 30 × (98 °C, 10 s; 59 °C, 30 s; 68 °C, 1 min); 68 °C, 7 min; 4 °C, ∞</td>
<td>Brandwagt et al. (2000)</td>
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<tr>
<td>R12 CAAGTAGTGCTGCCTACAG</td>
<td>nt 2017–1996</td>
<td>61</td>
<td>This study</td>
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<td>Primers to detect the ca. 400 bp-deletion in the 5′-UTR and a part of the 5′ ORF of <em>Ascl</em> (Figure 1)</td>
<td>F10 GAAACGATCAAACGTGTT</td>
<td>nt 178–198</td>
<td>56</td>
<td>98 °C, 2 min; 30 × (98 °C, 10 s; 56 °C, 30 s; 72 °C, 1 min); 72 °C, 7 min; 4 °C, ∞</td>
<td>Ago et al. (2016)</td>
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a Nucleotide position relative to that of accession #AF198177.
2.4. Mutations in Asc1

We sequenced the all of the Asc1 region of the tomato accessions except for 39 of the accessions from the INIA Collection and compared the sequences with that of the reference AAL-resistant SLL (Acc. #AF198177) [20–23].

Asc1 sequences of SLJ M5-3 and SLC PER018894, both of which were susceptible to AAL-toxin by the leaflet test, had the two-nucleotide deletion (nt 854_855del) in the second exon and generated a frameshift and possibly produced a non-functional protein (Figure 5 and Table 1). This two-bp deletion was identical with that reported for SLL cv. Aichi-first, an AAL-toxin susceptible cultivar [17].

SP PER018805, susceptible to AAL-toxin by the leaflet test, had a T-insertion (nt 931_932insT) in the second exon of Asc1, causing a frameshift that might generate a smaller, premature asc1 protein (Figure 5 and Table 1). This mutation in the Asc1 gene has not been reported previously. Although involvement of this mutation in AAL toxin-susceptibility in PER018805 can be genetically confirmed by outcrossing PER018805 with an AAL-resistant SP accession, the regulation of studies on wild tomatoes in Peru has prevented this experiment from being conducted.

Only five Mexican SLC accessions (M-UX, MC-5a, MC-5b and ML-1 in the TUAT Collection and LA 1623 in the TGRC Collection) had an Asc1 DNA sequence identical with #AF198177.

We found eleven kinds of missense mutations (509A>G, 569A>C, 570G>A, 572A>G, 617G>A, 807T>C, 836A>T, 911G>A, 1010A>C, 1366T>C, 1693T>G) in the Asc1 sequence in 31 accessions (Table 1). Many silent mutations were also detected in Asc1 nucleotide sequences of these accessions (Table 1).
Figure 5. (a) Nucleotide variations found in exon 2 of Asc1 (Figure 3). Identical nucleotides are highlighted in black in comparison to the reference sequence of SLL #AF198177 (resistant to AAL-toxin). SLL cv. Aichi-first (susceptible), SLJ M5-3 (susceptible) from Mexico and SLC PER018894 (susceptible) from Peru have the nt 854_855del mutation, and SP PER018805 (susceptible) from Peru has the nt 931_932insT mutation. (b) Deduced amino acid sequences of Asc1. The amino acid sequences were aligned using CLUSTALW [24]. Identical and similar amino acids are highlighted in black or gray, respectively, by GeneDoc [25]. * indicates termination. In comparison to the reference sequence of SLL #AF198177 (resistant to AAL-toxin), SLL cv. Aichi-first (susceptible), SLJ M5-3 (susceptible), SLC PER018894 (susceptible) and SP PER018805 (susceptible) produce smaller proteins that may be nonfunctional.

2.5. Phylogeny

The maximum likelihood (ML) phylogeny tree based on 18 COSII sequences is presented in Figure 6. The tree formed three clades supported by high bootstrap values, designated in this study as S1, S2 and S3. Clade S1 is composed only of Galápagos tomatoes, including SC and SG. Clade S2 is composed of SLL commercial cultivars, SLJ and SLC from Mexico and Peru. Clade S3 is composed of SLC and SP from Peru and Ecuador only. All of the tested SP accessions were in Clade S3.

