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APLICACIÓN DE HERRAMIENTAS FENOTÍPICAS, MOLECULARES Y GENÓMICAS PARA LA IDENTIFICACIÓN DE GENES CANDIDATOS EN TOMATE

por

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RESUMEN

Un programa de mejoramiento genético de tomate (*Solanum lycopersicum* L.) debe utilizar el conocimiento y la tecnología disponible en el desarrollo de estrategias eficientes para resolver diferentes problemas en la cadena de valor. En este trabajo partimos de cuatro fenotipos de interés comercial: i) tolerancia a ToCV (*Crinivirus*) ii) resistencia al herbicida Metribuzin iii) aumento del contenido de vitamina-C y iv) hábito de planta erecto (hoja erecta). Aplicando una estrategia de trabajo que integra herramientas a nivel fenotípico, molecular y genómico, nos propusimos avanzar en la identificación de genes candidatos. La estrategia comprendió los siguientes pasos: definición de la característica, estudio de su base genética, localización primaria del factor en el mapa físico, identificación de genes candidatos y validación genética. Se confirmó la presencia de cuatro alelos con efecto principal para los fenotipos descritos: *cvt*, *mtz* y *Vtc y Erl*. Para *cvt* y *mtz* se generó información suficiente para lograr su localización primaria. Para *Vtc* se avanzó en la identificación de dos genes candidatos que deberán ser confirmados y validados. *Erl* fue relacionado con un gen aún no caracterizado al que denominamos *SlTAC1*. La estrategia aplicada permitió avanzar con éxito en la identificación de genes candidatos, generando información que facilita el uso en mejoramiento de nuevos caracteres de interés para la producción de tomate. A la vez, se plantean nuevas hipótesis de investigación para complementar y profundizar el conocimiento generado.

Palabras clave: *Solanum lycopersicum*, caracterización de germoplasma, estudio de herencia, mapeo genómico

APPLICATION OF PHENOTYPIC, MOLECULAR AND GENOMIC TOOLS TO THE IDENTIFICATION OF TOMATO CANDIDATE GENES

SUMMARY

A tomato (Solanum lycopersicum L.) breeding program must use the knowledge and technology available for the development of efficient strategies to solve different problems in the crop value chain. In this work, we start with four phenotypes of commercial interest: (i) ToCV (Crinivirus) tolerance, (ii) resistance to the herbicide metribuzin, (iii) increase in the vitamin C content and (iv) erect growth habit (erectoid leaf). Applying a working model that integrates different tools at the phenotypic, molecular, and genomic level, we set out to to advance in the identification of candidate genes. The strategy included the following steps: definition of the trait, study of its genetic basis, primary location of the genetic factor in the physical map, identification of candidate genes and genetic validation. The presence of four alleles with a major effect in the described phenotypes was confirmed: *cvt*, *mtz* and *Vtc* and *Erl*. For *cvt* and *mtz,* sufficient information was generated to achieve its primary location. For *Vtc*, progress was made in the identification of two candidate genes that must be confirmed and validated. *Erl* was related to a gene not yet characterized, which we call *SlTAC1*. The applied strategy allowed to advance successfully in the identification of candidate genes, generating information that facilitates the use in tomato breeding of new interest traits. At the same time, new research hypotheses are proposed to complement and deepen the generated knowledge.

Keywords: *Solanum lycopersicum*, germplasm characterization, inheritance studies, genomic mapping

1. INTRODUCCIÓN

1.1. LA PRODUCCIÓN DE TOMATE

El tomate (*Solanum lycopersicum* L.; Solanácea) (Peralta y Spooner 2007), es en la actualidad el segundo cultivo hortícola en importancia del mundo, en términos de producción y volumen consumido. Su producción anual es 177.042 millones de kg (dato año 2016) y ha aumentado un 32 % en los últimos 10 años, generando una actividad económica directa de unos 100.000 millones de dólares y a la vez siendo la base de varias cadenas de valor y complejos agroindustriales (FAOSTAT, 2017). Desde el punto de vista alimenticio, si bien los aportes de compuestos nutricionales en comparación con otros productos vegetales son relativamente bajos, el hecho de ser un alimento universal ligado a la cultura de varios países, consumido en grandes volúmenes y bajo diferentes procesos, lo posicionan en un lugar estratégico.

En Uruguay el tomate es el principal cultivo hortícola después de la papa. En el año 2013/2014 se produjeron 37 mil toneladas de tomate de mesa para consumo en fresco, involucrando a un aproximado de 500 productores en todo el país (DIEA-DIGEGRA, 2015). La superficie destinada al rubro (2013/2014) se estima en 458 ha a nivel nacional, con un 60% del área correspondiente a cultivo protegido (DIEA-DIGEGRA, 2015). Durante el periodo 2002-2013 la producción de tomate de mesa tuvo una disminución del 60% en el número de productores y de un 50% en la superficie sembrada. Pese a esto, la producción total estimada tiende a ser constante con una variación marcada entre años, lo que sería explicado por un aumento en la productividad.

En el caso de tomate de mesa, es posible relacionar la producción nacional con el consumo, dado que históricamente las exportaciones e importaciones de fruta fresca son mínimas. A partir de esto, se calcula un consumo aproximado promedio para los últimos 10 años de 9 a 11 kg/hab/año (estimación personal en base a datos de producción). Además, se estima que el equivalente a 11 kg/hab/año se consumen en productos procesados (González-Arcos, 2005). Esto posiciona al tomate como uno de

los principales productos en la dieta de los uruguayos, con un papel protagónico y estratégico en el aporte de antioxidantes y vitaminas.

En cuanto a su producción existen dos zonas principales. La zona norte (Salto y Bella Unión), se especializó en el cultivo protegido con abastecimiento del mercado central de Montevideo durante los meses de invierno y primavera. La zona sur (Canelones y Montevideo), se especializó en el cultivo a campo con abastecimiento del mercado central durante los meses de verano y otoño. Actualmente en las dos zonas principales de producción se realizan cultivos protegidos mientras que los cultivos a campo se realizan solo en la zona sur. Según DIEA-DIGEGRA (2015) el 92% del volumen de producción de tomate de mesa proviene de cultivos protegidos.

1.2. EVOLUCIÓN DEL MEJORAMIENTO GENÉTICO DE TOMATE

Desde su domesticación en el continente americano (Peralta y Spooner, 2007) hasta los cultivares híbridos modernos, el tomate ha estado sometido a procesos de selección dirigidos por el hombre. Sin embargo, durante los últimos 150 años, el mejoramiento genético ha provocado grandes cambios en los principales cultivos que hoy conocemos, incluyendo el tomate. A finales del siglo XIX ya existían numerosos cultivares de tomate representando diferentes demandas de consumo. Todos eran líneas puras o mezclas de líneas puras. Como el tomate no se entrecruza fácilmente (especie autógama), las características de las líneas seleccionadas eran mantenidas de generación en generación por las propias familias de productores, de ahí el nombre derivado del inglés *heirloom* (= reliquia de familia) (Bai y Lindhout, 2007). Con el redescubrimiento de las leyes de Mendel en 1900, se daría inicio a la genética y una nueva era en el mejoramiento genético. Básicamente, el conocimiento generado durante esta etapa ha ido aumentando la capacidad del hombre de generar diversidad y la eficiencia del proceso de selección. En ese contexto, por diversos factores genéticos (herencia diploide, tolerancia a la homocigosis), reproductivos (generaciones cortas, facilidad y eficiencia de los cruzamientos) y moleculares (genoma pequeño, genes localizados en regiones contiguas de eucromatina) el tomate

ha sido utilizado como especie modelo en estudios de genética clásica y molecular (Shirasawa y Hirakawa, 2013). Esto ha permitido situar a un cultivo de importancia mundial a la vanguardia de los avances en el conocimiento genético y genómico.

A inicios del siglo XX varias empresas públicas y luego privadas comenzaron a trabajar en mejoramiento genético de tomate. Hasta mediados de siglo, los cultivares que se utilizaron eran líneas. A partir de las décadas de 1930 y 1940 son generados, evaluados y liberados varios cultivares híbridos en Europa, EUA y Asia (Atanassova y Georgiev, 2007). A partir de entonces esta técnica de mejoramiento se iría imponiendo hasta alcanzar la casi totalidad del mercado comercial en nuestros días. No solo por la ventaja comercial de generar una "protección biológica" del nuevo material genético obtenido, también por los beneficios de explotar la heterosis en caracteres complejos como el rendimiento (Atanassova y Georgiev, 2007), facilitar el proceso de acumulación de varios genes de herencia dominante (Scott y Gardner, 2007; Scott y Angell, 1998) y viabilizar la expresión de características cuyo interés comercial está en el heterocigoto (Scott, 1999; Kopeliovitch *et al*., 1979).

El tomate cuenta con varias especies silvestres relacionadas (Peralta y Spooner 2007), que exploran en forma natural diferentes ambientes del continente americano y son un potencial reservorio de diversidad. Esta diversidad comenzó a ser utilizada en mejoramiento genético de tomate a partir de la década del 1970 (Rick, 1979). De esta forma, el cultivo, que padecía los problemas de la estrecha base genética original, pasó a disponer de una gran capacidad de adaptación a los cambios que se producen en la etapa productiva, comercial y el consumo (Rick, 1982). Hoy en día, los cultivares modernos de tomate poseen numerosos genes aportados por especies silvestres que tienen que ver con resistencia a enfermedades y plagas, calidad de fruta y adaptación a ambientes extremos (Boiteux *et al*., 2016).

Hasta principios de 1980 los mejoradores se basaron exclusivamente en la selección fenotípica de los diferentes caracteres de interés, utilizando el conocimiento generado en materia de herencia, heredabilidad, varianzas y correlaciones genéticas e interacción genotipo *x* ambiente. Si bien hoy en día sigue estando en la base de los procesos de mejoramiento, la selección fenotípica genera varias condiciones limitantes

que, en la medida de superarlas, aumentan significativamente la eficiencia del proceso de mejoramiento. Por ejemplo: la escasa respuesta en caracteres de baja heredabilidad, la cosegregación de caracteres negativos ligados, la necesidad de utilizar grandes poblaciones y espacios, la necesidad de evaluar en diferentes ambientes, en momentos fenológicos determinados y de manejar bioensayos complejos de caracterización.

1.2.1. Genética molecular

El conocimiento generado por la genética molecular permite superar varias limitantes de la selección fenotípica. A partir de la década de 1980 se han generado diferentes herramientas biotecnológicas que aportan al mejoramiento del cultivo a nivel mundial, ya sea desde el soporte en la generación de conocimiento a nivel molecular de características de interés, hasta facilitando procesos de generación de diversidad y selección. Esto incluye el desarrollo de técnicas *in vitro* (regeneración de células, rescate de embriones, fusión somática) (Bhatia *et al*., 2004), transformaciones genéticas (USDA, 1994; Li *et al*., 2005) y amplias colecciones de mutantes (Emmanuel y Levy, 2002). En especial, grandes esfuerzos de la genética molecular en las últimas tres décadas se han concentrado en el desarrollo y uso de marcadores moleculares con el fin de facilitar el mapeo de genes de interés y su transferencia para líneas elite (Eshed y Zamir, 1995; Tanksley *et al*., 1996; Saliba-Colombiani *et al*., 2000). El tomate fue una de las primeras especies cultivadas para la cual los marcadores genéticos y mapas fueron desarrollados con fines de mejoramiento (Shirasawa y Hirakawa, 2013). Los marcadores moleculares basados en PCR y la selección asistida por marcadores sobrellevaron varias limitantes de la selección fenotípica (Foolad y Panthee, 2012).

1.2.1.1. Identificación de genes

Uno de los mayores objetivos de la genética aplicada al mejoramiento es la identificación y el aislamiento de genes principales o *major genes* (aquellos con efecto principal en la determinación del fenotipo) relacionados con caracteres de interés. La identificación de un gen genera en sí mismo marcadores moleculares (= marcador funcional o *functional markers*) que pueden ser usados para selección asistida y aumentar la eficiencia del proceso de selección de determinada característica (ver ejemplo en Gonzalez-Cendales *et al*., 2016). Además, la identificación del gen responsable de una característica genera conocimiento sobre sus bases genéticas, fisiológicas y moleculares, ayudando al entendimiento de esa y otras características relacionadas (ver ejemplo en Catanzariti *et al*., 2015).

Actualmente, el proceso de identificación de genes más utilizado se basa en la estrategia de genes candidatos (EGC). La EGC comienza con la propuesta/elección de genes de secuencia y función conocida que puedan corresponder con genes principales [de herencia mendeliana (HM), o cuantitativa (QTL)]. La hipótesis de trabajo asume que existe un polimorfismo a nivel molecular dentro del gen candidato relacionado (cosegregando) con la variación fenotípica de la característica.

Según Pflieger *et al*. (2001) la EGC pude dividirse en tres pasos: 1) Elección de genes candidatos. Este paso está limitado, por un lado, en la cantidad de genes secuenciados y de función biológica conocida que la especie en particular dispone. Por otro, en la información o conocimiento previo generado de la característica de interés, relativo a la función del gen (ej: tipo de proteína, pérdida o ganancia de función) y a su posición en el genoma basado en mapas genéticos y marcadores moleculares o fenotípicos ligados a genes conocidos. 2) Tamizado de genes candidatos. Este paso se basa en **(i)** la identificación de un polimorfismo asociado al gen candidato, y **(ii)** la asociación entre ese polimorfismo y la variación fenotípica de la característica. Dependiendo del número de genes candidatos con polimorfismo cercanos a la posición de la característica y del tipo de herencia será la precisión que podamos lograr en esta etapa. En el caso de HM se espera la ausencia de recombinación en estudios asociativos de media-alta resolución (más de 100 individuos). Para el caso de QTL,

dado que su posición y generalmente la medición de su efecto fenotípico es más imprecisa, se necesita un estudio asociativo estadístico para confirmar la cosegregación. 3) Validación del gen candidato. En este paso pueden realizarse análisis fisiológicos (actividad del gen, actividad de la proteína, o actividad enzimática) que sobre todo generan argumentos sobre el rol o la función del gen. Los experimentos de transformación con silenciamiento y sobreexpresión son los que finalmente prueban la identidad del gen candidato.

1.3. NUEVAS HERRAMIENTAS DISPONIBLES PARA LA IDENTIFICACIÓN DE GENES

En la última década, las herramientas disponibles para la identificación de genes candidatos han revolucionado este procedimiento. Sobre todo, a partir de la evolución de la eficiencia de los métodos de secuenciación.

1.3.1. Secuencia completa del genoma de tomate

En el año 2012, en un trabajo coordinado por investigadores de 14 países, se publicó la secuencia del genoma de referencia de la línea de tomate 'Heinz1706' (Tomato Genome Consortium, 2012). Esta información generó nuevas posibilidades para la investigación en lo que refiere a la identificación y localización de genes, estudios de función génica, diversidad genética y evolución, no solo en tomate sino también en forma comparativa con otras especies de solanáceas (Menda *et al*., 2013). Tanto la información generada sobre el genoma de tomate (secuencias, genes, mapas físicos y genéticos) así como su anotación (*International Tomato Annotation Group*, ITAG), datos de transcriptomas, proteínas, predicción de funciones y varios softwares de utilidad para bioinformática son administrados y están disponibles en los sitios web de *SOL Genomics Network* (http://solgenomics.net/) (Bombarely *et al*., 2010) y también en el *National Center for Biotechnology Information* (NCBI) (http://www.ncbi.nlm.nih.gov/). Además, en estos sitios se almacenan información sobre otras especies que permite hacer genómica comparativa.

1.3.2. Secuenciación de alto rendimiento

La secuenciación de nueva generación o *next-generation sequencing* (Shendure y Ji, 2008) aumenta significativamente la eficiencia y reduce los costos relativo a métodos anteriores. A partir de los avances en esta técnica, fue posible la culminación del proyecto de secuenciación del genoma completo de tomate en 2012. Con el primer modelo secuenciado ('Heinz 1706') y con la evolución de diferentes hardware y software capaces de almacenar, procesar y analizar cantidades significativas de datos, se facilitó la resecuenciación de diferentes genomas y transcriptomas. Aplicando esta herramienta, fue posible también el descubrimiento de innumerables variantes a través del genoma y el desarrollo del genotipado de larga escala.

1.3.2.1. Genotipado de larga escala

En la medida que se reducen los tiempos y costos de secuenciación (Poland y Rife, 2012), nuevas estrategias de genotipado hoy están disponibles. Con la resecuenciación de genomas fue posible la identificación de decenas de miles variantes (ejemplo SNP) a lo largo del genoma de tomate (Hamilton *et al*., 2012). Con el desarrollo paralelo de plataformas de genotipado (Gupta *et al*., 2008), que facilitan la caracterización de poblaciones a partir de marcadores seleccionados, fue posible la construcción de nuevos mapas genéticos de alta densidad (Sim *et al*., 2012). Su aplicación, permite localizar con precisión en el genoma una característica de interés facilitando el proceso de selección de genes candidatos (Rodríguez *et al*., 2013).

Otras estrategias de genotipado basadas en secuenciamiento de alto rendimiento están siendo actualmente utilizadas. El objetivo de la estrategia es identificar polimorfismos (*single nucleotide polymorphisms* – SNPs) y en simultáneo recabar información genómica de una población de interés. Para eso, se utiliza la secuenciación de fragmentos determinados de ADN con más o menos representación total del genoma de los individuos (o grupos de individuos) de una población. Es el caso del genotipado por secuenciamiento (*Genotyping by sequencing* – GBS). La metodología

es actualmente aplicada para genotipar a gran escala varias especies, ya sea que dispongan o no de genomas de referencia (Elshire *et al*., 2011; Kim *et al*., 2016). Estas metodologías pueden utilizarse para generar mapas genéticos de alta densidad o directamente para mapear características/genes individuales. Es el caso de la aplicación de la metodología RAD-seq para el reposicionamiento de un QTL relacionado con la resistencia a *Phythohphtora infestans* (Chen *et al*., 2014), la metodología SALF-seq (Sun *et al*., 2013) para el mapeo del gen *Cf-19* relacionado a la resistencia a *Cladosporium fulvum* (Zhao *et al*., 2016) y la metodología BSA-seq (Win *et al.,* 2017) para el mapeo del gen recesivo *ty-5* que confiere resistencia a begomovirus (Wang *et al*., 2018).

1.3.2.2. Secuenciación, mapeo y cuantificación del perfil de transcriptos

El transcriptoma es el conjunto completo de transcriptos de una célula y su cuantificación, asociado a un estado de desarrollo y condición ambiental dados. La tecnología de secuenciación de alto rendimiento permitió generar herramientas como el RNA-seq (Wang *et al*., 2009) capaces de mapear y cuantificar transcriptomas con niveles de eficiencia (tiempo, costo) muy altos. Utilizando RNA-seq, junto con un diseño experimental adecuado, pueden identificarse transcriptos diferencialmente expresados ante un estímulo ambiental y en determinado tejido vegetal, con lo que esta herramienta puede utilizarse especialmente para seleccionar genes candidatos por función, con el beneficio que, a la vez, de contarse con un genoma de referencia disponible, estos genes también son mapeados. Es el caso de la selección de genes candidatos para el control de la forma del fruto de tomate (Sun *et al*., 2015) y la identificación del gen *I-7* que confiere resistencia a *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) razas 1, 2 y 3 (Gonzalez-Cendales *et al*., 2016).

1.3.3. Edición genómica: nueva herramienta para el tamizado y la validación de genes candidatos

El desarrollo de herramientas de edición genómica mediante nucleasas secuencia-específicas está avanzando con velocidad (Osakabe y Osakabe, 2015). En 2014 se demostró la aplicabilidad del complejo nucleoproteico CRISPR/Cas9 para modificar el genoma vegetal de tomate (Brooks et al., 2014). Estas técnicas permiten generar mutaciones solo en sitios específicos dentro de un gen. Esto permite, por ejemplo, poder generar alelos mutantes defectuosos (= pérdida de función) con alta precisión y eficiencia (Zsögön *et al*., 2017, 2018) y es esperable que en el futuro se pueda manipular su regulación (Lowder *et al*., 2015). Esta herramienta es muy eficiente tanto para la selección como para la validación de genes candidatos (Huang *et al*., 2018; Tang *et al*., 2018)

1.4. OBJETIVOS DEL TRABAJO

Un programa de mejoramiento genético debe utilizar el conocimiento generado y la tecnología disponible en el desarrollo de estrategias eficientes (factor tiempo, factor costo) para resolver problemas de las diferentes etapas de la cadena de valor. En este trabajo se plantea el estudio de cuatro características de interés para el mejoramiento de tomate: **(i)** tolerancia a ToCV (capítulos 2 y 3), **(ii)** resistencia al herbicida metribuzin (capítulo 4), **(iii)** aumento del contenido de vitamina-C (capítulo 5) y **(iv)** crecimiento erecto (Capítulo 6). Aplicando un modelo de trabajo que integra herramientas a nivel fenotípico, molecular y genómico se pretende avanzar en la identificación de genes candidatos con efecto principal en la determinación de estas características. La estrategia comprende los siguientes pasos: definición de la característica, estudio de su base genética, localización primaria del factor en el mapa físico, identificación de genes candidatos y validación genética. La realización de este trabajo permitirá desarrollar nuevas herramientas de investigación funcionales al mejoramiento genético de la especie. Además, permitirá ampliar el conocimiento

existente sobre los cuatro caracteres seleccionados, facilitar su uso en mejoramiento genético y generar nuevas hipótesis de trabajo para futuros esfuerzos de investigación.

1.4.1. Presentación de capítulos

La tesis se compone de siete capítulos además de la presente revisión (**capítulo 1**). Los capítulos 2 al 6 corresponden con artículos científicos escritos de acuerdo a las normas detalladas en *Instructions for Authors* de la revista Euphytica (Springer Nature). La descripción general de los mismos se detalla a continuación:

El **capítulo 2** tiene el objetivo de identificar dentro de germoplasma de tomate (sección *Lycopersicon*) fuentes de resistencia/tolerancia al *Tomato chlorosis virus* (ToCV). Para eso se analizan datos fenotípicos y se utilizan técnicas moleculares de identificación del virus en una serie de tres experimentos desarrollados en Uruguay y Brasil. Este capítulo fue publicado como:

González-Arcos M, Fonseca MEN, Arruabarrena A, Lima MF, Michereff Filho M, Moriones E, Fernández-Muñoz R, Boiteux LS (2018) Identification of genetic sources with attenuated *Tomato chlorosis virus*-induced symptoms in *Solanum* (section *Lycopersicon*) germplasm. Euphytica 214:178.

El **capítulo 3** avanza en la descripción de la base genética de un factor de tolerancia al *Tomato chlorosis virus* (ToCV), partiendo de la línea de *S. lycopersicum* LT05 identificada en el capítulo 2. Se realizan estudios fenotípicos y moleculares dentro de diferentes poblaciones segregantes, se asocia un primer marcador molecular basado en PCR y se discuten funciones de posibles proteínas relacionadas. Este capítulo será propuesto para publicar como:

González-Arcos M, Fonseca MEN, Arruabarrena A, Lima MF, Boiteux LS. First report of a molecular marker associated with a tomato genetic factor controling a tolerant reaction to *Tomato Chlorosis Virus.*

El **capítulo 4** describe la base genética de la "resistencia a metribuzin" en tomate y avanza en la localización cromosómica de la característica. Se analizan datos fenotípicos, moleculares y genómicos dentro de diferentes poblaciones segregantes y se discuten funciones de posibles proteínas relacionadas. Este capítulo será propuesto para publicar como:

González-Arcos M, Fonseca MEN, Arruabarrena A, Correia NM, Boiteux LS. Phenotypic expression, inheritance, and chromosome location of *mtz*: a recessive locus that confers high-levels of metribuzin tolerance in tomato.

El **capítulo 5** avanza sobre la identificación de un factor genético relacionado con el aumento del contenido de ácido ascóribico (vitamina C) en fruta madura de tomate. El estudio confirma la localización cromosómica de este factor y discute posibles genes candidatos a partir de la información existente. Este capítulo será propuesto para publicar como:

González-Arcos M, Fonseca MEN, Boiteux LS. An introgression associated with the *Sw-5* locus in tomato chromosome 9 produce an increase of ascorbic acid content in mature fruits.

El **capítulo 6** describe la base genética del fenotipo "hoja erecta" en tomate e identifica un gen candidato responsable de la característica. Para eso se analizan datos fenotípicos en diferentes poblaciones segregantes para luego utilizar información genómica y molecular en la identificación y selección de genes candidatos. Este capítulo fue publicado como:

González-Arcos M, Fonseca MEN, Zandonadi DB, Peres LEP; Arruabarrena A, Ferreira DS, Kevei Z, Mohareb F, Thompson AJ, Boiteux LS (2019). A loss-of-function allele of a TAC1-like gene (*SlTAC1*) located on tomato chromosome 10 is a candidate for the Erectoid leaf (*Erl*) mutation. *Euphytica.* 215(5), 95

El **capítulo 7** es destinado a las conclusiones y perspectivas del trabajo.

2. IDENTIFICATION OF GENETIC SOURCES WITH ATTENUATED *Tomato chlorosis virus*-INDUCED SYMPTOMS IN *Solanum* (SECTION *Lycopersicon*) GERMPLASM *

2.1. ABSTRACT

The whitefly-transmitted *Tomato chlorosis virus* (ToCV) (genus *Crinivirus*) is associated with yield and quality losses in field and greenhouse-grown tomatoes (*Solanum lycopersicum*) in South America. Therefore, the search for sources of ToCV resistance/tolerance is a major breeding priority for this region. A germplasm of 33 *Solanum* (*Lycopersicon*) accessions (comprising cultivated and wild species) was evaluated for ToCV reaction in multi-year assays conducted under natural and experimental whitefly vector exposure in Uruguay and Brazil. Reaction to ToCV was assessed employing a symptom severity scale and systemic virus infection was evaluated via RT-PCR and/or molecular hybridization assays. A subgroup of accessions was also evaluated for whitefly reaction in two free-choice bioassays carried out in Uruguay (with *Trialeurodes vaporariorum*) and Brazil (with *Bemisia tabaci* Middle-East-Asia-Minor1 – MEAM1 = biotype B). The most stable sources of ToCV tolerance were identified in *S. habrochaites* PI 127827 (mild symptoms and low viral titers) and *S. lycopersicum* 'LT05' (mild symptoms but with high viral titers). These two accessions were efficiently colonized by both whitefly species, thus excluding the potential involvement of vector-resistance mechanisms. Other promising breeding sources were *S. peruvianum* (sensu lato) 'CGO 6711' (mild symptoms and low virus titers), *S. chilense* LA1967 (mild symptoms, but with high levels of *B. tabaci* MEAM1 oviposition) and *S. pennellii* LA0716 (intermediate symptoms and low level of *B. tabaci* MEAM1 oviposition). Additional studies are necessary to elucidate the genetic basis of the tolerance/resistance identified in this set of *Solanum* (*Lycopersicon*) accessions.

Key words: virus resistance, whitefly resistance, tolerance, virus screening

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2.2. INTRODUCTION

Whitefly (*Hemiptera: Aleyrodidae*)-transmitted viruses of the genus *Crinivirus* (family *Closteroviridae*) belong to the alphavirus-like supergroup of single-stranded, positive-sense RNA plant viruses with large (15.3–17.6 kb), complex, and segmented genomes (Mongkolsiriwattana et al. 2016). Two crinivirus species have been reported infecting tomato (*Solanum lycopersicum* L.) across the world: *Tomato chlorosis virus* (ToCV) and *Tomato infectious chlorosis virus* (TICV) (Hanssen et al. 2010; Navas-Castillo et al. 2014). Among them, ToCV is considered an emerging tomato virus with global impact associated with significant yield and quality losses in all continents (Navas-Castillo et al. 2011, 2014). The host range of tomato-infecting criniviruses includes a wide array of domesticated and weed species, which may function as natural reservoirs of these viruses and their vectors (Wintermantel and Wisler 2006; Tzanetakis et al. 2013; Orfanidou et al. 2016). In South America, ToCV has been reported thus far as the only crinivirus infecting tomatoes (Barbosa et al. 2011; Arruabarrena et al. 2014; 2015) as well as other Solanaceae species (Fonseca et al. 2016; Boiteux et al. 2018) and associated weeds (Fonseca et al. 2013; Arruabarrena et al. 2015; Boiteux et al. 2016).

ToCV is transmitted in a semi-persistent, non-circulative manner by at least five whitefly species: *Trialeurodes abutilonea*, *T. vaporariorum*, *Bemisia tabaci* New World (*B. tabaci* NW species, formerly biotype A), *B. tabaci* Middle-East-Asia-Minor 1 (*B. tabaci* MEAM1 species, formerly biotype B), and *B. tabaci* Mediterranean (*B. tabaci* MED species, formerly biotype Q) (Wintermantel and Wisler 2006; Navas-Castillo et al. 2014). Some of these vectors can acquire the criniviruses very rapidly, and they can transmit them very efficiently (Wintermantel and Wisler 2006; Shi et al. 2018).

Tomato plants infected with ToCV develop characteristic yellowing symptoms similar to those induced by mineral (especially magnesium) deficiency. In early infection stages, the symptoms are more evident in the basal and middle leaves, while the apical region appears normal (Wisler et al. 1998). ToCV-induced symptoms

in tomatoes often include interveinal foliar yellowing, leaf brittleness, reduced plant vigor, earlier senescence, and delay in fruit ripening (Tzanetakis et al. 2013). The viral latent period may vary from three to four weeks depending on the environmental conditions. Once the disease progresses, purple and necrotic areas may appear in leaves associated with the interveinal discoloration, contributing to the premature leaf senescence (Tzanetakis et al. 2013). Crinivirus infection remains confined to the phloem tissues, and most of the symptoms are the result of phloem plugging by large virus-associated inclusion bodies, which interfere with the normal nutrient transport in infected plants (Wisler and Duffus 2001). The decrease in the photosynthetic activity is the major cause of yield and quality losses in infected solanaceous hosts (Navas-Castillo et al. 2000; Fortes et al. 2012). Another peculiar feature of ToCV is its ability to interact with other viral tomato pathogens, altering the symptom expression or even leading to complete breaking-down of natural resistance factors of their host plants (García-Cano et al. 2006; Wintermantel et al. 2008).

In tomato, two major mechanisms have been identified to prevent or reduce infection by whitefly-transmitted viruses: (1) resistance/tolerance factors against viral pathogens after their invasion of the host cells and (2) resistance/tolerance factors interfering with virus-transmission efficiency by their vectors (Rodríguez-López et al. 2012; Rakha et al. 2017). Regarding resistance to the insect vector, it was demonstrated that the accumulation of defense compounds in glandular leaf trichomes interferes with the whitefly ability to visit, feed and colonize tomato accessions with these structures, which could reduce primary and secondary spread of whitefly-transmitted viruses (Mutschler and Wintermantel 2006; Rodríguez-López et al. 2011, 2012). The indirect effect of these insect-defense compounds on criniviruses incidence was observed in open field trials carried out in the Southern coast of California. A significant delay in TICV infection was observed in tomato accessions less preferred by *T. vaporariorum*, including *S. pennellii* LA0716 (an acylsugar-producing accession with type IV glandular trichomes) and *S. habrochaites* (with type VI trichomes and accumulation of methyl-ketones and sesquiterpenes) (Mutschler and Wintermantel 2006). The *S. pimpinellifolium* accession 'TO-937' (which has type IV glandular trichomes and acylsugar accumulation) was found to be tolerant to mites (Fernández-Muñoz et al.

2003), *B. tabaci* MED (Rodríguez-López et al. 2011), and *B. tabaci* MEAM1 (Silva et al. 2014). However, the broad-spectrum tolerance to pests of *S. pimpinellifolium* 'TO-937' was unable to avoid ToCV transmission and infection according to distinct field observations in Uruguay, Brazil, and Spain (García-Cano et al. 2010) as indicated by the high susceptibility levels of this accession.

In relation to resistance and/or tolerance to criniviruses, some promising genetic sources were found in *Solanum* (section *Lycopersicon*) germplasm. Stable levels of resistance to ToCV were identified in the lines '802-11-1' [derived from selfing the line 'IAC-CN-RT', obtained from an interspecific cross of *S. lycopersicum* × *S. peruvianum* (lato sensu) LA444-1] and the line '821-13-1', which was derived from *S. chmielewskii* LA1028 after two generations of selfing (García-Cano et al. 2010). Even though some plants displayed systemic infection, '802-11-1' and '821-13-1' exhibited significant resistance to ToCV accumulation and attenuated ToCV-induced symptoms (i.e. virus-infected plants displaying only mild symptoms). In addition, a subgroup of *S. chilense*, *S. lycopersicum*, and *S. corneliomulleri* accessions exhibited good levels of resistance under field conditions. However, the resistant reaction of these accessions was not stable in controlled inoculation assays under greenhouse conditions (García-Cano et al. 2010). More recently, superior levels of ToCV resistance were identified in *S. habrochaites* (PI 127826 and PI 134417), *S. peruvianum* (LA444-1 and LA0371) and several *S. lycopersicum* × *S. peruvianum* hybrids (e.g. 'IAC-68F-22-2-24-1', 'IAC-CN-RT', 'IAC-14-2-49', and 'IAC-14-2-85') in free-choice assay using viruliferous *B. tabaci* MEAM1 (Mansilla-Córdova et al. 2018). When these accessions were inoculated at non-choice vector conditions, only 'IAC-CN-RT' was confirmed as being resistant to ToCV.

Even after these pioneering breeding efforts so far there are no commercial tomato varieties with either resistance or tolerance to criniviruses. The present work is an attempt to combine the information generated by a diverse set of greenhouse assays as well as some field observations in distinct tomato crop seasons as well as in two distinct geographic regions of South America (Uruguay and Central Brazil). The present work describes a characterization of a wide range of differential reactions of a *Solanum* (*Lycopersicon*) germplasm collection (comprising cultivated and wild

species) to ToCV and against two of its vectors (*T. vaporariorum* and *B. tabaci* MEAM1) in multi-year trials conducted under natural and experimental whitefly vector exposure.

2.3. MATERIALS AND METHODS

***Solanum* (section *Lycopersicon*) germplasm –** A germplasm collection of 33 *Solanum* (*Lycopersicon*) accessions from diverse origins (Table 1) was used in a set of five independent bioassays. This germplasm comprised 16 commercial tomato hybrids from distinct seed companies, two open pollinated (OP) cultivars, six breeding lines, and ten wild accessions belonging to the species S. *pimpinellifolium* (two accessions), *S. chilense* (one accession), *S. peruvianum* (one accession), *S. pennellii* (one accession), and *S. habrochaites* (four accessions). This germplasm was evaluated in three independent bioassays for ToCV reaction (collectively named as ToCV trials) and in two independent free-choice bioassays for whitefly reaction (which were collectively named as WF trials).

Table 1 List of *Solanum* (section *Lycopersicon*) accessions evaluated for *Tomato chlorosis virus* (ToCV) reaction in three different bioassays (ToCV trails) in Uruguay and Central Brazil.

***Solanum* (*Lycopersicon*) accession**	**Origin**	**ToCV trials**
S. chilense LA1967	Tomato Genetics Resource Center	3[x]
S. habrochaites PI 126445	USDA-GRIN	1, 3
S. habrochaites PI 126925	USDA-GRIN	1
S. habrochaites PI 127827	USDA-GRIN	1, 2, 3
S. habrochaites PI 134417	USDA-GRIN	3
S. lycopersicum 'BRS Nagai'	F_1 hybrid (Embrapa–Agrocinco)	1
S. lycopersicum 'BRS Portinari'	F_1 hybrid (Embrapa–Agrocinco)	1
S. lycopersicum 'Cetia'	F_1 hybrid (Clause)	1
S. lycopersicum 'Santa Clara' (*sw*-5/*sw*-5)	OP commercial cultivar	3
S. lycopersicum 'Compack'	F_1 hybrid (De Ruiter)	1
S. lycopersicum 'Dominique'	F_1 hybrid (Hazera)	1
S. lycopersicum 'Elpida'	F_1 hybrid (Enza Zaden)	1, 2
S. lycopersicum 'Gostomiel'	F_1 hybrid (Syngenta)	3
S. lycopersicum 'Ivanhoé'	F_1 hybrid (Rijk Zwaan)	1
S. lycopersicum 'Kumato'	F_1 hybrid (Syngenta)	3
S. lycopersicum 'Santa Clara (*Sw*-5/*Sw*-5)'	Tospovirus R inbred line	1, 2, 3
S. lycopersicum 'LT05'	Inbred line (INIA–Uruguay)	1, 2, 3
S. lycopersicum 'LT17'	Inbred line (INIA–Uruguay)	1, 2, 3
S. lycopersicum 'Matrero'	F_1 hybrid (Seminis)	1, 2
S. lycopersicum 'Montenegro'	F_1 hybrid (Rijk Zwaan)	1
S. lycopersicum 'Nemo Netta'	F_1 hybrid (Nirit)	1
S. lycopersicum 'Paronset'	F_1 hybrid (Syngenta)	1
S. lycopersicum 'Swanson'	F_1 hybrid (De Ruiter)	1, 2
S. lycopersicum 'Torry'	F_1 hybrid (Syngenta)	1, 2
S. lycopersicum 'TX 468 RG'	Begomovirus R inbred line	3
S. lycopersicum 'Tyerno'	F_1 hybrid (Syngenta)	1
S. lycopersicum 'Viradoro'	OP Cultivar (Embrapa–Brazil)	3
S. lycopersicum × *S. pimpinellifolium* 'BC3F3'	Inbred line (CSIC–Spain)	3
S. lycopersicum × *S. pimpinellifolium* 'BC5F5'	Inbred line (CSIC–Spain)	3
S. pennellii LA0716	Tomato Genetics Resource Center	3
S. peruvianum (sensu lato) 'CGO 6711'	International Germplasm	1
S. pimpinellifolium PI 126931	USDA-GRIN	1
S. pimpinellifolium 'TO-937'	CSIC–Spain	1, 2, 3

[x] Numbers correspond to the experiments in which each acession was evaluated: 1= fall 2013 trial in Uruguay, 2= fall 2014 trial in Uruguay, 3= fall 2015 trial in Brazil.