The accessions SLJ M5-3 and SLC PER018894, susceptible to AAL-toxin and carrying the identical mutation (nt 854_855del) in Asc1 as SLL cv. Aichi-first, were in Clade S2 with SLL cv. Aichi-first. The SP accession, PER018805, susceptible to AAL-toxin and with a mutation (nt 931_932insT) in Asc1, was placed in Clade S3.

The topology of the ML tree did not contradict that of the BI tree (Figure S3).
Figure 6. A maximum likelihood (ML) tree based on 18 COSII sequences of tomato accessions estimated using Modeltest-NG ver. 0.1.6 [26] and RAxML-NG v. 1.0.0 [27]. *S. arcanum* and *S. neorickii* were used as outgroups. The bootstrap values were calculated after 1,000 bootstrap replicates. The data sets of SG (LA 0317), SC (LA 1450), SLC (LA 1673), SP LA 1581, *S. arcanum* (LA 2185) and *S. neorickii* (LA 1326) are from [10] and are indicated with a § symbol in the tree. AAL-toxin susceptible accessions are highlighted with a black background. SC, *S. cheesmaniae*; SG, *S. galapagense*; SLC, *S. lycopersicum* var. *cerasiforme*; SLJ, *S. lycopersicum* var. *lycopersicum* “jitomate criollo”; SLL, *S. lycopersicum* var. *lycopersicum* (commercial varieties); SP, *S. pimpinellifolium*.

3. Discussion

In this study, we evaluated 119 ancestral tomato accessions for their susceptibility to AAL-toxin produced by *Aal*. Only three accessions, an SLJ from Mexico, an SLC from Peru and an SP from Peru, were susceptible to AAL-toxin; the others were resistant. The number of AAL-toxin susceptible accessions was less than expected. This is the first time that AAL-toxin susceptible SLJ and SLC have been reported.

Among the three AAL-toxin susceptible accessions, SLJ M5-3 sampled from Mexico and SLC PER018894 from Peru had a frameshift mutation (nt 854_855del) identical to that found in SLL cv. Aichi-first, also an AAL-toxin susceptible accession. As SLC is thought to be the oldest progenitor of present-day commercial cultivars and SLJ is an intermediate tomato between SLC and present-day commercial SLL, both of Asc1 genes with the frameshift mutation (nt 854_855del) and without the frameshift seemed to have been passed down throughout the history of tomato domestication and modern breeding.

As SP and its derivative species, SC and SG, have been collected from the Galápagos Islands and the Hawaiian Islands [11], it has been proposed that SP seeds were carried to the islands from the South American mainland by the Humboldt Current. Interestingly, all SC and SG accessions from the Galápagos Islands evaluated so far are AAL-toxin susceptible and have a ca. 400-bp deletion in *Asc1* [17]. We inferred that the genetic diversity of SP, including the *Asc1* gene, is rich in areas considered to be the center of origin of this species. One of the strains having the ca. 400-bp deletion in *Asc1* was carried to Galápagos Islands by the Humboldt Current to establish SC and SG there. We also hypothesized that the original SP strains having the ca. 400-bp deletion in *Asc1* still survive in South America, the proposed center for the origin of tomatoes. Therefore, we sequenced...
Asc1 from 23 SP accessions (Table 1). Contrary to our expectations, no SP accession with ca. 400-bp deletion has been found. It is possible that we have not yet identified the place of origin of the SP that crossed the ocean to the Galápagos Islands.

Although the diversity of Asc1 among the accessions seemed not as rich as expected (Table 1), we found that PER018805, one of the SP accessions from Lambayeque in Northwestern Peru, had a frameshift mutation, nt 931_932insT, in the second exon of Asc1 (Table 1). This mutation generates the production of a smaller (97 aa.) and possibly premature asc1 protein (Figure 5) and is reported here first.

Sequencing of Asc1 identified the frequent presence of missense mutations (509A>G, 569A>C, 572A>G, 617G>A, 807T>C, 836A>T, 911G>A, 1010A>C, 1366T>C, 1693T>G) that did not affect the susceptibility to AAL-toxin (Table 1).

Since the stem canker disease pathogen Aal has not been reported in South America, susceptibility/resistance to Aal or to AAL-toxin may not be a factor in the selection of Asc1 mutations. These findings suggested that if we analyze more accessions of SP, we may find accessions having more diverse Asc1 sequences.

Silent mutations were frequently detected in introns and exons. Especially 1306T>G in the third intron was common in SLJ (2 among the 2 accessions sequenced), SLC (46 among 51), SP (22 among 23) and both of the SLL commercial varieties (Table 1), suggesting that the Asc1 sequence of SLL #AF198177 used as a reference in this study was not an ideal standard type.

From 2000 to 2019, we tried to isolate *Alternaria* spp. from the tissues of ancestry tomato accessions in Chile, Ecuador, Mexico and Peru. We examined SP, SLC, SLJ, SLL, *S. chilense*, *S. peruvianum*, *S. penellii* and samples of the surrounding air and soil. Although we obtained hundreds of *Alternaria* spp., no isolate causing stem canker in tomato was found (data not shown) [28]. We have studied the co-evolution of tomato and tomato wilt pathogen, *F. oxysporum f. sp. lycopersici* [3,5]. Tomato and the stem canker pathogen *Aal* seem also likely to be a good model system for co-evolution analysis.

The ML phylogeny tree (Figure 6) formed three clades (S1–S3). Galápagos tomatoes, SC and SG, all of which are susceptible to AAL-toxin and have the ca. 400-bp deletion in Asc1, were grouped together as Clade S1, in agreement with a previous report [11].

Clade S2 is composed of SLC from Mexico and Peru, SLJ from Mexico and SLL commercial cultivars (Figure 6). Although the number of accessions tested in this study is small, clade S2 seems to support the hypothesis that the present commercial tomato (SLL) was established from SLC via SLJ. Our finding was consistent with the report by Raziferd et al. (2020) [10]. Clade S2 includes AAL-susceptible SLC (PER018894), SLJ (M5-3) and SLL cv. Aichi-first, AAL-resistant SLC (BRC016 and ML-1) and SLL cv. Momotaro-8. All three of the AAL-susceptible accessions had the identical frameshift mutation (nt 854_855del) in Asc1, which again suggested that Asc1 with and without the nt 854_855del frameshift mutation have been passed down throughout the history of tomato domestication and modern breeding from SLC to SLL. The mutation was found only in clade S2.

All of the tested SP accessions from Peru and Ecuador were grouped in Clade S3. Clade S3 also includes SLC accessions from Peru and Ecuador. Identification of SP and SLC in this study was based on the morphological characteristics first detailed by Darwin et al. (2003) [9]. SP and SLC are often very similar in morphology, and there have been many discussions on how to classify them correctly [9–11]. Our phylogeny based on the COSII complex region again indicated that SP and SLC are genetically indistinguishable (Figure 6). JAE036, JAE037 and CPN032 constituted a subclade (S3a) supported with a bootstrap value of 89. The accessions LA1581, CCY152, and PER018805, all of which were collected in Lambayeque Province in different years, constituted another subgroup (S3b) with a bootstrap value of 86 (Figures 1 and 6). Interestingly one of the accessions, PER018805 was susceptible to AAL-toxin and had a newly identified mutation (nt 931_932insT) in Asc1. From the phylogenetic tree, this mutation appears to have occurred independently within this subclade.
Most of the accessions in Clade S3 were collected from Cajamarca, La Libertad and Lambayeque Regions, which are geographically close, suggesting that this northwestern area might be the center of origin for tomatoes. Moreover, the two Peruvian SLC accessions, BRC016 and PER018894, in Clade S2 were collected in Lima and Huanuco Provinces, respectively, both of which are in central Peru. These results suggest that these SLCs had already formed an evolutionary branch to SLL, and, moreover, the SLC in central Peru was likely the germplasm brought to Mesoamerica.