Evaluation criteria employed to characterize the reaction of *Solanum* (*Lycopersicon*) germplasm to ToCV - The accessions of *S. pimpinellifolium* 'TO-937' (García-Cano et al. 2010) and the *S. lycopersicum* 'Santa Clara *Sw*-5/*Sw*-5' were employed as susceptible controls in all bioassays for reaction to ToCV. Three criteria

employed to evaluate the reaction to ToCV are described below: **(1) Assessment of ToCV-induced symptom severity –** this evaluation was carried out employing a visual scale for assessment of virus-induced symptom severity (developed in the present work; Supplementary Material A1) where: 0 = no visible symptoms, 1 = interveinal chlorosis restricted to basal leaves, 2 = interveinal chlorosis in basal and middle leaves, presence of some necrotic areas, 3 = intense interveinal chlorosis and overall yellowing, with presence of necrotic areas. **(2) Evaluation of systemic ToCV infection via reverse transcription polymerase chain reaction (RT-PCR) –** Total plant RNA was extracted from foliar tissue with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Total RNA was diluted in 50 µL of DEPC-treated water. Virus detection by RT-PCR was carried out essentially as described by Dovas et al. (2002). One step RT-PCR was performed using the HS-11/HS-12 primer pair, which amplifies a fragment of ≈ 587 bp corresponding to the heat shock protein (HSP-70) gene homolog. This genomic region is highly conserved across Crinivirus species. RT-PCR reaction was performed using the following conditions: a first step at 42°C for 60 minutes, second step at 50°C for 2 minutes, third step at 94°C for 5 minutes, 35 cycles subdivided in step #1: 30 seconds at 95°C, step #2: 30 seconds at 43°C, and step #3: 15 seconds at 72°C, followed by a final extension step (72°C for 2 minutes). Afterwards, ToCV infection was confirmed by assaying the RT-PCR products as templates in multiplex nested-PCR with the TIC-3/TIC-4 and ToC-5/ToC-6 primer pairs (Dovas et al. 2002). These assays allowed the determination of TICV-specific (≈ 263 bp) and ToCV-specific (≈ 463 bp) amplicons. Nested RT-PCR assays were performed using the following conditions: a first denaturizing step at 95°C for 1 minute, 40 cycles subdivided in 20 seconds at 95°C, 15 seconds at 60°C, and 10 seconds at 72°C, followed by one final extension step (72°C for 2 minutes). The final RT-PCR products were separated by 1.5% agarose gel electrophoresis in 0.1X TBE buffer and stained with ethidium bromide to determine presence/absence of the target DNA. Direct Sanger sequencing was carried out (at the Genomic Analysis Lab, CNPH, Brasília–DF, Brazil) with a subset of gel-purified PCR amplicons (PureLink PCR micro kit®; Invitrogen, Carlsbad, CA, USA) in order to confirm the identity of the putative ToCV-specific bands. **(3) Analysis of systemic**

ToCV infection via dot-blot and tissue-print hybridization – Molecular hybridization assays with a ToCV RNA-specific probe were performed using a previously described methodology (García-Cano et al. 2006). In the first assay for assessment of ToCV reaction (ToCV trial #1), dot-blot hybridization was implemented with a standardized drop of total RNA from each plant placed on a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). Total RNA of the plant was extracted from leaves from the middle section of the plants using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). For standardization, RNA of each sample was diluted or concentrated so that each drop contained 1 μg of total RNA in a volume of 1.5 μL. For the ToCV trial #2, tissue-print hybridization was implemented using fresh cross sections of petioles obtained from basal leaves of each plant, which were directly squash-blotted on the membrane. For the ToCV trial #3, tissue-print hybridization was also implemented from cross-sections of leaf petioles using basal and middle leaves of each evaluated plant (two leaves per plant), which were also directly squash-blotted on the membrane. The membranes with RNA drop or tissue prints were oven-fixed at 80°C for 2 hours and then hybridized with a ToCV RNA specific probe containing a fragment of the ToCV coat protein gene. The probe was synthesized from total RNA using M380/M381 ToCV-specific primers (Fortes et al. 2012), which amplify a fragment of 436 bp. The fragment was cloned in pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA). To generate the antisense RNA probe labeled with dioxigenin-11-UTP, the linearized plasmid (containing the 436 bp amplicon) was subjected to *in vitro* transcription with T7 RNA polymerase. Chemiluminescent detection was done with CDP-Star Detection Reagent (Roche Diagnostics GmbH, Mannheim, Germany). The probe synthesis, hybridization and chemiluminescent detection were performed using the DIG-labeling and detection kit (Roche Diagnostics GmbH, Mannheim, Germany) and following the protocols supplied by the manufacturer. Development of the chemiluminescent reaction and analysis of blots were performed by a ChemiDoc XRS+ System (Bio-Rad; Hercules, CA, USA).

Screening *Solanum* (*Lycopersicon*) germplasm for reaction to ToCV in Uruguay (ToCV trial #1) - This bioassay was conducted with 23 *Solanum* accessions (Table 1) during the fall season of 2013 under conditions of natural ToCV infection in a plastic greenhouse in Salto (Uruguay). Healthy seedlings were grown in an insect-proof glasshouse and 30 days after sowing five plants of each accession (with four fully expanded true leaves) were transplanted direct to the soil under the plastic house. No insecticide applications were employed during the entire assay in order to promote an abundant presence of ToCV-carrying *T. vaporariorum* adults. Previous experience indicates that, under these experimental conditions, high levels of ToCV infection are observed, following the virus spread from either infected susceptible commercial tomato crops or weed hosts in the surrounding areas (Arruabarrena et al. 2014, 2015). Disease severity was rated in all individual plants at 100 days after planting (DAP) using our previously described visual symptom scale. At this evaluation time, five plants of the most susceptible and five plants of the most resistant accessions were analyzed for systemic ToCV infection by RT-PCR. Also, five plants of a selected group of accessions were analyzed by dot-blot hybridization.

Evaluation of a selected subgroup of *Solanum* (*Lycopersicon*) accessions for reaction to ToCV in Uruguay (ToCV trial #2) - This experiment was carried out in Salto (Uruguay) during the fall season of 2014 under similar conditions as described in the ToCV trial #1. A selected group of nine *Solanum* (*Lycopersicon*) accessions that displayed contrasting reactions to the virus in the ToCV trial #1 (Table 1) was re-evaluated to confirm the germplasm responses previously observed and to study the rate of symptom development. Healthy seedlings were grown in an insect proof glasshouse and 30 days after sowing ten plants of each accession (at the four-leaf growth stage) were transplanted to 5 L-pots (filled with sterile substrate) and kept under a plastic house. Three plots (three to four plants) per genotype were employed in a complete randomized design. Disease severity was rated on individual plants using our visual symptom scale, and average plot ratings were calculated for each evaluation date (50, 70, and 90 DAP). The presence as well as the crinivirus identity was confirmed by RT-PCR. The systemic presence of ToCV in the plants was evaluated

by tissue-print hybridization in duplicates along with the first and the last visual evaluation of the symptoms (i.e. 50 and 90 DAP).

Evaluation of a selected subgroup of *Solanum* (*Lycopersicon*) accessions for reaction to ToCV in Brazil (ToCV trial #3) - This experiment was conducted in Brasilia–DF (Central Brazil) during the fall season of 2015 under greenhouse in a region where ToCV occurrence is endemic (Macedo et al. 2014). A selected group of six *Solanum* (*Lycopersicon*) accessions that displayed contrasting reactions to ToCV in the previous assays and ten novel accessions of breeding interest were evaluated (Table 1). Healthy seedlings were grown in an insect proof glasshouse and 20 days after sowing (i.e. at stage of two fully expanded true leaves) were placed inside a greenhouse containing eggplant and tomato plants with severe ToCV symptoms (Fonseca et al. 2016) and harboring a high population of viruliferous *B. tabaci* MEAM1 adults, which is the only whitefly vector species present thus far in Central Brazil (Blawid et al. 2015). Ten days later, seedlings were transplanted in 5 L-pots with a mixture of soil and commercial substrate on the definitive greenhouse. No insecticide applications were employed inside the greenhouse, aiming to promote higher levels of natural infection and spread of ToCV by allowing abundant presence of *B. tabaci* MEAM1 adults. Three plots per accession (six plants each) were organized in a completely randomized design. Disease severity was rated on individual plants using our visual symptom scale, and average plot ratings were calculated at 70 DAP. The systemic presence of the virus was evaluated in two central plants of each plot (six plants per accession) along with the last evaluation of symptoms (done at 70 DAP) by tissue-print hybridization. In addition, Sanger sequencing was carried out (at the Genomic Analysis Lab, CNPH, Brasília–DF, Brazil) with a subset of gel-purified PCR amplicons (PureLink PCR micro kit®; Invitrogen, Carlsbad, CA, USA) in order to confirm the identity of the putative ToCV-specific bands.

Evaluation of a selected subgroup of *Solanum* (*Lycopersicon*) accessions to whitefly species infestation (WF trails) - A subgroup of accessions was evaluated in two independent bioassays for reaction to distinct whitefly species. Two experiments

were carried out: **(1) Evaluation of *Solanum* (*Lycopersicon*) accessions to *T. vaporariorum* in Uruguay (WF trial #1) –** The same subgroup of accessions evaluated in the ToCV trial #2 were also evaluated for *T. vaporariorum* oviposition in a free-choice bioassay as a way to estimate potential differences of insect preference among accessions. Evaluation was performed under natural infestation by counting of whitefly eggs at 70 DAP over the same plants of ToCV trial #2. The two central plants of each plot (in the first block) and one plant of each plot (in the second and third blocks) were evaluated providing a total of four plants (replicates) per accession. From each plant, the first fully developed leaf counted from the apex was taken and ten disks of 0.8 cm in diameter were removed from the foliar tissue. The counting of eggs was performed on the abaxial surface of each disc using a stereo-microscope. Values of the same plant were averaged and expressed as the number of eggs per cm^2 of leaf. **(2) Evaluation of *Solanum* (*Lycopersicon*) accessions to *B. tabaci* MEAM 1 in Brazil (WF trial #2) –** The same subgroup of accessions evaluated in ToCV trial #3 was also evaluated for *B. tabaci* MEAM 1 oviposition in a free-choice bioassay. Tomato seedlings were transplanted to pots filled with 5 L of sterile substrate. Thirty-days after sowing, ten plots per treatment (one plant each) were arranged in a completely randomized design within a greenhouse. Heavily *B. tabaci* MEAM 1-infested cucumber (*Cucumis sativus* L.) plants with adults and fourth instar nymphs were placed into the greenhouse for 24 hours. After that, cucumber plants were removed from the substrate and left on the benches for three days in order to promote aviruliferous whitefly movement towards tomato plants under evaluation. Twenty-one days after exposure to the insects, eggs on the leaves were counted in the laboratory with the help of a stereo-microscope. The counting was performed in the abaxial surface of leaf disks (0.8 cm in diameter), which were excised from three different leaflets per plant (= six disks per plant). Countings of the same plant were averaged and expressed as the number of eggs per cm^2 of leaf tissue.

Statistical analyses - For the assessment of the plant reaction to ToCV, the ordinal symptom severity values (0 to 3) were expressed by the average of the mean values of each replicate. In the ToCV trial #2 and ToCV trial #3, the nonparametric

Kruskal-Wallis analysis of variance was implemented, followed by non-parametric multiple comparisons of ranks used for testing significant difference among treatments (Zar 1984). In the ToCV trial #2, disease progress curves were constructed based on symptom ratings, and the area under the symptom progress curve (AUSPC) was determined using the following formula: AUSPC = Σ [(Si + $S(i+1)$)($T(i+1) - vTi$)]/2 with Si = mean symptom score values at date i, and Ti = time (in days) at date i. AUSPC was used as the single variable for disease progression description. For WF preference trials, due to lack of independence of the treatments in vector free-choice tests, the nine tomato accessions evaluated for *T. vaporariorum* oviposition in Uruguay and the 16 accessions evaluated for *B. tabaci* MEAM1 oviposition in Brazil were ranked (within each replication) from the least preferred to the most preferred accession (Menezes et al. 2005). The summation of the ranks obtained for each accession in relation to the potential maximal ranking (either nine or 16) was calculated. The Friedman's test for block design was performed to determine whether there were differences among tomato accessions based upon the rank summation (Conover 1998). Multiple comparisons based upon rank summation differences were conducted between pairs of tomato accessions, using the sequential Holm's adjustment for significance level (Holm 1979).

2.4. RESULTS

Screening of *Solanum* (section *Lycopersicon*) germplasm for reaction to ToCV in Uruguay (ToCV trial #1) – Twenty-three *Solanum* (section *Lycopersicon*) accessions were initially evaluated for ToCV reaction under natural infection conditions in Salto, Uruguay (Table 2). The vector (*T. vaporariorum*) population was high and ToCV spread was uniform. This was somewhat expected for this region during the time of the year when the bioassay was carried out. Typical symptoms of interveinal chlorosis (initially restricted to basal leaves) were observed as soon as 45 DAP. In susceptible accessions, the symptoms progressed to chlorosis in the middle leaves and finally in apical leaves as well (Fig. 1). Necrotic areas were observed associated with the yellowing leaf sectors by the end of the experiment. The five plants

of the susceptible controls (*S. lycopersicum* 'Santa Clara *Sw*-5/*Sw*-5' and *S. pimpinellifolium* 'TO-937') displayed typical disease symptoms with severity ranging from medium-high (1.8 ± 0.1) to high (2.6 ± 0.2), respectively (Table 2). Plants of the accessions with the lowest (= *S. habrochaites* PI 127827) and highest (= *S. lycopersicum* 'Matrero') levels of symptom severity displayed systemic virus infection as indicated by RT-PCR assays (Table 2, Fig. 2 a). Among the 17 *S. lycopersicum* accessions under evaluation, a broad range of symptom severity was observed, from mild (attenuated) symptoms restricted to the basal leaves (e.g. 'LT05', 'Swanson', 'BRS Nagai', and 'Elpida') to overall chlorosis and yellowing with necrotic areas (e.g. 'Tyerno', 'Matrero', 'Montenegro', and 'Cetia'). Among wild species accessions, *S. habrochaites* PI 127827, *S. habrochaites* PI 126445, and *S. peruvianum* 'CGO 6711' displayed mild chlorosis symptoms in some plants, while *S. pimpinellifolium* PI 126931, *S. habrochaites* PI 126925, and *S. pimpinellifolium* 'TO-937' displayed severe symptoms in all plants. The dot-blot hybridization with a ToCV RNA-specific probe is shown for three accessions classified as having high symptom severity and four accessions classified as mild symptom severity (Table 2, Fig. 2 b). Strong hybridization signals were detected in *S. lycopersicum* plants in both high (e.g. 'Matrero' and 'Santa Clara *Sw*-5/*Sw*-5') and mild (e.g. 'LT05') symptom severity groups of accessions. In the wild species, some plants with low hybridization signals (Fig. 2 b) were observed in high symptom severity accessions (e.g. *S. pimpinellifolium* 'TO-937') as well as in the mild symptom severity accessions (e.g. *S. habrochaites* PI 127827, *S. habrochaites* PI 126445, and *S. peruvianum* 'CGO 6711')

Table 2 Evaluation to *Tomato chlorosis virus* (ToCV) reaction of a collection of commercial hybrids, breeding lines, and wild *Solanum* (section *Lycopersicon*) germplasm under natural infection conditions in a greenhouse in Salto, Uruguay (ToCV trial #1). Fall, 2013.

***Solanum* (*Lycopersicon*) accession**	**Symptom severity[x]**
S. habrochaites PI 127827	0.1 ± 0.1
S. lycopersicum 'Swanson'	0.2 ± 0.1
S. habrochaites PI 126445	0.5 ± 0.0
S. lycopersicum 'BRS Nagai'	0.5 ± 0.0
S. lycopersicum 'LT05'	0.5 ± 0.0
S. lycopersicum 'Elpida'	0.6 ± 0.1
S. peruvianum 'CGO 6711'	0.6 ± 0.2
S. lycopersicum 'Paronset'	0.8 ± 0.1
S. lycopersicum 'Compack'	0.9 ± 0.3
S. lycopersicum 'Ivanhoé'	1.0 ± 0.0
S. lycopersicum 'Dominique'	1.2 ± 0.2
S. lycopersicum 'Nemo Netta'	1.4 ± 0.2
S. lycopersicum 'Torry'	1.6 ± 0.2
S. lycopersicum 'LT17'	1.7 ± 0.1
S. lycopersicum 'BRS Portinari'	1.7 ± 0.3
S. lycopersicum 'Santa Clara (*Sw*-5/ *Sw*-5)'	1.8 ± 0.1
S. pimpinellifolium PI 126931	2.0 ± 0.0
S. lycopersicum 'Cetia'	2.2 ± 0.2
S. habrochaites PI 126925	2.4 ± 0.1
S. lycopersicum 'Montenegro'	2.4 ± 0.2
S. pimpinellifolium 'TO-937'	2.6 ± 0.2
S. lycopersicum 'Matrero'	2.7 ± 0.1
S. lycopersicum 'Tyerno'	2.7 ± 0.1

[x] Evaluation carried out at 100 days after planting, where 0 = absence of ToCV-induced symptoms and 3 = severe ToCV-induced symptom expression. Results are mean ± standard error for five plants.

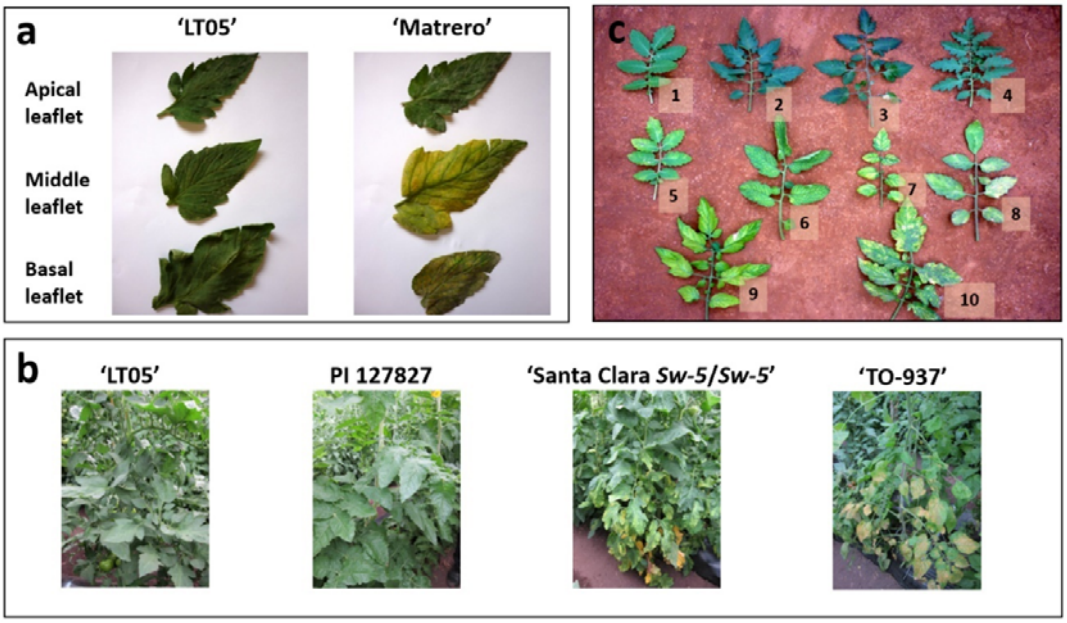

Fig. 1 (a) Trial #1: Carried out at 100 days after sowing, displaying differences of *Tomato chlorosis virus* (ToCV)-induced symptoms in the leaflets of the tolerant line *S. lycopersicum* 'LT05' (symptom severity value of 0.5 ± 0.0) and the susceptible F_1 hybrid 'Matrero' (symptom severity value of 2.7 ± 0.1). Position of the leaflet in the plant is indicated at the left of the figure. **(b)** Trial #2: Carried out at 70 days after sowing, displaying peculiar aspects of the ToCV-tolerant accessions *S. lycopersicum* 'LT05' and *S. habrochaites* PI 127827 in contrast with the susceptible standards (*S. pimpinellifolium* 'TO-937' and *S. lycopersicum* 'Santa Clara *Sw*-5/*Sw*-5'). **(c)** Trial #3: Carried out at 70 days after sowing, displaying differences in ToCV-induced symptoms in the middle leaves of distinct accessions: **(1)** *S. habrochaites* PI 127827; **(2)** *S. lycopersicum* 'LT05'; **(3)** *S. lycopersicum* 'Gostomiel'; **(4)** *S. chilense* LA1967; **(5)** *S. habrochaites* PI 134417; **(6)** *S. lycopersicum* 'Santa Clara *Sw*-5/*Sw*-5'; **(7)** *S. pimpinellifolium* 'TO-937'; **(8)** *S. lycopersicum* × *S. pimpinellifolium* 'BC5F5'; **(9)** *S. lycopersicum* 'LT17', and **(10)** *S. lycopersicum* 'Viradoro'.

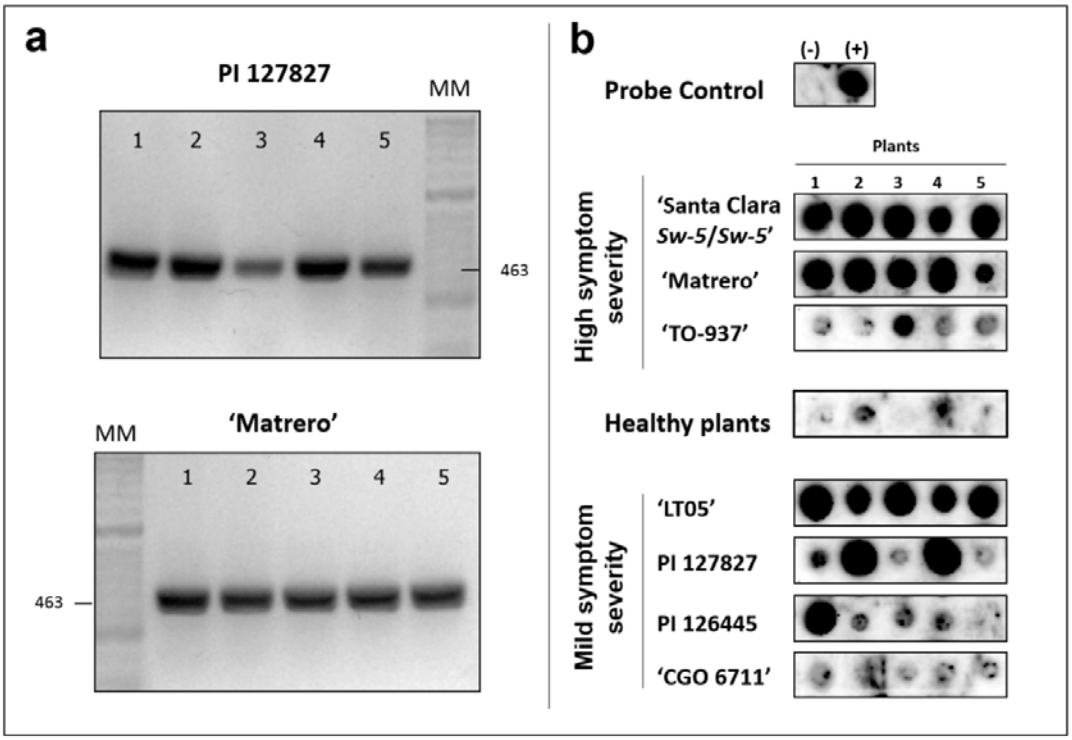

Fig. 2 (a) Evaluation of *Solanum* (section *Lycopersicon*) accessions for reaction to *Tomato chlorosis virus* (ToCV at 100 days after planting. All five plot plants of *S. habrochaites* PI 127827 (with mild symptom severity = tolerant) and *S. lycopersicum* 'Matrero' (high symptom severity = sensitive) were analyzed for ToCV systemic invasion by reverse transcription-polymerase chain reaction (RT-PCR). The expected (≈ 463-bp) ToCV-specific amplicons were observed in all plants. (**b)** Evaluation carried out at 100 days after planting. All five plants of each plot of the three accessions classified as sensitive and four accessions classified as tolerant were analyzed for presence of ToCV by a standardized dot-blot hybridization using a ToCV-derived probe. All drops contain 1.0 μg of total host plant RNA.

Evaluation of selected *Solanum* (*Lycopersicon*) germplasm for reaction to ToCV in Uruguay (ToCV trial #2) – Nine accessions evaluated in ToCV trial #1 were selected for a second assay in order to confirm the first observations and to improve the characterization of the potential resistance/tolerance mechanisms. In this

assay, the natural population of the vector (*T. vaporariorum*) was present in moderate levels. Each evaluated plant was analyzed for presence of the virus by tissue-blot hybridization. All accessions displayed 100% of the plants with systemic ToCV infection, except for *S. habrochaites* PI 127827, *S. pimpinellifolium* 'TO-937', and *S. lycopersicum* 'Santa Clara *Sw*-5/*Sw*-5', which each had eight plants infected out of ten (Table 3, Supplementary Material A2). Assessments carried out at 90 DAP are summarized in Table 3. As for the ToCV trial #1, the susceptible controls (*S. pimpinellifolium* 'TO-937' and *S. lycopersicum* 'Santa Clara *Sw*-5/*Sw*-5') displayed high levels of symptom severity with values of 2.4 ± 0.1 and 2.1 ± 0.1, respectively. Taking this reference, it was possible to identify three accessions ('LT05', PI 127827 and 'Elpida') with significantly lower symptom severity level at the end of the evaluation period, and were classified as having high tolerance. 'Matrero' and 'LT17' did not differ from the sensitive controls. 'Swanson' and 'Torry' could not be differentiated from either the highly tolerant or the sensitive accessions, and these were classified as having intermediate levels of tolerance. The AUSPC values indicated that the highly tolerant accessions had a significantly slower symptom development rate (i.e. lower AUSPC values) relative to the susceptible controls (Table 3).

Table 3 Evaluation of reaction to *Tomato chlorosis virus* (ToCV) and whitefly (*Trialeurodes vaporariorum*) oviposition in nine selected *Solanum* (section *Lycopersicon*) accessions under greenhouse conditions and natural infection. Salto, Uruguay. (**ToCV trial #2**, Fall, 2014).

***Solanum* (*Lycopersicon*) accession**	**No. of infected plants/ Total no. of evaluated plants [w]**	**AUSPC [x]**		**Symptom severity[y]**		**Eggs/cm² [z]**
S. lycopersicum 'LT05'	10/10	3.3 ± 1.7	a	0.1 ± 0.1	a	1.25 ± 0.24
S. habrochaites PI 127827	8/10	5.0 ± 5.0	a	0.2 ± 0.2	a	0.75 ± 0.31
S. lycopersicum 'Elpida'	10/10	10.8 ± 2.5	ab	0.3 ± 0.1	ab	1.30 ± 0.10
S. lycopersicum 'Swanson'	10/10	14.6 ± 3.4	ab	0.4 ± 0.1	abc	1.00 ± 0.22
S. lycopersicum 'Torry'	10/10	27.5 ± 8.0	abc	0.8 ± 0.3	abcd	1.10 ± 0.58
S. lycopersicum 'LT17'	10/10	49.6 ± 5.4	bc	1.7 ± 0.2	bcd	0.55 ± 0.38
S. lycopersicum 'Matrero'	10/10	50.0 ± 9.8	bc	1.8 ± 0.4	bcd	1.73 ± 0.37
S. lycopersicum 'Santa Clara (*Sw*-5/ *Sw*-5)'	8/10	65.4 ± 5.6	c	2.1 ± 0.1	cd	0.90 ± 0.47
S. pimpinellifolium 'TO-937'	8/10	72.1 ± 4.1	c	2.4 ± 0.1	d	0.70 ± 0.17

[w] Evaluation carried out at 50 and 90 days after planting. Plants were analyzed for presence of ToCV by tissue-print hybridization using a ToCV-derived probe.

[x] Area Under Severity Progress Curve (AUSPC). Values are displayed as mean ± standard error of three replicates. Treatments within the column followed by different letter are significantly different ($p<0.05$) by non-parametric multiple comparisons of ranks after Kruskal–Wallis test. ($H = 22.73$; $p = 0.0037$).

[y] Evaluation carried out at 90 days after planting, where 0 = no conspicuous ToCV-induced symptoms; 3 = severe ToCV-induced symptoms. Values are mean ± standard error of three replicates. Treatments within the column followed by different letter are significantly different ($p<0.05$) by non-parametric multiple comparisons of ranks after Kruskal–Wallis test. ($H = 21.94$; $p=0.0046$).

[z] Whitefly (*Trialeurodes vaporariorum*) eggs per cm^2 of leaflet. Values are mean ± standard error of four plants (replicates). No statistical differences between ranks sums of accessions were observed for the Friedman's test ($Fr = 10.271$; $p = 0.246520$).

Evaluation of *Solanum* (*Lycopersicon*) germplasm for reaction to ToCV in Brazil (ToCV trial #3) – This bioassay was carried out in Brasília–DF (Brazil) under controlled inoculation conditions with high *B. tabaci* MEAM1 pressure (with an average of more than 30 whitefly adults per plant) at early (seedling) growth stage.

This assay was composed of 11 accessions previously evaluated in trials in Uruguay plus a subgroup of five accessions of breeding interest that were not previously evaluated (Table 4). ToCV infection symptoms evolved rapidly and the final evaluation was carried out at 70 DAP. At that time, tissue-blot hybridization signals indicated that all six plants of each *Solanum* (*Lycopersicon*) accession under evaluation were positive for systemic presence of ToCV (Table 4, Supplementary Material A3). High levels of symptom severity were confirmed once again in *S. lycopersicum* breeding line 'Santa Clara *Sw*-5/*Sw*-5' and *S. pimpinellifolium* 'TO-937' (both used as susceptible controls). On the other hand, it was possible to confirm again the low disease severity observed in plants of the *S. lycopersicum* 'LT05' and *S. habrochaites* PI 127827. Plants of *S. pennellii* LA0716, *S. habrochaites* PI 126445, and *S. chilense* LA1967 displayed low symptom severity under these conditions. Interestingly, plants of the *S. lycopersicum* cultivars 'Kumato' and 'Gostomiel' (carrying the "*green-flesh*" – *gf* mutation) displayed medium to low symptom severity. The remaining *S. lycopersicum* accessions evaluated in this assay were found to be susceptible to ToCV, including two *S. lycopersicum* × *S. pimpinellifolium* lines ('BC3F3' and 'BC5F5') derived from an introgression program aiming to incorporate the insect tolerance traits (high acylsugar and type IV glandular trichomes) from *S. pimpinellifolium* 'TO-937' into cultivated tomato cv. 'Moneymaker' (Rodriguez-López et al. 2011, Escobar-Bravo et al. 2016).

Table 4 Evaluation of reaction to *Tomato chlorosis virus* (ToCV) and of whitefly (*Bemisia tabaci* MEAM 1) oviposition in 16 *Solanum* (section *Lycopersicon*) accessions. Assays carried out under greenhouse conditions in Brasilia–DF, Brazil (**ToCV trial #3**). Fall, 2015.

***Solanum* (*Lycopersicon*) accession**	**No. of infected plants/No. of total analyzed plants [x]**	**Symptom severity[y]**		**Eggs/cm² [z]**	
S. pennellii LA0716	6/6	0.2 ± 0.1	a	0.006 ± 0.003	d
S. habrochaites PI 126445	6/6	0.3 ± 0.2	ab	25.85 ± 8.90	a
S. chilense LA1967	6/6	0.3 ± 0.1	ab	21.83 ± 5.78	ab
S. lycopersicum 'LT05'	6/6	0.5 ± 0.1	ab	10.86 ± 1.34	abc
S. habrochaites PI 127827	6/6	0.6 ± 0.3	abc	8.38 ± 2.00	abc
S. lycopersicum 'Gostomiel'	6/6	0.9 ± 0.1	abcd	15.36 ± 3.86	abc
S. lycopersicum 'Kumato'	6/6	1.2 ± 0.1	abcde	9.91 ± 2.79	abc
S. habrochaites PI 134417	6/6	1.4 ± 0.1	abcde	4.24 ± 1.93	cd
S. lycopersicum × *S. pimpinellifolium* 'BC3F3'	6/6	2.0 ± 0.2	bcde	8.41 ± 3.06	abc
S. lycopersicum 'TX 468 RG'	6/6	2.1 ± 0.2	cde	23.28 ± 6.99	ab
S. pimpinellifolium 'TO-937'	6/6	2.3 ± 0.1	cde	5.62 ± 2.29	bcd
S. lycopersicum 'LT17'	6/6	2.4 ± 0.2	de	6.81 ± 2.34	abc
S. lycopersicum × *S. pimpinellifolium* 'BC5F5'	6/6	2.6 ± 0.1	e	7.75 ± 1.47	abc
S. lycopersicum 'Viradoro'	6/6	2.6 ± 0.1	e	24.10 ± 6.60	ab
S. lycopersicum 'Santa Clara (*Sw*-5/*Sw*-5)'	6/6	2.6 ± 0.0	e	10.12 ± 1.38	abc
S. lycopersicum 'Santa Clara (*sw*-5/*sw*-5)'	6/6	2.7 ± 0.1	e	17.04 ± 3.42	ab

[x] Evaluation carried out at 70 days after planting. The two central plants from each plot (six plants) were analyzed for presence of ToCV by tissue-print hybridization using a ToCV-derived probe. [y] Evaluation carried out at 70 days after planting, where 0 = no conspicuous ToCV-induced symptoms; 3 = severe ToCV-induced symptoms. Values are displayed as mean ± standard error of three replicates. Treatments within the column followed by different letter are significantly different ($p<0.05$) by non-parametric multiple comparisons of ranks after Kruskal–Wallis test ($H = 40.86$; $p = 0.0003$). [z] Whitefly (*Bemisia tabaci* MEAM 1) eggs per cm^2 of the sampled leaflet. Values are displayed as mean ± standard error for ten plants (replicates). Different letters within the column indicate significant difference between the rank sum of each treatment (Friedman multiple pair-wise test, followed by sequential Holm adjustment: $p<0.05$). ($Fr = 63.560$; $p<0.0001$).

Evaluation of *Solanum* (*Lycopersicon*) germplasm to *T. vaporariorum* in Uruguay (WF trial #1) – The same subgroup of accessions tested in the ToCV trial #2 was additionally estimated for whitefly preference by evaluation of oviposition

(Table 3). Accessions displayed varying levels of *T. vaporariorum* oviposition. However, the differences observed among these accessions were not significant. For the accessions under evaluation, there was no significant correlation between ToCV symptom severity and *T. vaporariorum* preference based upon oviposition.

Evaluation of *Solanum* (*Lycopersicon*) germplasm to *B. tabaci* MEAM 1 in Brazil (WF trial #2) – A subgroup of 16 tomato accessions with contrasting reactions to ToCV were also evaluated against *B. tabaci* MEAM 1 in a free-choice bioassay in Brazil (Table 4). The *S. pennellii* accession LA0716 displayed the highest level of resistance to *B. tabaci* MEAM1 based on oviposition ($6.0 \times 10^{-3} \pm 3.0 \times 10^{-3}$ eggs / cm2), but this value was not statistically different from oviposition rates of *S. pimpinellifolium* 'TO-937' and *S. habrochaites* PI 134417 with 5.62 ± 2.29 and 4.24 ± 1.93 eggs / cm^2, respectively. However, it was not possible to differentiate these latter two accessions from a final subgroup of materials that were characterized by presenting the highest levels of oviposition (high susceptibility). 'TO937' was only significantly better than the accession PI 126445, whereas *S. habrochaites* PI 134417 displayed lower ovipositon levels than five accessions (Table 4).

2.5. DISCUSSION

To date, there is no commercial tomato cultivar reported with adequate levels of resistance to ToCV. This may be a result of a reduced priority in breeding for resistance against this virus when compared with the solid efforts conducted for begomoviruses (genus *Begomovirus*, family *Geminiviridae*) resistance due to their prevalence in all major tomato-producing regions around the world (Giordano et al., 2005; Pereira-Carvalho et al. 2015). In addition, for most tomato farmers, ToCV infection is often misdiagnosed as being induced by nutritional deficiency with no conspicuous effects on fruit yield and quality. More recently, after the massive deployment of begomovirus resistant hybrids (Boiteux et al. 2007), the losses induced by ToCV infection are becoming more evident. In this new scenario, growers and the tomato agribusiness sector are demanding genetic solutions to this problem. However,

tomato breeding programs are facing some difficulties since the currently available sources of resistance (García-Cano et al. 2010; Mansilla-Córdova et al. 2018) are not easily incorporated into elite commercial material.