4. Materials and Methods

4.1. Plant Materials

The Solanum accessions used in this study are listed in Table 1. From the TUAT Collection (Laboratory of Plant Pathology, Tokyo University of Agriculture and Technology (TUAT), Fuchu, Tokyo) [5], two SLJ and four SLC accessions from Mexico and two SLC and two SP accessions from Ecuador were used. No SP or SLJ accessions were collected in Mexico or Ecuador, respectively.

The UNALM-TUAT Collection (La Molina, Peru) of Peruvian tomatoes is composed of 41 SLC and 19 SP accessions sampled from 2016 to 2019. Details about this collection are described in the Results section.

From the INIA Collection (La Molina, Lima, Peru) 12 SLC and 29 SP accessions sampled from Ayacucho, Cajamarca, Cusco, Huanuco, Lambayeque, Lima, and Ucayali regions of Peru were used (Table 1).

SC (LA 0437 and LA 0521) and SG (LA 0438 and LA 0528), which are susceptible to Aal and its culture extract that contains AAL-toxin, were obtained from the TGRC Collection, C.M. Rick Tomato Genetics Resource Center (Davis, CA, USA). Three additional SLC accessions (LA 1456, LA 1632 and LA 1909) and one SP (LA 3123) accession from the TGRC Collection were used as references.

Cultivated tomato, SLL cvs. Momotaro-8 (Takii & Co., Kyoto, Japan) and Aichi-first (Matsunaga Seed, Konan, Aichi, Japan) were also used as references. Momotaro-8 is a cultivar that is resistant to Aal and to its culture extract containing the AAL-toxin. In contrast, cv. Aichi-first is susceptible to Aal and the culture extract (Figure S1) [14].

For the accessions from the TUAT, TUAT-UNALM and TGRC Collections and the commercial cultivars, three to five seeds were sown in sterilized soil (Nippi Engei Baido; Nihon Hiryo Co, Chuo, Tokyo, Japan) in plastic pots (7 cm in diameter) and were grown in a greenhouse maintained at around 28 ºC for about three weeks. Leaflets (or folioles) were harvested. For the accessions in the INIA Collection, leaflets were harvested from plants grown in a greenhouse for about three weeks at INIA (La Molina, Peru) and the INIA Donoso Agriculture Experiment Station (Huaral, Peru).

4.2. Fungal Isolate and the Preparation of Culture Extracts Containing the AAL-Toxin

Alternaria alternata f. sp. lycopersici As-27 (Aal) maintained in the Laboratory of Plant Pathology, Tottori University, Tottori, Japan was used in this study [29,30]. The isolate is the pathogen responsible for tomato stem canker disease and also produces AAL-toxin [22]. The isolate was maintained on V-8 juice agar medium [31] in the dark at 28 ºC and was used to prepare culture extracts.

Culture extracts of Aal containing the AAL-toxin were prepared following a published protocol [32] with a slight modification. Briefly, Aal was cultured in a modified Richard’s liquid medium (1 L) at room temperature for two weeks. The mycelium was removed by filtration using filter paper (No. 1, Toyo Roshi Kaisha, Chiyoda, Tokyo, Japan), and the filtrate was lyophilized using a freeze-dryer (VD-500, TAITEC Co., Koshigaya, Saitama, Japan), dissolved into 100 mL 70% (v/v) acetonitrile and used as the Aal culture extract containing the AAL-toxin. We assessed the presence of the toxin by bioassay using cv. Aichi-first by the same manner described in 4.3.
4.3. AAL-Toxin Susceptibility Assay

The test was conducted following previously reported procedures with a slight modification [30,32,33]. Briefly, a droplet (3 µL) of the Aal culture extract was pipetted onto a 3 mm square filter paper (No. 2, Toyo Roshi Kaisha) and air dried to vaporize acetonitrile. Three-week old tomato leaflets were detached, and the abaxial side of each leaflet was wounded slightly by rubbing with a paper towel. A droplet (30 µL) of sterilized distilled water was applied to the leaflet wound, and the filter paper containing the culture extract was placed on the water droplet. Filter paper to which a droplet (3 µL) of sterile distilled water (SDW) had been applied was used as the control. The treated leaflets were placed in a humid square petri dish (140 × 100 × 14.5 mm, Eiken Chemical, Taito, Tokyo, Japan) and maintained at 25 °C for three days. Development of veinal necrosis on the leaflet was evaluated using SLL cv. Aichi-first (susceptible to AAL-toxin and presenting veinal necrosis) and cv. Momotaro-8 (resistant to AAL-toxin and presenting no symptoms) as positive and negative controls, respectively.