The present work summarizes our breeding efforts to identify and characterize potential novel sources of resistance/tolerance to ToCV, involving an extensive set of bioassays (under both natural and controlled viruliferous and aviruliferous whitefly exposure) with a diverse *Solanum* (*Lycopersicon*) germplasm collection. These assays were carried out in vegetable-producing areas in South America where severe ToCV infections are reported in tomatoes as well as in several other cultivated and weed Solanaceae species (Arruabarrena et al. 2014, 2015; Macedo et al. 2014; Fonseca et al. 2016; Boiteux et al. 2018).

The use of terms related to the response of plants to a virus infection was taken from Cooper and Jones (1983). In this way, when we describe the behavior of virus in the plant (e.g. viral titer) we will refer to resistance/susceptibility, whereas when we describe the response of the plant to the disease (e.g. symptom severity) we will refer to tolerance/sensitivity. Consistent infections were observed in the sensitive controls (which were included in all independent trials), confirming the high ToCV pressure in across all assays. Most of the cultivated tomato (*S. lycopersicum*) accessions were systemically infected by ToCV and displayed high to medium symptom severity levels (= sensitivity). However, it was possible to identify the *S. lycopersicum* inbred line 'LT05' (high tolerance in ToCV trial #1, #2 and #3) and in a second step the commercial F_1 hybrids 'Elpida' (high tolerance in ToCV trial #1 and #2) and 'Swanson' (high tolerance in ToCV trial #1 and intermediate in #2). All *S. lycopersicum* were preferred by *T. vaporariorum* based upon oviposition values, indicating that tolerance to ToCV found in some accessions is likely not to be related to vector resistance mechanisms. For these tolerant accessions, it was possible to observe, in the conditions described for ToCV trial #2, a lower rate of symptom progression when compared with more sensitive accessions.

Previous studies have identified a subgroup of *S. lycopersicum* accessions with high levels of field tolerance to ToCV-induced symptoms expression in Spain (García-Cano et al. 2010). However, this tolerance was not expressed under high vector

pressure conditions. Based upon our experiments, we cannot exclude the possibility that the lower severity of symptoms observed in 'Elpida' and 'Swanson' would be stable under either high disease pressure conditions or under longer crop production cycles. However, the *S. lycopersicum* breeding line 'LT05' confirmed the low severity of ToCV-induced symptoms across all bioassays corresponding to three different environmental conditions. In this case, all evaluated plants displayed strong hybridization signals in the dot-blot assays, indicating systemic infection of ToCV and, therefore, we discarded the potential involvement of resistance mechanisms related to limitation of virus infection (i.e. multiplication and transport within the plant). In this way, employing the concept defined by Cooper and Jones (1983), we identify a stable source of tolerance to ToCV-induced symptom development in the inbred line 'LT05'. This inbred line may be particularly a source of ToCV tolerance for breeders, based on its high and stable levels of ToCV tolerance and due to the fact that it is a cultivated tomato (*S. lycopersicum*) which will facilitate its employment in genetic improvement programs. Future studies would be necessary to investigate the genetic basis of the ToCV tolerance in 'LT05' and the expected impact of this trait on tomato fruit yields.

In the ToCV trial #3 carried out in Brazil, two *S. lycopersicum* accessions 'Kumato' and 'Gostomiel' were identified with low to medium levels of symptom severity (Table 4). Coincidently, these two cultivars have the presence (in homozygous condition) of the *green-flesh* (*gf*) gene (Kerr 1956), which is located on the long arm of the tomato chromosome 8 (Kerr 1958). The *gf* gene encodes for the STAY-GREEN (SGR) protein, which is necessary for chlorophyll degradation. In the case of this mutation, its major effect includes the inhibition of chlorophyll degradation (Akhtar et al. 1999). Therefore, it would be interesting to further investigate if the *gf* gene could be responsible for the attenuation of the ToCV-induced symptoms by limiting the severe manifestation of foliar chlorosis, which is typically associated with this virus infection in susceptible tomatoes.

In wild tomato species, some stable phenotypes with low symptom severity were identified in *S. habrochaites* PI 127827 and PI 126445. In the case of *S. habrochaites* PI 127827, individual plants with contrasting responses were identified after analyses with RT-PCR and dot-blot hybridization assays. RT-PCR indicated the

prevalent systemic presence of ToCV, but some plants display very low signals in the hybridization assay (Fig. 2 a, b). Therefore, it would be interesting to investigate if some mechanism of virus resistance could still be segregating in this accession as it is an allogamous species that can display a high variability as well as heterozygosity. Further studies from inbred lines generated by self-pollination of resistant plants (i.e. the ones with low viral accumulation) should be conducted to evaluate this aspect. The potential involvement of vector-resistance mechanisms was discarded, since *S. habrochaites* PI 127827 was susceptible to *T. vaporariorum* colonization in WF trial #1 (not different to other accessions) and *B. tabaci* MEAM1 colonization in WF trial #2 (not different to the sensitive subgroup of accessions), whereas *S. habrochaites* PI 126445 was susceptible to *B. tabaci* MEAM1 colonization based upon the results of the WF trial #2.

Other wild species accessions that displayed low symptom severity were *S. peruvianum* 'CGO 6711' (in the ToCV trial #1), *S. chilense* LA1967, and *S. pennellii* LA0716 (in the ToCV trial #3). In the case of *S. peruvianum* 'CGO 6711', it is interesting to note that the analysis of individual plants under evaluation by standardized dot-blot hybridization indicated very low hybridization signals in all five samples (Fig. 2 b). Again, this could be related to some mechanism of resistance to virus accumulation (multiplication and/or movement within the plant). In this case, we cannot yet discard the potential involvement of vector resistance mechanisms, as *S. peruvianum* 'CGO 6711' could not be included in the WF trials. Therefore, further studies are needed with this accession. No standardized viral titer evaluations were performed for *S. chilense* LA1967 and *S. pennellii* LA0716. However, *S. chilense* LA1967 displayed high levels of *B. tabaci* MEAM1 susceptibility based upon oviposition values (Table 4). Thus, its low level of ToCV symptom severity is likely unrelated to vector resistance. *Solanum pennellii* LA0716 was the only accession that showed a clear-cut resistance for *B. tabaci* MEAM1 infestation (Table 4). Therefore, it is probably that the mild ToCV symptoms observed *S. pennellii* LA0716 could be related, at least in part, to extreme resistance to *B. tabaci* MEAM1. It is also important to point out the light green leaf color of *S. pennellii* LA0716, which makes difficult a precise assessment of ToCV symptoms in plants derived from this germplasm. In fact,

ToCV symptoms in *S. pennellii* LA0716 is manifested, in general, by an even lighter greenish leaf color rather than the typical chlorosis and leaf patches observed in other *Solanum* species. This is the first report of *S. pennellii* as possible source of tolerance to ToCV, although it has already been reported as displaying a significant delay of TICV symptom expression, which was also associated with its low whitefly preference (Mutschler and Wintermantel 2006).

The wild species *S. peruvianum*, *S. chilense*, and *S. habrochaites* have been reported as sources of ToCV resistance in previous works (García-Cano et al. 2010; Mansilla-Córdova et al. 2018). In fact, the accession 'IAC-CN-RT5' (hybrid from *S. lycopersicum* cv. 'Angela Gigante' × *S. peruvianum* LA0444-1) and the line '802-11-1' (derived from two cycles of selfing and selection of 'IAC-CN-RT5') displayed some levels of resistance (low systemic infection and reduced virus multiplication), coincident with our preliminary results for *S. peruvianum* 'CGO 6711' in ToCV trial #1.

The accessions of *S. pimpinellifolium* evaluated here have consistently shown high levels of ToCV symptom severity, in particular *S. pimpinellifolium* 'TO-937', the material chosen as our sensitive reference. However, standardized dot-blot hybridization analysis showed low hybridization signals in some of *S. pimpinellifolium* 'TO-937' plants (Fig. 2 b), which could be related to levels of resistance to virus infection (multiplication and/or movement within the plant) or some mechanism of interference with the vector, which in our case was confirmed for *B. tabaci* MEAM1 (Table 4), corroborating previously results obtained for *B. tabaci* MED in Spain (Rodríguez-López et al. 2011) and for *B. tabaci* MEAM1 in Brazil (Silva et al. 2014). Thus, *S. pimpinellifolium* 'TO-937' could have an interesting case of extremely high ToCV sensitivity, with higher levels of symptom severity even with low viral titers, which might merit further investigation.

Mansilla-Córdova et al. (2018), studying the tolerance to ToCV in a set of commercial tomato cultivars, found that most of the evaluated materials did not display significant yield and quality losses. However, some cultivars were highly sensitive, showing reductions up to 58.1% and 71.9% in total fresh weight and fruit yield, respectively. Perhaps, this cultivar-specific trait of high sensitivity to ToCV infection

(as observed for *S. pimpinellifolium* 'TO-937') might be also present in some of the currently available commercial tomato hybrids. The unaware use of these highly sensitive accessions could be an important factor in the emergence of ToCV for many tomato-producing regions around the globe. A similar phenomenon was observed in the epidemiology of *Tomato torrado virus* (ToTV), another whitefly-transmitted RNA virus, which is emerging in some regions of the world (Navas-Castillo et al. 2014).

In conclusion, the present work allowed the identification of a subset of stable sources of ToCV tolerance and some novel promising sources of virus resistance (*sensu* Cooper and Jones 1983). Among the stable sources, we highlight the *S. lycopersicum* breeding line 'LT05' (with high level tolerance to ToCV-induced symptoms even displaying high viral titers) and *S. habrochaites* accessions PI 127827 and PI 126445 (whose reactions may involve some mechanisms of restriction to virus accumulation). We also reported *S. peruvianum* 'CGO 6711' as a potential source of high resistance levels, which was associated with low viral titers. However, we were not able to exclude the potential involvement of vector resistance mechanisms of *S. peruvianum* 'CGO 6711' since it was not included in our WF trails due to the low amount of seeds available. Other tolerant accession, *S. chilense* LA1967, may have more likely resistance mechanism to the virus since it displayed high levels of *B. tabaci* MEAM1 infestation. In the other hand, *S. pennellii* LA0716 might have its tolerance to ToCV associated with interference with the vectors, in agreement with previous works with TICV (Mutschler and Wintermantel 2006). It will be of breeding interest to carry out additional inheritance and genomic mapping studies with these promising sources aiming to elucidate if they share similar tolerance/resistance mechanisms with previously identified sources or if they are under a less complex genetic control.

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3. FIRST REPORT OF A MOLECULAR MARKER ASSOCIATED WITH A TOMATO GENETIC FACTOR CONTROLLING A TOLERANT REACTION TO *Tomato Chlorosis Virus*

3.1. ABSTRACT

Thus far, *Tomato chlorosis virus* (ToCV) is the only *Crinivirus* species reported infecting tomatoes in South America. Typical viral symptoms are interveinal leaf yellowing and early senescence, resulting in reduced photosynthetic capacity. Our previous work identified accessions of wild and cultivated tomato species with high levels tolerance to the development of ToCV-induced symptoms. To study the genetic basis of this trait, a highly tolerant tomato (*Solanum lycopersicum*) breeding line 'LT05' was crossed with the highly susceptible *S. pimpinellifolium* accession 'TO-937'. Six experimental populations (P_R, P_S, F_1, BCP_R, BCP_S and F_2) derived from this interspecific cross were inoculated (at seedling stage) employing viruliferous *Bemisia tabaci* MEAM1 adults. Inoculated plants were then transplanted to individual pots for phenotypic characterization. Chlorophyll content was indirectly assessed at 60 days after planting (DAP) using a Minolta SPAD-meter. Evaluation of ToCV symptoms was conducted at 120 DAP based upon a symptom expression grade system varying from 0 (tolerant) to 3 (highly susceptible). Presence of systemic ToCV infection was evaluated by tissue blot hybridization. Even though all F_2 plants showed systemic ToCV infection, distinct levels of symptoms expression were observed, with the line 'LT05' displaying very mild symptoms (severity index = 1.2), whereas 'TO-937' exhibited overall severe yellowing (severity index = 2.93). High SPAD reading units were strongly correlated with attenuated ToCV symptoms expression. Segregation ratio of these two traits revealed a genetic control by a major recessive gene/locus. This crinivirus tolerance locus was tentatively named as *cvt* (= *crinivirus tolerance*). A PCR-based marker in association with attenuated ToCV symptoms expression were identified via bulked segregant analysis. This information could be used as an input for future works about the location of this factor in the tomato genome. The simple genetic control confirmed here indicated that the development of ToCV-tolerant tomato cultivars is a feasible breeding objective.

Keywords: Crinivirus, tolerance, *Solanum lycopersicum*, candidate gene

3.2. INTRODUCTION

Tomato chlorosis virus (ToCV) and *Tomato infectious chlorosis virus* (TICV) (TICV) are the two species of the genus *Crinivirus* (family Closteroviridae) which are able to infect cultivated tomatoes (*Solanum lycopersicum* L.). ToCV has been reported in several regions of the world infecting more than 40 host species, including crops and weeds (Arruabarrena et al. 2015, Boiteux et al. 2016, Boiteux et al. 2018, Fonseca et al. 2013, Fonseca et al. 2016, Navas-Castillo et al. 2000, Orfanidou et al. 2016). Thus far, ToCV is the only *Crinivirus* species reported infecting tomatoes in South America (Arruabarrena et al. 2014, Barbosa et al. 2011). The outbreaks of ToCV in tomato crops worldwide can be explained by the wide host range of this virus as well as by its semi-persistent, non-circulating transmission by multiple whitefly species such as *Trialeurodes abutilonea*, *T. vaporariorum*, *Bemisia tabaci* (NW1 and NW2 species = former biotype A), *B. tabaci* Middle East-Asia Minor1 (MEAM1 = biotype B), and *B. tabaci* Mediterranean subgroup Q (MED Q = biotype Q) (Hanssen et al. 2010, Navas-Castillo et al. 2011, Navas-Castillo et al. 2014, Wintermantel and Wisler 2006).

Visible ToCV symptoms begin 3-4 weeks after infection, initially at the older basal leaves and then evolving towards the apex. In general, virus-induced symptoms are similar to a mineral nutrition deficiency and include internerval chlorosis, overall yellowing, necrotic leaf spots and leaflet deformations (Wintermantel and Wisler 2006). The majority of the commercial tomato hybrids did not display significant effects on fruit fresh weight and yield. However, some highly-sensitive cultivars may present reductions of up to 70% in total fresh weight and 50% fruit yield (Mansilla-Córdova et al. 2018). In sweet-pepper (*Capsicum annuum* L.), yield losses may vary from 45 to 75% (Fortes et al. 2012). In addition, these viruses have the peculiarity of interacting with other viruses within the host plant cells, altering levels of resistance and/or symptom expression (Karyeija et al. 2000, García-Cano et al. 2006, Mukasa et al. 2006, Susaimuthu et al. 2008, Wintermantel et al. 2008, Cuellar et al. 2011).

Several sources of resistance to criniviruses were identified and a wide array of genetic factors were characterized in the germplasm of many host species. Resistance

to *Lettuce infectious yellows virus* (LIYV) in lettuce germplasm was identified in accessions of the wild species *Lactuca saligna* L. (McCreight, 1987). The resistance to LIYV was found to be controlled by either a single dominant locus denominated *Lyi* (McCreight 2000) or by multiple genes with expression strongly depedent upon the environmental conditions (Haley 1990). In melon (*Cucumis melo* L.) resistance to *Cucurbit yellowing stunting disorder virus* (CYSDV) was found to be under control of either a dominant locus named as *Cys* (Lopez-Sesé and Gomez-Guillamon 2000) or by a recessive locus (McCreight and Wintermantel 2011). The genetic basis of resistance to CCYV detected in novel melon germplasm sources has not yet been determined (Okuda et al. 2013). In cucumber (*Cucumis sativus* L.), two sources of resistance to CYSDV were identified (Aguilar et al. 2006). In addition, seven accessions were identified with resistance to CYSDV in a broad-based *Cucumis* germplasm collection (Eid et al. 2006). The genetic basis of these resistance sources has not yet been determined. No sources of HR (Hypersensitive Response) resistance were found in lettuce, melon, and cucumber germplasm. The major mechanism associated with these genes is a significant attenuation of the virus-induced symptoms, sometimes associated to a lower viral multiplication within the host plant cell and sometimes associated with antioxenotic factors against the whitefly vector.

The management of *Crinivirus* species is usually carried out by chemical control of the vector populations, especially in early crop stages. This achieved in many cases reduction or delay of incidence and severity of symptoms. However, given the ToCV transmission efficiency, which includes a few hours acquisition and transmission period (Wisler and Duffus 2001; Wintermantel and Wisler 2006), these measures may not be effective in all cases. Commercial tomato cultivars with resistance and/or tolerance to *Crinivirus* species have not been reported thus far. In this context, breeding efforts aiming for the identification of resistance sources to *Crinivirus* were recently initiated via screening germplasm collections of cultivated tomato and related wild species accessions.

Two resistance mechanisms can reduce the impact of *Crinivirus*: vector resistance and virus resistance. Regarding to vector resistance, it was demonstrated that the presence of acylsucrose in tomato leaves produced by type IV glandular

trichomes interferes with the whitefly ability to feed and colonize the host, which could interfere significantly in the primary and secondary dispersion of the *Begomovirus* TYLCV (Rodríguez-López et al. 2011, 2012). For criniviruses, preliminary open field trials at the southern coast of California demonstrated a significant delay in TICV infection in accessions of *S. pennellii* and *S. habrochaites* which produced acylsucrose and were less preferred by the whitefly *T. vaporariorum* (Mutschler and Wintermantel 2006). The foliar acylsucrose-producing accession of *S. pimineliffollium* ('TO-937') displayed large-spectrum tolerance to mites, tomato leaf miner, and whitefly species (Fernandez-Muñoz et al. 2003, Resende et al. 2006; Silva et al. 2014). However, different field observations in Uruguay, Brazil, and Spain indicate that *S. pimineliffollium* ('TO-937') is highly susceptible to ToCV.