To conserve genetic resources, wild tomato seeds cannot be transported from Peru, and, moreover, Aal, the stem canker pathogen that has not invaded Peru, could not be transported into Peru; thus, we have not conducted Aal-inoculation tests using wild tomatoes.

4.4. Tomato Genomic DNA Extraction

Genomic DNA from each tomato accession was purified from leaflets by a cetyltrimethylammonium bromide (CTAB) protocol [34]. Freeze-dried leaflets were powdered using a mortar and pestle and dissolved in 700 µL of CTAB buffer (2.0% (w/v) CTAB, 0.1 M Tris-HCl pH 8.0, 0.02 M EDTA pH 8.0, and 8.2% (w/v) NaCl in Milli-Q water) containing 0.5% (v/v) β-mercaptoethanol, and incubated at 65 °C for 45 min with occasional mixing by gentle swirling. To each tube, an aliquot (700 µL) of chloroform:isoamyl alcohol=24:1 (v:v) (CIA) was added, mixed by inversion to form an emulsion, and centrifuged at 10,000 × g for 10 min at room temperature. The aqueous phase was harvested and added to 60 µL of 10× CTAB buffer. After mixing, the samples were again extracted with CIA (700 µL), mixed by inversion, and centrifuged in the same conditions. The aqueous phase was combined with isopropanol (500 µL), mixed well to precipitate DNA and centrifuged for 30 min at room temperature. After centrifugation the supernatant layer was removed carefully, and the precipitated DNA was twice washed with 99% ethanol (500 µL). The DNA pellet was air-dried and dissolved in 50 µL of Milli-Q water.

4.5. PCR

The reference nucleotide sequence of SLL Asc1 is archived in the GenBank database under accession #AF198177. The SLL Asc1 gene is composed of 6 exons (nts 505–645, 791–1017, 1106–1261, 1340–1525, 1616–1800, and 1889–1920) that encode a protein composed of 308 amino acids. In this report the nucleotide positions are assigned in reference to this accession unless otherwise stated.

Primer set BASC87/R12 (Table 2 and Figure 3) was used to amplify a fragment of ca. 1600 bp encoding Asc1. The reaction mixture (10 µL) contained 40 ng of gDNA, 0.4 nmol of each primer, 1× Buffer (Toyobo, Osaka, Japan), 0.2 nmol each dNTP (Toyobo) and 0.2 U of KOD plus NEO polymerase (Toyobo). The thermal conditions are presented in Table 2.

To detect a specific deletion of ca. 400 bp that includes the 5′ UTR and part of the 5′ ORF of Asc1 as found in SC and SG [17], the primer set F10/R10 (Table 2 and Figure 3) was used. The reaction mixture (10 µL) contained 40 ng of gDNA, 0.3 µM each primer, 1× Ex-Taq buffer (Takara Bio, Kusatsu, Shiga, Japan), 200 µM each dNTP and 0.25 U of Ex-Taq polymerase (Takara Bio).

The amplicons were separated in a 1% (w/v) agarose gel by electrophoresis using TAE buffer and were visualized by staining with 0.5 µg/mL ethidium bromide.
4.6. DNA Sequencing

Amplicons obtained with the primer set BASC87/R12 were purified using ExoSAP-IT (Thermo Fisher Scientific, Santa Clara, CA, US), attached to a fluorescent dye by STeP PCR [35], and sequenced with an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). For each accession three individual PCR reactions and three times sequencing for each reaction by both directions were performed. When the sequences obtained were not identical, we performed additional PCR/sequencing and the sequence was finalized by “majority vote”.

The obtained sequences of Asc1 were aligned with that of #AF198177 (2457 bp) as the reference sequence using Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets MEGA7 [36] and GeneStudio.exe [37]. Deduced amino acid sequences were obtained using EMBOSS Six pack [38].

4.7. Phylogenetic Analysis of Tomato Accessions

The phylogenetic relationships among the 14 tomato accessions (indicated with an asterisk in Table 1) and the reference accessions were analyzed based on their conserved orthologous set (COSII) of nuclear loci [11]. Eighteen COSII markers for each accession were amplified by PCR using the primer sets, sequenced, and combined [11]. Details about the primers and PCR conditions are described in Table S1. The combined sequences of the tested accessions and the reference sequences of six Solanum spp., including SC, SG, SLC, SP, S. arcanum, and S. neorickii in the GenBank databases (Table S2), were subjected to phylogenetic analyses using MEGA7 and MAFFT version 7 [39] (https://mafft.cbrc.jp/alignment/server/index.html, accessed on September 16, 2020). All gaps in the alignment were ignored in the following analyses. The phylogenies were estimated using two methods including maximum likelihood ML [40] and Bayesian inference (BI) [41]. The data obtained for S. arcanum (LA 2185) and S. neorickii (LA 1326), both of which are accessions in the TGRC Collection, were used as the outgroups [11].

ML analysis was evaluated with Modeltest-NG ver. 0. 1. 6 [26] using Akaike Information Criterion (AIC). ML phylogeny was estimated using RAxML-NG v. 1.0.0 [27] that allows each partition (each COSII) to have its own model and parameters. Modeltest-NG determined the appropriate substitution model for each respective COSII region (Table S3). To evaluate the stability of the clade on the optimal tree, a bootstrap analysis was performed with 1000 bootstrap replicates. Each branch was statistically estimated by a bootstrap (BS) test in ML analysis and posterior probability (PP) in BI analysis.

BI phylogenetic analysis also was performed using MrBayes version 3. 2. 7a [42]. Model parameters for DNA data were chosen according to the criteria described above. Tree searching using MrBayes was performed for 1,000,000 generations with trees sampled every 100 generations. A conservative burn-in period was determined, and only post burn-in trees were saved. Finally, the posterior probabilities of each branch were calculated.

5. Conclusions

AAL toxin- susceptible SP and SLC were found in this study for the first time, and that the nt 931_932insT mutation found in SP may confer AAL-toxin susceptibility is the novel report.

Moreover, in Clade S2, we found two AAL-toxin susceptible accessions (SLC PER018894 and SLJ M5-3) that had the nt 854_855del mutation in Asc1. The mutation was identical to that of cv. Aichi-first, an AAL-toxin susceptible commercial cultivar of SLL. This finding suggested that this deletion mutation in Asc1 might have passed down throughout the history of tomato domestication and modern breeding from SLC to SLL.

Since plant breeding is usually carried out by crossing with wild species, conserving the rich genetic resources of wild species is an important issue. We suggest that several wild tomato genetic resources have influenced the transition and breeding of tomatoes so far and that rich genetic resources will continue to play an important role in the future breeding of this globally important crop.
Supplementary Materials: The following are available online at https://www.mdpi.com/2223-7747/10/1/47/s1, Figure S1: Standardization of pathogenicity in Alternaria alternata tomato pathotype As-27 (Aal) and leaf necrosis bioassay for AAL-toxin.; Figure S2: Leaflet bioassay of all accessions used in this study for AAL-toxin from a culture extract of Aal.; Figure S3: Bayesian inference (BI) tree based on 18 COSII sequences of tomato accessions estimated using MrBayes version 3. 2. 7a [42]; Table S1: COSII nucleotide primers used in this study referred from Rodriguez et al. 2009 [11]; Table S2: GenBank accession numbers of 18 COSII regions nucleotide sequence; Table S3: Model analysis of maximum likelihood (ML) and Bayesian inference (BI).


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

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