Stable resistance levels to ToCV were identified in the accessions '802-11-1' (inbred line derived from the interspecific cross *S. lycopersicum x S. peruvianum* 'LA444-1') and '821-13-1' (*S. chmielewskii*) (García-Cano et al. 2010). Both accessions exhibited significant levels of tolerance to ToCV under natural and controlled inoculation conditions. In addition, some accessions from *S. chilense*, *S. lycopersicum* and *S. corneliomulleri* exhibited an interesting level of resistance under field conditions. However, this resistance was not stable under controlled inoculation conditions. The differences exhibited between natural and controlled conditions can be explained by differences in the inoculum pressure or some kind of vector resistance in these genotypes (García-Cano et al. 2010). The resistant reaction to ToCV infection in *S. peruvianum* 'LA444-1' and *S. peruvianum* LA371 as well as in two lines (IAC68F-22-2-24-1' and 'IAC-CN-RT5' = '802-11-1') derived from *S. lycopersicum x S. peruvianum* LA444-1 was confirmed in a study carried out in Brazil (Mansilla-Córdova et al. 2018). In addition, novel sources of resistance to ToCV infection in found two *S. habrochaites* accessions: PI 127826 and PI 134417 (Mansilla-Córdova et al. 2018). In a previous work performed by our research group (González-Arcos et al. 2018) we were able to identify sources of tolerance to ToCV-induced symptoms development in accessions of *S. habrochaites, S. chilense*, *S. peruvianum*, and *S. pennellii*. A wide array of responses observed in this germplasm suggested the presence of distinct ToCV tolerance/resistance mechanisms. Within *S. lycopersicon*,

germplasm, the breeding line ‘LT05’ was identified as a stable source of tolerance, even under relatively high vector pressure conditions. This accession displayed systemic ToCV infection and no differences were found in viral accumulation levels (titers) when compared to highly susceptible accessions (González-Arcos et al. 2018). It was not possible to demonstrate a lower vector preference related to the higher levels of tolerance displayed by LT05’. Thus, tolerance corresponds with a low disease symptoms expression. In addition, an intense foliar green color is observed at different development stages of ‘LT05’ plants, which generates the hypothesis that the low expression of symptoms (tolerance) is related to a higher concentration or a lower degradation of chlorophyll. The main purposes of the present work were to study the genetic basis and to generate a preliminary mlecular information useful for the chromosomal location of the genetic factor controlling a tolerant reaction to ToCV in the tomato line ‘LT05’.

3.3. MATERIALS AND METHODS

Plant material and trials – The breeding line *S. lycopersicon* ‘LT05’ (tolerant to ToCV and with intense green leaf color) and the accession of *S. pimpinellifolium* ‘TO-937’ (sensitive for ToCV and light green leaf color) were used as contrasting parents in controlled crosses. Four experimental populations [F_1, Back-cross (BC) to LT05, BC to TO-937 and F_2] were obtained and evaluated in the first trial (Trial #1) located in Brasília-DF (Brazil) during the fall of 2015. Seventeen F_3 families were obtained via self-pollination from selected individual F_2 plants displaying different levels of symptom expression. These F_3 families (with 10 plants each) were evaluated together with the parental ‘LT05’ and ‘TO-937’ in a second trial (Trial #2) located in Salto (Uruguay) during the fall of 2016.

Trials development – For Trial #1, four adult scarlet eggplants (*S. aethiopicum* var. *gilo*) showing typical foliar symptoms (Fonseca et al., 2016) were used as the original ToCV source. The presence of systemic ToCV infection in the scarlet eggplants was confirmed by RT-PCR. A virus-free colony of *Bemisia tabaci* MEAM1 was multiplied on cabbage (*Brassica oleracea*) plants and then placed in screen-cages

together with the infected scarlet eggplants for two days. The viruliferous whiteflies were transfered to other screen-cages along with the tomato seedlings in a two-true-leaves stage. For six days the levels of whiteflies were increased to reach an average of 40 adult whiteflies per plant. The plants together with the flies were kept for others seven days inside the cage with at least two daily manual movements of the leaves aiming to promote both the mobility of the vector and uniform ToCV inoculation. The transplant was performed under a screenhouse in a four-true-leave stage on single 5L-pots filled with sterile substrate. As growing media, a mixture of commercial peat and soil were used in equal parts. Twenty plants of 'LT05', 20 of 'TO-937', 20 of the F_1 generation, 36 of BC to LT05, 33 of BC to TO-937 and 170 of F_2 generation were transplanted. The plants were conducted with one stem and during their development no insecticide applications were carried out aiming to promote a high *B. tabaci* MEAM1 population and efficient ToCV spread across the plants under evaluation. Plats were evaluated for leaf green color intensity at 60 days after planting (DAP). At 120 DAP plants were evaluated for ToCV symptom expression. During this evaluation, sample of five 'LT05', five TO-937' plants and five F_1 hybrid plants as well as 15 categorized F_2 plants (*viz*. five tolerant, five intermediate, and five sensitive plants) were selected in order to analyze the systemic invasion of ToCV by tissue-blot hybridization.

Trial #2 was conducted under plastic greenhouse and natural infection conditions in Salto (Uruguay) in a region where ToCV outbreaks were endemic (Arruabarrena et al. 2014, 2015). Six F_2 plants of Trial #1 were chosen within each of three assigned categories: (1) high intensity of leaf green color / low level of ToCV symptoms expression, (2) medium intensity of leaf green color / medium level of ToCV symptoms expression, (3) low intensity of leaf green color / high level of ToCV symptom expression. Then, a total of 18 F_3 families (10 plants each) were used together with ten plants of the contrasting parents ('LT05' and 'TO-937'). The plants were cultivated directly on the soil and were conducted with one stem. No insecticide applications were carried out aiming to promote a high *T. vaporariorum* population and efficient ToCV spread across the plants under evaluation. At 60 DAP plants were evaluated for leaf green color intensity. At 120 DAP, plants were evaluated for ToCV

symptom expression. In this same evaluation, all plants of 'LT05', 'TO-937' and eight F_3 families were analyzed for ToCV systemic presence by tissue-blot hybridization.

Evaluation of leaf green color intensity – The leaf green color intensity was measured using a portable chlorophyll meter SPAD-502 (Minolta Corporation, Ltd., Osaka, Japan). The measurement was performed on individual plants. For each plant, four SPAD values (SPAD-units) were taken using as target the four terminal leaflets of the first fully developed leaf counting from the apex. For the non-segregating populations ('LT05', 'TO-937' and F_1) a total of 20 plants were measured on Trial #1 and ten plants for Trail #2, from which the average and the confidence interval (95%) were calculated for each genotype. These data were used to elaborate a leaf green intensity scale (low, medium, high) with which the plants of the segregant populations (BC-LT05, BC-TO-937, F_2 and F_3 progenies) were characterized. For each plant of the segregating populations, four SPAD values were also obtained from which the mean and the standard error were calculated. From that value, each plant was assigned in one of the categories described above.

Evaluation of ToCV symptom severity – The following visual scale developed by González-Arcos et al. (2018) was used: 0 = no visible symptoms, 1 = intervenial chlorosis in basal leaves, 2 = intervenial chlorosis in basal and middle leaves, presence of some necrotic spots, 3 = intervenial chlorosis and yellowing generalized, with presence of necrotic spots. To facilitate the evaluation, intermediate values were used in some cases. To evaluate the segregant populations, a category of symptom expression level was arbitrarily assigned to each plant with the following phenotypic classes: from 0 to 1.5 = low; 2 = medium; 2.5 to 3 = high.

ToCV detection via reverse transcription polymerase chain reaction (RT-PCR) – Total plant RNA was extracted from foliar tissue with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Total RNA was diluted in 50 µL of DEPC-treated water. Virus detection by RT-PCR was carried out essentially as described by Dovas et al. (2002). One step RT-PCR was performed

using the HS-11/HS-12 primer pair, which amplifies a fragment of ≈ 587 bp corresponding to the heat shock protein (HSP-70) gene homolog. This genomic region is highly conserved across *Crinivirus* species. RT-PCR reaction was performed using the following conditions: a first step at 42 °C for 60 minutes, second step at 50 °C for 2 minutes, third step at 94 °C for 5 minutes, 35 cycles subdivided in step #1: 30 seconds at 95°C, step #2: 30 seconds at 43 °C, and step #3: 15 seconds at 72°C, followed by a final extension step (72 °C for 2 minutes). Afterwards, ToCV infection was confirmed by assaying the RT-PCR products as templates in multiplex nested-PCR with the TIC-3/TIC-4 and ToC-5/ToC-6 primer pairs (Dovas et al. 2002). These assays allowed the identification of TICV-specific (≈ 263 bp) and ToCV-specific (≈ 463 bp) amplicons. Nested RT-PCR assays were performed using the following conditions: a first denaturizing step at 95 °C for 1 minute, 40 cycles subdivided in 20 seconds at 95 °C, 15 seconds at 60 °C, and 10 seconds at 72 °C, followed by one final extension step (72 °C for 2 minutes). The final RT-PCR products were separated by 1.5% agarose gel electrophoresis in 0.1X TBE buffer and stained with ethidium bromide to determine presence/absence of the target DNA. Direct Sanger sequencing was carried out (at the Genomic Analysis Lab, CNPH, Brasília–DF, Brazil) with a subset of gel-purified PCR amplicons (PureLink PCR micro kit®; Invitrogen, Carlsbad, CA, USA) in order to confirm origin of the putative ToCV-specific bands.

ToCV detection by tissue-blot hybridization – Hybridization assays (with a ToCV RNA-specific probe) were performed using the methodology elaborated by García-Cano et al. (2006). Tissue-print hybridization was implemented from fresh petiole sections of basal, middle, and apical leaves of each plant, which were directly squash-blotted on the membrane. The membranes with RNA drop or tissue prints were oven-fixed at 80 °C for 2 hours and then hybridized with a ToCV RNA specific probe containing a fragment of the ToCV coat protein gene. The probe was synthesized from total RNA using M380/M381 ToCV-specific primers (Fortes et al. 2012), which amplify a fragment of 436 bp. The fragment was cloned in pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA). To generate the antisense RNA probe labeled with dioxigenin-11-UTP, the linearized plasmid (containing the 436 bp

amplicon) was subjected to in vitro transcription with T7 RNA polymerase. Chemiluminescent detection was done with CDP-Star Detection Reagent (Roche Diagnostics GmbH, Mannheim, Germany). The probe synthesis, hybridization and chemiluminescent detection were performed using the DIG-labeling and detection kit (Roche Diagnostics GmbH, Mannheim, Germany) and following the protocols supplied by the manufacturer. Development of the chemiluminescent reaction and analysis of blots were performed by a ChemiDoc XRS+ System (Bio-Rad; Hercules, CA, USA).

Identification of molecular markers linked to the tolerance trait – Bulked Segregant Analysis (BSA) methodology (Michelmore et al. 1991; Tanksley et al. 1995) was used for the initial search for molecular markers linked to the ToCV-tolerance trait. BSA allows the rapid identification of molecular markers linked to a specific gene or region. For that, two DNA samples (bulks) of the individuals from the F_2 segregating population were used. Each bulk was composed by an equimolar amount of DNA from five F_2 individuals sharing the phenotype of interest: tolerant to ToCV-induced symptom development (bulk tolerant) and sensitivity to ToCV (bulk sensitive). Both bulks were screened along with the contrasting parents 'LT05' and 'TO-937' for a set of 400 RAPD primers (Operon Technologies Inc., Alameda, California, USA). The polymorphic markers found were validated employing individuals of the F_2 population to confirm the linkage with the characteristic of interest (see the stragegy for BSA application in Boiteux et al. 2000). For DNA extraction, fresh leaf samples were collected from the apex of each individual plant. The total DNA of each plant was extracted according to the methodology of 2X CTAB with modification implemented by Boiteux et al. (1999). The PCR reagents for the characterization with RAPD markers were placed in a final volume of 12.5 μL using 5.95 μL Milli-Q water, 1.25 μL of 10X Buffer *Taq* Polymerase (100 mM Tris- HCl, pH 8.3 and 500 mM KCl, Invitrogen), 0.6 μL of $MgCl_2$ (50 mM, Invitrogen); 0.5 μL of dNTPs (2.5 mM, Invitrogen); 2 μL of primer, 0.2 μL *Taq* DNA polymerase (Invitrogen, São Paulo, SP, Brazil), and 2 μL of DNA template. The amplification conditions were the following: an initial stage of denaturation at 94 °C for 2 minutes,

followed by 35 cycles of denaturation at 94 °C for 30 seconds, anneling at 36 °C for 1 minute and extension at 72 °C for 1.5 minutes and a final extension step at 68 °C for 10 minutes. For the generated amplicon visualization, 5 µL of bromophenol blue + glycerol was added to each sample and then applied on an agarose gel (1.5%) containing 1.5% of ethidium bromide. The DNA fragments were separated by electrophoresis in TBE buffer (45 mM Tris-Borate and 1 mM EDTA) at 120 v for a period of 2.5 to 4 hours. The gels were photodocumented on ultraviolet light.

Data analysis - A Chi-squared test was applied to: **(a)** fit the segregation ratios of the F_2 population with Mendelian segregation models, and **(b)** to confirm the linkage between the tolerant trait and the molecular markers, searching for a statistical difference from a 3:1 segregation ratio (independent) in a sub-groups of tolerant and sensitive F_2 genotypes. In all cases a probability level (P-value) is given as the value for the null hypothesis.

3.4. RESULTS

Green leaf color intensity and ToCV symptoms expression (Trial #1) – The screenhouse used for Trial #1 was soon populated with whiteflies *B. tabaci* MEAM1, which contributed to an efficient ToCV dispersion among different individuals as indicated by the levels of infection in the susceptible control plants. Up to 60 DAP, typical ToCV symptoms of chlorosis, yellowing and anthocyanin accumulation areas were not yet apparent. However, differences in leaf green color intensity among plants and populations could be visualized (Fig 1a). At 60 DAP, a measurement of the green leaf color intensity was carried out using a portable chlorophyll meter (SPAD). As shown in Fig 2a, the two parents were contrasting: 'LT05' displayed higher green leaf color intensity (higher average SPAD units) whereas 'TO-937' displayed low green leaf color intensity (lower average SPAD units). The F_1 hybrid plants displayed intermediate green leaf color intensity in relation to the parental lines. The variation

among the 20 plants evaluated was very low, with CV (%) ranges of 4.5, 6.6, and 4.7 for ‘LT05’, ‘TO-937’ and F_1 plants, respectively.

At 120 DAP, chemiluminescent detection showed uniform and strong ToCV signals for all evaluated plants, confirming the uniform infection levels and systemic invasion of the virus (Fig 1b). At 120 DAP, plants were also evaluated for ToCV symptom severity using a grading system. Again, the parental genotypes displayed contrasting reactions, with ‘LT05’ displaying very incipient yellowing symptoms restricted to the basal leaves (average severity index of 1.2), while ‘TO-937’ exhibit symptoms of generalized yellowing and chlorosis as well as areas of anthocyanin accumulation in the leaves (average severity index of 2.93). The F_1 hybrid plants showed a reaction much closer to ‘TO-937’ although somewhat less intense (average severity index of 2.45) (Fig 1c, Fig 2b).

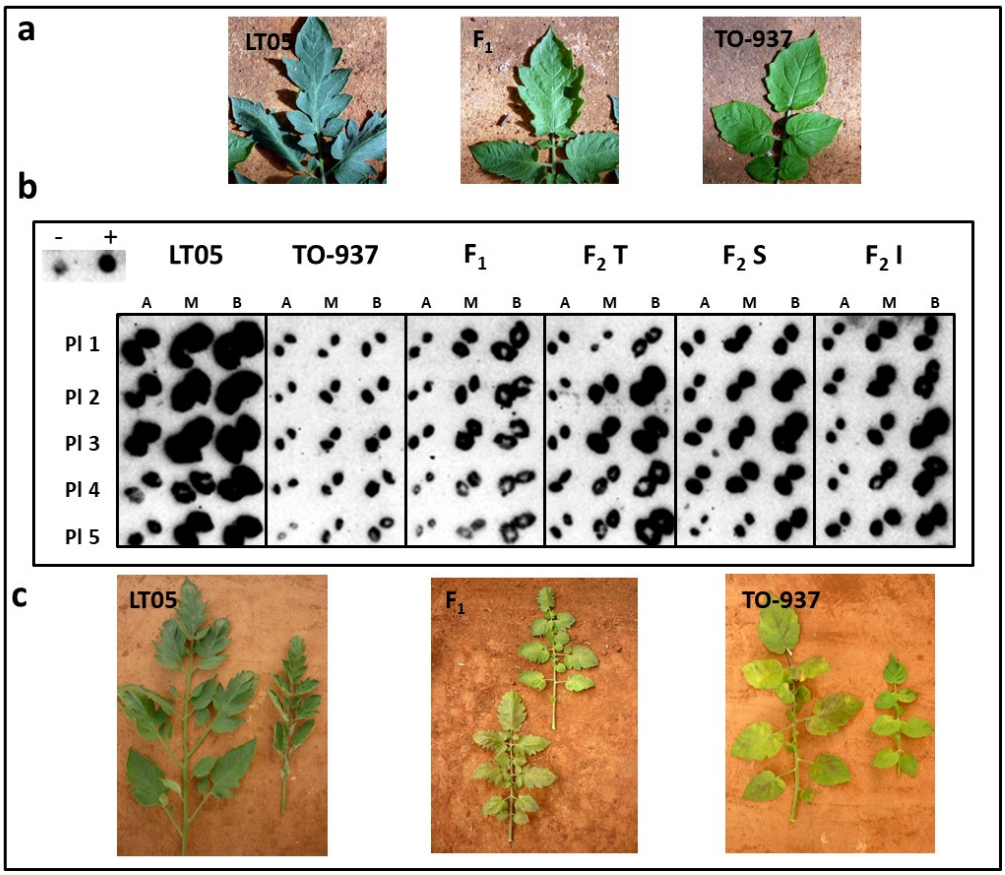

Fig 1 (**a)** Differences in intensity of green color in terminal leaflets of the completely developed apical leaf at 60 days afer planting – DAP. (**b)** Autoradiograph for ToCV hybridization results of positively charged nylon membranes with squash blots of leaf petiole cross sections (two squash blots per leaf petiole) performed at 120 DAS in five platns per genotype (15 in F_2 population) and distinct leaf positions (A = apical, M = middle and B = basal), with plant number indicated at the left of the figure. (**c)** Differences in severity of ToCV symptoms expressed in leaves of representative plants of the parental lines 'LT05', 'TO-937' and the resulting F_1 generation.

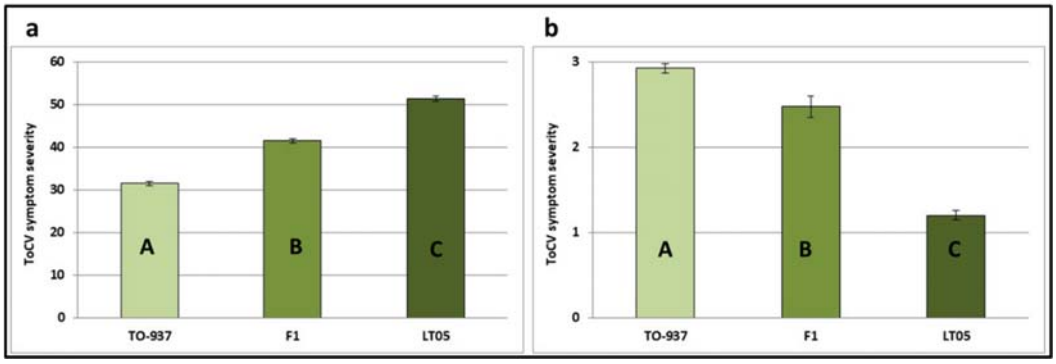

Fig 2 (a) Average SPAD values at 60 days after sowing. Different letters indicate significant statistical differences using LSD-Fisher ($p<0.0001$). **(B)** Average severity grade of *Tomato chlorosis virus* (ToCV)-induced symptoms at 120 days after sowing. Data obtained for non-segregating populations ('LT05', 'TO-937' and the F_1). Different letters indicate significant statistical differences using LSD-Fisher ($p<0.0001$).

The segregant populations BC to 'LT05' (BCP1), BC to 'TO-937' (BCP2) and F_2 were evaluated using an ordinal scale conversion of three values, with which the individuals of each population were located according to their leaf green color intensity at 60 DAP and their ToCV symptoms expression level at 120 DAP (Fig 3).The leaf green color intensity trait at 60 DAP fit to the segregating ratio of 1:1, 1:1 and 1:2:1, for BCP1, BCP2 and F_2 with X^2 of 0.44; 0.03 and 0.86, respectively. This indicates a strong fit for a single semi-dominant gene.

For ToCV symptom expression level, all three populations showed high association between individuals evaluated with intense green color and then presented low level of symptom expression. In the case of BCP1 population, this association was maintained for low green intensity and high symptom expression level, medium green intensity and medium symptom expression level. However, for the BCP2 and F_2 populations, the association between these two levels for both variables was not clear. Assuming that, there is an overlap between low and medium levels of symptom expression. This putative overlap is also the major explanation for the smaller

difference of symptom expression observed between the F_1 hybrid and the sensitive parental line 'TO-937'. Therefore, the observed segregation fit better to a recessive gene model, adjusting for ratios of 1:1 (X^2=2.68), 1:0 and 3:1 (X^2=0.20), for the populations BC_1, BC_2 and F_2, respectively.

To verify that the variables leaf green color intensity and symptom expression level are dependent, a contingency table analysis was performed using the 170 individuals of the F_2 population and their three-level ordinal scale category for each variable. The dependence of the variables was found to be highly significant ($p < 0.0001$), confirming that symptom expression level is related to the leaf green color intensity. We tentatively name this locus as *cvt* (= *crinivirus tolerance*).

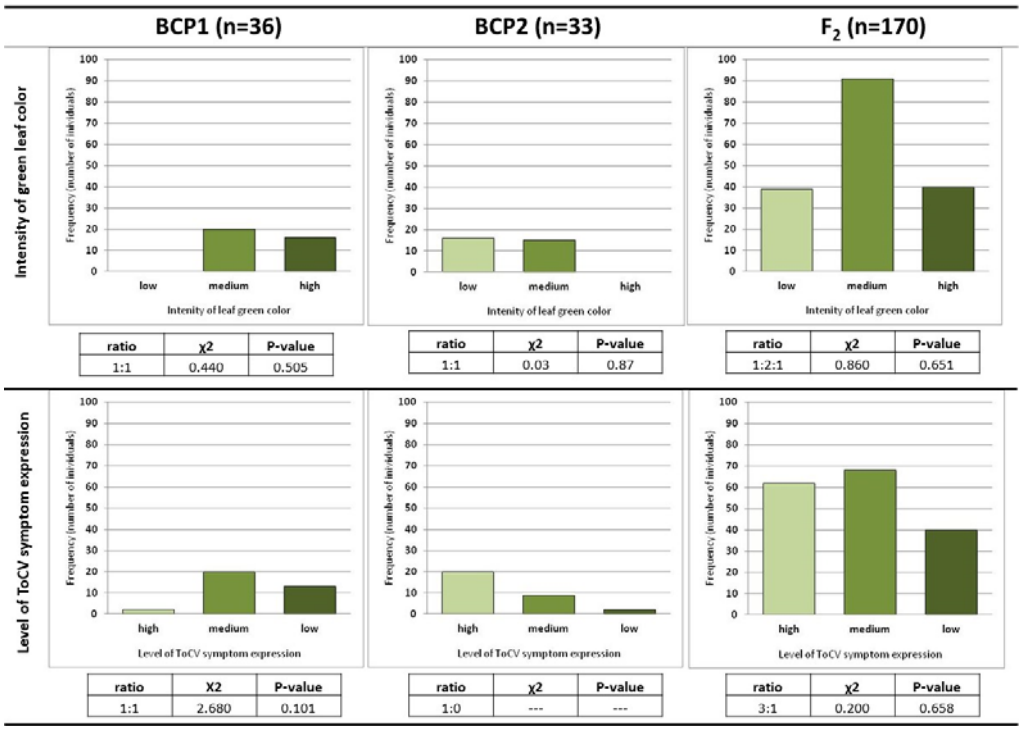

Fig 3 Frequencies for each phenotypic class in leaf green color intensity and ToCV symptom expresion level in the segregating populations BC_1, BC_2 and F_2.

F_2 progeny test (Trial #2) – Table 1 shows the results of the evaluation of leaf green color intensity and expression of ToCV symptoms. The parental lines were again

in sharp contrast for both traits (leaf green color intensity and for subsequent evaluation in ToCV symptom expression level). At 60 DAP, plants were healthy and vigorous, with no clear symptoms of ToCV infection. The results for leaf green color intensity were not clear. F_3 families derived from F_2 plants with high green leaf color intensity showed individuals of medium and high green leaf color intensity. The F_3 families derived from F_2 plants with low green color intensity segregate for low, medium and in same cases (F_3-99 and F_3-100) high green color intensity. At 120 DAP, all plants tested showed strong hybridization signals, confirming the systemic presence of ToCV and its uniform distribution within and among plants (Fig 4a). All sensitive control plants of 'TO-937' showed severe symptoms of yellowing, chlorosis, and anthocyanin accumulation areas. Within the F_3 families, the level of ToCV symptom expression was in the expected range for the five families derived from F_2 plants with high intensity of leaf green color and low expression of ToCV symptoms: F_3-93, F_3-94, F_3-95, F_3-97 and F_3-98. Individuals were classified in the group of low ToCV symptom expression (within the range of 0 to 1.5 in our scale). Only four out of 47 individuals displayed intermediate symptom expression (scale level = 2). For the six families derived from F_2 plants with low green color intensity and high symptom expression level, families F_3-99, F_3-100 and F_3-102 did not segregate, displaying only plants with severe symptom expression. Only three plants of the F_3-102 family showed a medium level of symptom expression. On the other hand, families F_3-101, F_3-103 and F_3-104 showed a clear segregation, with plants being distributed in different categories of ToCV symptom expression level. Finally, for the six F_3 families derived from F_2 plants with medium green leaf color intensity and medium ToCV symptom expression level, all plants segregated in the different categories except for the F_3-105 family that displayed nine out of 10 individuals in severe levels of symptom expression. This segregation pattern within each sub-group of F_3 families was in agreement with a recessive inheritance model for the ToCV-tolerance factor. Fig 4b shows the appearance of selected medium leaves representative of the contrasting parents ('LT05' and 'TO-937') and the same selected F_3 families.

Table 1 Distribution of plants within contrast parents and 17 F_3 families in three categories of leaf green color intensity and *Tomato chlorosis virus* (ToCV)-induced symptoms.

	Original F_2 phenotype level		Numbers of plants with:					
			Leaf green intensity at 60 DAP			ToCV symtpoms expression level at 120 DAP		
Genotype/Family	**Leaf green color intensity**	**ToCV symtom expression level**	**low**	**medium**	**high**	**low**	**medium**	**high**
LT05		----	0	0	9	9	0	0
TO-937		----	8	2	0	0	0	10
F_3-93	high	low	0	2	7	10	0	0
F_3-94	high	low	0	6	3	9	0	0
F_3-95	high	low	0	6	4	10	0	0
F_3-97	high	low	0	1	9	6	3	0
F_3-98	high	low	0	2	8	9	1	0
F_3-99	low	high	9	1	0	0	0	10
F_3-100	low	high	6	4	0	0	0	10
F_3-101	low	high	0	0	8	3	2	3
F_3-102	low	high	0	8	1	0	3	7
F_3-103	low	high	0	3	7	3	3	4
F_3-104	low	high	0	8	0	1	3	5
F_3-105	medium	medium	5	5	0	0	1	9
F_3-106	medium	medium	2	8	0	3	7	0
F_3-107	medium	medium	2	7	0	5	1	3
F_3-108	medium	medium	1	8	1	1	4	5
F_3-109	medium	medium	3	7	0	2	4	4
F_3-110	medium	medium	6	4	0	1	5	4

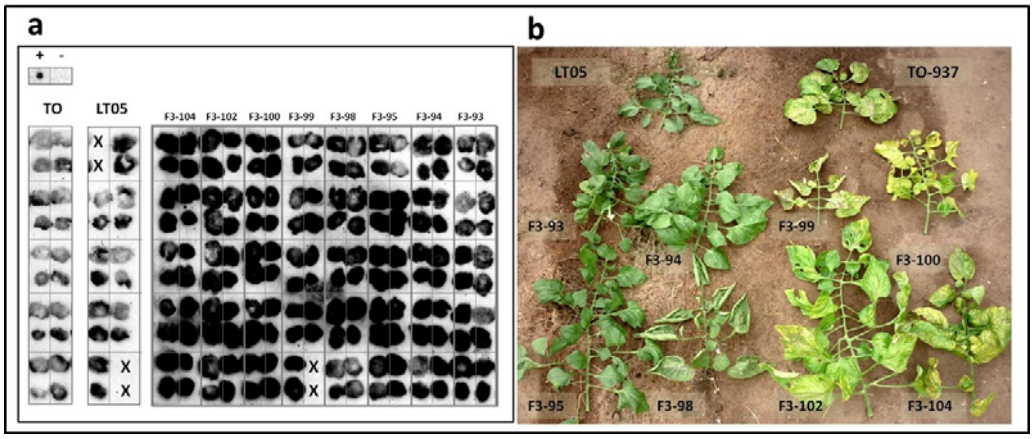

Fig 4 (a) Autoradiograph of *Tomato chlorosis virus* (ToCV) hybridization assays employing positively charged nylon membranes with squash blots of leaf petiole cross sections (two squash blots per leaf petiole) performed at 120 days after sowing (DAS) in 10 plants of 'LT05', 10 of 'TO-937' and eight selected F_3 families. **(b)** Differences in ToCV-induced symptoms expressed in middle leaves of representative plants sampled from the parental lines 'LT05', 'TO-937' as well as plants of eight selected F_3 families.

Identification of RAPD markers linked to the ToCV tolerance trait – A total of 400 RAPD primers with known sequence were tested. Twelve primers showed polymorphism in the original BSA. Six primers confirmed the presence of polymorphic amplicons in a second round and were then validated employing 11 sensitive and 11 tolerant F_2 individuals, including those individuals present in the original bulks. The primer OPO-14 generated a polymorphic amplicon of ≈ 450 bp, (derived from the tolerant parent 'LT05') linked in coupling phase with the *cvt* locus (Fig 5). This marker was named as OPO14-450. To confirm the linkage between OPO14-450 and *cvt*, a subgroup of 39 tolerant and 42 sensitives individuals from the F_2 population were analyzed. Since *cvt* is a recessive allele, it would be expected to find the presence of OPO14-450 in the tolerant subgroup. For the subgroup of sensitive genotypes, given the dominant nature of RAPD it would be expected to find some

individuals with presence of the marker, but never in a typical 3:1 proportion of an independent dominant trait.

Therefore, the observed frequencies for each sub-sample of sensitive and tolerant F_2 individuals were compared with frequencies expected for a 3:1 ratio (Table 2).

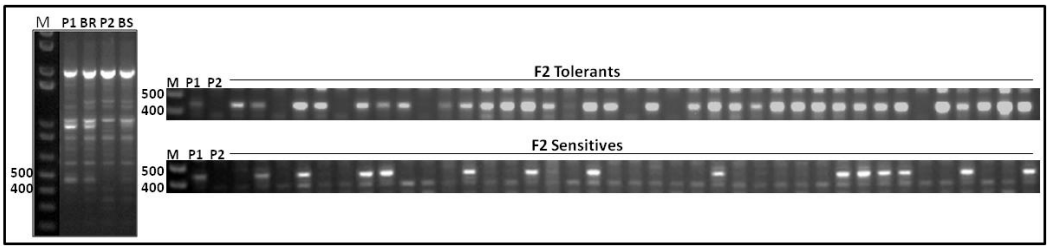

Fig 5 Agarose gels (1.5%) used for the detection of polymorphic marker OPO14-450 in the original bulked segregant analysis (BSA) composed by 'LT05' (P_1), 'TO-937' (P_2), bulk of resistant plants – BR (an equimolar DNA mixture of five individuals F_2 with tolerant phenotype) and bulk of susceptible plants – BS (an equimolar DNA mix of five F_2 individuals with sensitive phenotypes) and detection of marker OPO14-450 in a set of tolerant and sensitives F_2 individuals.

Table 2 Expected (assuming independence) and observed frequencies for segregation of the dominant RAPD marker OPO14-450 in 160 categorized F_2 individuals for ToCV tolerance.

	F_2 individuals			
	Sensitive: n=42		**Tolerant: n=39**	
	marker present	**marker absent**	**marker present**	**marker absent**
Expected (3:1)	31.5	10.5	29.25	9.75
Observed	15	27	33	6
X^2 (P-value)	34.57 (<0.00001)		1.92 (0.166)	

The analysis showed that the X^2 value for a 3:1 ratio in the resistant (n = 39) subgroup was high, fitting better for proportions of 6:1 (X^2 =0.04). Therefore, in these subgroups of F_2 genotypes there is a tendency to present higher proportions than expected in a condition of independence of the traits (3:1). This was confirmed by observing the subgroup of sensitive genotypes (n = 42), where the absence of the marker was not in agreement to a condition of independence, adjusting better for a ratio of 1:2 (X^2 =0.11) So, it is assumed that the marker OPO14-450 is linked in coupling phase with the locus *cvt*, both positioned on the same chromosome.

3.5. DISCUSSION

The first objective of the present work was to characterize the genetic basis of the low levels of ToCV-induced symptom expression of the *S. lycopersicum* 'LT05' inbred line observed in several trials in different locations in South America (González-Arcos et al. 2018). The reaction of the line *S. lycopersicum* 'LT05' is characterized by mild disease symptoms even though the pathogen (ToCV) is able to multiply withing the host cell and be detected systemically. This condition is classified by Cooper and Jones (1983) as a typical tolerant reaction. In assays carried out by our group it was confirmed that 'LT05' plants display low level ToCV-induced symptoms even displaying systemic presence of ToCV as indicated by strong hybridization signals. Therefore, it was not possible to relate this tolerance with lower viral titers (according to an indirect estimation measure like blot-hybridization) or with some mechanism of interference with the vector (González-Arcos et al. 2018). In this context, our working hypothesis was that tolerant plants do not show symptoms due to attributes related to the maintenance of the overall chlorophyll content, which can be measured by the intensity of green leaf color, a striking feature of the 'LT05' acession (Fig 1a).

Our results (Fig 2 and 3) showed that the level of ToCV symptom expression (measured at 120 DAP) can be explained by a single recessive gene, which we tentatively name *cvt* (= *crinivirus tolerance*; being cvt^+ the wild type allele). This recessive inheritance is clearly observed in Fig 2b where the levels of symptom

expression of 'TO-937' and the F_1 are very close, with an average of 2.93 and 2.48 respectively. To confirm this, some F_3 families from different F_2 plants representative of each category were evaluated (Table 1 and Fig 4). Segregation for the levels of symptom expression was as expected. Plants F_2 with low expression of symptoms (i.e. homozygous recessive *cvt*/*cvt*) generated stable progenies (without segregation) with low level of symptoms expression. On the other hand, F_2 plants with high symptoms expression (cvt^+/cvt^+ or cvt/cvt^+) showed stable progenies (no segregation) with high symptoms expression or segregating for different categories. Finally, F_2 plants with medium level of symptoms expression (cvt/cvt^+ or cvt^+/cvt^+) displayed segregating progenies for all three categories ranging from stable (= no segregation) to severe ToCV-induced symptom expression. Thus, these results are in agreement with the hypothesis of a single recessive gene controlling the ToCV tolerance observed in the 'LT05' line.

In relation to the leaf green intensity trait (measured at 60 DAP), the results of the first trial fit a model of a gene with incomplete dominance. The F_1 reaction was classified as an intermediate phenotypic value between the two contrating parents (Fig 2A and Fig 3). To demonstrate that the effect on leaf green color intensity and ToCV symptoms expression is determined by the same *cvt* gene, we performed a contingency table analysis for 170 individuals of the F_2 family, confirming a highly significant relationship between both variables. However, in the F_2 progeny test, neither the model of a gene with partial dominance nor the relationship between the two variables was clear. As can be seen in Table 1, plants with high level intensity of leaf green color at 60 DAP may derive in high and medium level of symptoms expression at 120 DAP, as well as medium intensity of green leaf color can derivate in high, medium, or low level of symptoms expression. This result generated the hypothesis that, in fact, the factor that is related to the expression of ToCV symptoms is not chlorophyll content (measured as green intensity at 60 DAP in Trial#1), but chlorophyll degradation capacity. Therefore, plants that initially have a more intense green color could degrade more chlorophyll under stress than plants of a less intense green. This hypothesis may also explain the adjustment of this variable in Trial#1, both for the model of single semi-dominant gene and for its relationship with the variable symptoms expression

level. Probably, the leaf green color intensity measured at 60 DAP was performed under overall mild environmental conditions (e.g. mild cold and nutritional stress) or for the yet incipient expression of ToCV symptoms. With moderate stress levels, the effect of *cvt* can be classified as semi-dominant. However, with stronger levels of environmental stress, the effect is more adequately classified as recessive (i.e. two copies of the *cvt* allele are necessary to delay the degradation of chlorophyll and show reduce ToCV symptoms expression at 120 DAP). Therefore, our hypothesis is that the plants of the first trial, at the time of measuring the intensity of the leaf green color (60 DAP), already had symptoms of chlorophyll degradation, which was not the generalized situation for the second experiment employing F_3 families.

In cucurbits, sources of resistance against distinct criniviruses have been identified. In general, major genes contributing in symptoms attenuation to crinivirus in cucurbits are associated either with lower virus replication rates within the host plant tissues or with antioxenotic factors affecting the vector efficiency in colonizing its host (López-Sesé et al. 2000, McCreight1 2000, Marco et al. 2003, Aguilar et al. 2006, McCreight1 and Wintermantel 2011). Complete (i.e immune-like) resistance to crinivirus in cucumber and melon is uncommon. In tomato germplasm, the only study related to the genetic basis of ToCV resistance was carried out with the inbred line '821-13-1' (derived from the accession *S. chmielewskii* LA 1028) (Garcia-Cano et al. 2010). The line '821-13-1' exhibited significant tolerance to disease symptoms development. This tolerant reaction was stable even after challenging plants with very high virus pressure. Nevertheless, ToCV could be detected systemically infecting plants. A quantitative genetic analysis suggested an oligogenic resistance to ToCV in '821-13-1', determined by a major locus with partial dominance (the F_1 between a susceptible line displayed an intermediate resistance) and modified by epistatic additive × dominance interactions with other minor genes.

Another objective was to generate a preliminary molecular information related to the genetic factor. Despite the low number of individuals analyzed, it was possible to identify the RAPD marker OPO14-450 linked in coupling phase with the tolerance factor (Fig. 5, Table 2). In each of the phenotypic subgroups analyzed of F_2 individuals (resistant and sensitive), strong deviations were observed regarding the independent

segregation of a dominant marker (3:1). Therefore, it is possible to affirm that the OPO14-450 marker and the genetic factor are in the same chromosome. Based on that, it should be possible to clone and sequence the amplicon, and then to locate it on the tomato physical map. Strategies based on the identification of RAPD markers by bulked-segregant analyses to generate associated genomic information (related to amplicon sequence) have been used at different times to locate different traits in tomato genetic and physical maps (Chagué et al., 1996; De Giovanni et al., 2004; Truong et al., 2015). Since the low number of individuals analyzed and the condition of a dominant marker linked to a recesive trait, it was not possible to calculate accurately the number of recombinants and, therefore, the genetic distance between traits. In any case, knowledge of the chromosome where the factor is positioned is a very useful element to restrict options in a comparative genomic study within a candidate gene approach strategy (see application in González-Arcos et al., 2019). If necessary, with the available genomic information, it would be possible to design a new set of molecular markers to carry out a fine mapping and thus discard nearby candidate genes by location.

Complementaryly, any available information about the function of the protein encoding the candidate gene would be useful. In this way, our results indicated that 'LT05' plants are systemically infected by ToCV. However, the tolerance determined by the *cvt* locus on the 'LT05' line acts to attenuate visible symptoms of ToCV, associated with loss of photosynthetic capacity due to yellowing, chlorosis and necrotic spots. González-Arcos et al. (2018) observed similar reaction with commercial cultivars harboring the homozygous *green-flesh* mutation (*gf*) (Kerr 1956), located in the long arm of the tomato chromosome 8 (Kerr 1957). The *gf* locus codes for the STAY-GREEN (SGR) protein, which controls the inhibition of chlorophyll degradation (Akhtar et al. 1999).

In the STAY-GREEN phenotypes, the operative mechanisms are related to either the delay of chlorophyll degradation by modification in catabolism/synthesis of pigments or by slowing down the entire senescence process (Thomas and Howarth 2000). Similar phenotypes have been selected during the last decades in crops such as sorghum, corn, wheat, barley, and cowpea (Thomas and Ougham 2014). The selection

of STAY-GREEN phenotypes has been carried out aiming to develop lines with improved levels of adaptation to a wide range of environmental stresses related to temperature and drought (Vijayalakshmi et al 2010; Jordan et al., 2012). Kusaba et al. 2013 summarizes several molecular mechanisms related to stay-green phenotypes. Mutations in genes that encode chlorophyll-related proteins result in different types of STAY-GREEN phenotypes. In addition, hormones such as cytokinin, ethylene, auxin, abscisic acid, jasmonic acid, brassinosteroids, and strigolactones are related to the senescence process in plants and in many cases are responsible for STAY-GREEN functional phenotypes. Some families of transcription factors have several senescence-inducible members. This is the case of the NAC family (Balazadeh et al 2011) and WORKY (Zentgraf et al., 2010) which, due to both negative and positive regulation, can participate in the plant senescence process and be directly responsible for STAY-GREEN phenotypes.

A recent study at the transcriptome level relate the tomato symptoms induced by ToCV showed the positive or negative regulation of several genes related to the senescence process, including transcription factors such as NAC, WORKY, bHLH, BAG as well as the regulation of different hormonal processes and programmed cell death (Seo et al. 2018). Therefore, we speculate that the *cvt* locus might be involved with some molecular process that leads to a lower levels of chlorophyll degradation and, therefore, a lower expression of the ToCV-associated symptomatology. This does not ensure that the virus effects in other non-visible symptoms, such as fruit yield or internal fruit quality, are also being diminished. Likewise, this kind of tolerance may not avoid synergistic interactions of ToCV with other viruses (e. g. TSWV) in mixed host cell infections (García-Cano et al. 2006).

Finally, identifying the *cvt* locus could help to determine the molecular basis of symptom attenuation but, in addition, our interest will focus on the factor that determine the sensitivity. Our study employed *S. pimpinellifolium* 'TO-937', which shows an extreme sensitivity to ToCV (González-Arcos et al. 2018; Garcia-Cano 2010). In this context, it will also be useful to study the genetic determination of this high ToCV sensitivity trait and its possible association with the very sensitive reactions previously identified in several commercial cultivars (González-Arcos et al. 2018,

Mansilla-Córdova et al. 2018). This sensitive related factor could explain the worldwide emergence and the recent outbreaks of ToCV across tomato-producing of Europe and Americas (Navas-Castillo et al. 2014).

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4. PHENOTYPIC EXPRESSION, INHERITANCE, AND CHROMOSOME LOCATION OF *mtz*: A RECESSIVE LOCUS THAT CONFERS HIGH-LEVELS OF METRIBUZIN TOLERANCE IN TOMATO

4.1. ABSTRACT

Metribuzin is a largely used herbicide in fresh-market and processing tomato (*Solanum lycopersicum* L.) crops conducted under open field conditions. Tomato growers usually apply metribuzin in pre-transplant or at early developmental crop stages. However, even at recommended doses and application times, many tomato cultivars may display (especially at seedling stage) some levels of toxicity to metribuzin. This metribuzin toxicity may be intensified in sandy soils as well as in rainy days and under sprinkler irrigation when water may wash of the herbicide from the foliage and the excess could be absorbed by the root system. In this context, the deployment of tomato cultivars with naturally higher levels of metribuzin tolerance could facilitate weed management strategies. In the present work, we use the inbred line 'UGA1113-MT (= 'CNPH-0498') that was able to tolerate up to 2.88 Kg.ha-1 of metribuzin via foliar spraying at four-true-leave stage without showing any conspicuous damage in its vegetative development or in yield. Inheritance studies were carried out using populations derived from controlled crosses between two contrasting parental lines: 'Viradoro' (highly sensitive to metribuzin) and 'CNPH-0498'. The reciprocal F_1 hybrids, the segregating F_2 population as well as F_2:F_3 families were obtained. Twenty days after planting, the commercial product Sencor® was sprayed at the dose of 2 L.ha-1 (0.96 kg.ha-1 of metribuzin) under greenhouse conditions. The F_1 generation was found to be as sensitive as 'Viradoro'. The F_2 generation (n = 515 plants) showed a segregation pattern of 126 tolerant to 389 sensitive individuals, indicating that a single nuclear recessive gene is controlling this trait. We tentatively named this locus as *mtz*. A linked molecular marker in association with *mtz* locus was identified via bulked segregant analysis. The sequence of this polymorphic amplicon allowed its location on tomato physic map. This preliminary information could be useful for future efforts in a candidate gene approach strategy for the *mtz* locus.

Key words: herbicide, tolerance, *Solanum lycopersicum*, candidate gene

4.2. INTRODUCTION

Metribuzin (4-amino-6-tert-butyl-3-(methylthio)-as-triazin-5(4H)-one) is a member of the herbicide family known as 'Triazonas', which are potent photosynthesis inhibitors due to their ability to disrupt the electron flux in photosystem II, the first protein complex in the light-dependent oxygenic photosynthesis (Roberts and Hutson 1999). Metribuzin is a hydrophilic compound that is assimilated by either plant root systems through diffusion from the soil or by the foliage via cuticles (Roberts and Hutson 1999). The systemic movement of the metribuzin in the plant is done via xylem. Once reaching the chloroplasts, metribuzin is coupled to the plastoquinone QB binding site in D1 protein, preventing the normal electron transport from plastoquinone QA to plastoquinone QB in portein D2 (Senseman and Armbrust 2007).

The site of metribuzin action is shared with other groups of herbicides such as phenylcarbamates, pyridazinones, triazines, uracils, benzothiadiazinones, nitriles, phenylpyridazines, amides, and ureas (Senseman and Armbrust 2007). The difference in the mechanism of action of these groups of herbicides is their specific binding site in D1 protein, resulting in the obstruction of the plastoquinone QB coupling. The disruption of the electron flow causes an accumulation of simple oxygen and the consequent destruction of chlorophyll and lipids due to oxidative stress (Preston and Mallory-Smith 2001, Vencill et al. 2012). Symptoms induced by metribuzin in sensitive plants include chlorosis followed by necrosis of leaves. In foliar applications, lipid membrane peroxidation occurs so rapidly that sometimes the symptoms evolve directly to necrosis.

Metribuzin is employed as a pre-emergence and post-emergence herbicide in a wide array of crops including soybean [*Glycine max* (L.) Merr.] (Tuti and Das 2011); wheat (*Triticum aestivum* L.) (Zand et al. 2011); potato (*Solanum tuberosum* L.) (Mukherjee et al. 2012); carrot (*Dacus carota* L.) (Sasnauskas et al. 2010), and sweet potato (*Ipomoea batatas* L. Lam.) (Harrison and Dukes 1996). In tomatoes (*Solanum lycopersicum* L.), metribuzin is largely used for both fresh-market and processing crops conducted under field conditions (Stephenson et al. 1976, Morse 1999, McNaughton 2013). Tomato growers usually apply a pre-transplant dose combined

with s-metolachlor ranging from 0.288 to 0.480 kg.ha^{-1}. Two weeks after seedling transplant, when some weeds start emerging in the field, an additional application is often made at doses ranging from 0.192 to 0.384 kg.ha^{-1}, depending on the predominant weed species as well as in the weed population density (Correia 2015).

At recommended doses and application times, a wide range of tomato cultivars display relative selectivity to metribuzin. However, many tomato cultivars may display some levels of toxicity to metribuzin (Phatak and Collin 1970, Fortino and Splittstoesser 1974, Stephenson et al. 1976, Gawronski 1983). In addition, under some environmental conditions, sensitivity could manifest even at the recommended doses. The tomato crop is more sensitive to metribuzin at early stages of plant growth (Fortino and Splittstoesser 1974) and during low-light conditions (Phatak and Stephenson 1973). Metribuzin applications followed by rain or by sprinkler and center-pivot irrigation may increase toxicity since the plants could absorb via roots the excess of herbicide from the foliage. This effect is intensified in sandy soils. For these reasons, to prevent damage, growers usually choose to make several successive applications with reduced concentrations up to the full-recommended dosage. In this context, the deployment of tomato cultivars with naturally higher levels of metribuzin tolerance could facilitate weed management strategies.

Some mutations in the D1 protein can modify the binding site of plastoquinone QB, preventing the coupling of the herbicide molecule without affecting the plastoquinone binding site. This is the main mechanism of site-specific resistance reported to the photosystem II inhibitor herbicides (Oettmeier 1999, Powles and Yu 2010). Protein D1 is encoded by the *psbA* chloroplast gene, so the inheritance of this type of resistance is maternal. Within this mechanism, the mutation which causes the substitution Ser-264-Gly, conferring resistance to atrazine across different plant species was particularly studied (Hirschberg and McIntosh 1983). This type of site-specific mechanism can also generate cross-resistance with other herbicides from the same chemical family (Vencill et al. 2012). Another type of resistance (non-site specific) is based on the ability of the plant to metabolize the active ingredient of the herbicide. Three enzymatic systems are involved in herbicide metabolizing processes in plants: glutathione-transferase, aryl-acylamidase and cytochrome-P450-

monoxygenases. Most examples of herbicide resistance by increased plant metabolism are explained by the enzyme cytochrome-P450-monoxygenasa (Preston and Mallory-Smith 2001). An increase in the activity of these enzymes, either by overproduction or increased catalytic capacity, results in higher levels of metabolization and thus higher levels of resistance in several plant species (Powles and Yu 2010).

In diploid potatoes the sensitivity to metribuzin is determined by a single recessive gene/locus (De Jong 1983), whereas a single dominant gene controlling resistance to other members of Triazones such as atrazine and simazine in cultivated soybean (Edwards et al. 1976), wild soybeans (Kilen and He 1992) and maize (Grogan et al. 1963) was found. The resistance to metribuzin in durum wheat cultivars has been determined by multiple genes with additive effect (Villarroya et al. 2000). In induced mutants of lupine (*Lupinus angustifolius* L.), two independent semi-dominant genes with additive effect were identified controlling metribuzin resistance (Si et al. 2011).

In tomatoes, Stephenson et al. (1976) worked with the tolerant variety 'Fireball', determining that it could tolerate twice the concentration of metribuzin to produce the same damages as in the sensitive cultivar 'H7492'. The authors demonstrated that metribuzin root absorption and translocation to leaves were equal and therefore this was ruled out as the potential resistance factor. However, at 96 hours of application, the foliar rates of metabolic detoxification were twice in the tolerant variety. In subsequent works, Souzamachado et al. (1982) determined that tolerance to metribuzin from 'Fireball' is determined by a major gene with possible effects of modifying genes. The study was done under laboratory conditions using a nutrient solution supplemented with metribuzin (Souza Machado et al. 1978). Inheritance studies were performed with data at 15 days of application, using a visual phytotoxicity scale as well as the height and dry weight of seedlings. The segregation pattern found was more likely in accordance with a recessive gene hypothesis. Smith et al. (1989) worked with the tolerant line 'UGA-1113MT' (= PI 204976 acession, derived from a cross between *S. lycopersicum* and *S. pimpinellifolium*) that could tolerate up to 16 times the recommended dose of 1.12 Kg.ha-1 (Phatak and Jaworski 1985). The tolerance to metribuzin was partly explained by an increase in the rate of herbicide metabolism by the N-glucosidic route. The main metabolite found in leaves was

glucose conjugated with metribuzin. The hybrid between tolerant and sensitive variety resulted in intermediate levels of metabolic activity. This mechanism of detoxification had already been reported in tomato by Frear et al. (1983). The increase in metabolic rate would be explained either by a higher activity of the soluble enzyme UDP-glucose: metribuzin N-glucosyltransferase, described later by Davis et al. (1991), or by an increase in the availability of its substrate (simple carbohydrates). The last mechanism would explain the observed increase in herbicide sensitivity under low-light conditions. N-glucosyl conjugates have been reported as metabolites of several herbicides in plants: chloraben, dinoben, metribuzin, picloram, propanil, and pyrazon (Lamoureux et al. 1991).

The genetic basis of 'UGA1113-MT' metribuzin tolerance (measured from a visual phytotoxicity scale) was studied by different authors via root applications. The results, even though not very accurate, showed the action of a recessive gene (Phatak and Emmatty 1987) and later the action of an incomplete recessive gene with the possible presence of modifiers (Liu and Phatak 1993). In our experimental conditions, preliminary work with the line 'UGA1113-MT' (= 'CNPH-0498') found that it is able to tolerate, by aerial spraying and in four-true-leave stage, up to 2.88 Kg.ha-1 of metribuzin, without showing any visible damage in the vegetative development or yield reduction. Even at doses of 4.88 Kg.ha-1, plant death was not observed. In the same experiment, the reference variety 'Viradoro' displayed considerable yield losses and showed phytotoxicity symptoms at doses of up to 0.12 Kg.ha-1 (Correia et al. 2018). In this context, the main objectives of the present work were to characterize the phenotypic expression of the levels of metribuzin tolerance in the inbred line 'UGA1113-MT' (= 'CNPH0498'), to study the genetic basis of this trait and to determine the chromosomal location of the tolerance factor(s) in the reference tomato genome map.

4.3. MATERIALS AND METHODS

Plant material - The tolerant tomato line 'CNPH0498' (= 'UGA1113-MT') and the susceptible open pollinated cultivar 'Viradoro' (Giordano et al. 2000) were used as contrasting genetic materials in the phenotypic evaluation to metribuzin and in the

inheritance studies. ‘Viradoro’ was used as female parent. Controlled crosses between the two contrasting materials were carried out and the F_1, F_2 and F_3 families were obtained (Table 1).

Table 1 Parental lines and segregating populations used for metribuzin tolerance studies.

Code	Description	Number of plants
P_1	‘CNPH-0498’ (tolerant)	20
P_2	‘Viradoro’ (sensible)	20
F_1	P_1 *x* P_2	20
F_2	F_1 ⊗	515
F_3	Individual F_2⊗	480[x]

[x]32 F_3 families of 15 plants each.

Evaluation of metribuzin tolerance levels – For the metribuzin tolerance evaluation, 20 plants of ‘CNPH-0498’, 20 plants of ‘Viradoro’, 20 plants of the population F_1 and 515 plants of the population F_2 were used. The populations were sown in plastic pots filled with sterile substrate. Thirty days after planting, the seedlings were transplanted to their final pots of 5 L capacity in a screenhouse. Twenty days after planting, the commercial product Sencor® was sprayed at the dose of 2 L.ha-1 (0.96 kg.ha-1 of metribuzin). At the time of spraying the plants of ‘CNPH-0498’ and ‘Viradoro’ displayed four fully-expanded leaves. Five days after spraying, the response of each plant was measured employing the following binary scale: 0 = absence of phytotoxicity symptoms and 1 = presence of phytotoxicity symptoms. The spray dose and the binary scale were adjusted according to the data obtained in a previous work conducted by Correia et al. (2018), where the dose of 0.96 kg.ha-1 of metribuzin allowed the best visual discrimination between the two contrasting tomato accessions. In order to confirm the observations 32 F_3 families of 15 plants each, derived from selfing of 16 F_2 individuals with scale = 0 (tolerant) and 16 F_2 plants with scale =1 (sensitive), were also evaluated in the same way as described above.

Identification of linked molecular markers – A strategy based upon bulked segregant analysis (Michelmore et al. 1991) was employed for the initial search for molecular markers linked to metribuzin tolerance/sensitivity. This methodology allows a rapid "chromosome landing" and subsequent identification of molecular markers linked to a specific gene or region associated with a given phenotype. For that, two DNA samples (bulks) of the seven contrasting individuals of the segregating population F_2 were used. Each bulk was constituted by an equimolar amount of DNA from seven F_2 individuals displaying the phenotype of interest: tolerance to metribuzin (= bulk tolerant) and sensitivity to materibuzin (= bulk sensitive). Both bulks were screened along with the contrasting parents for a set of 500 RAPD primers (Operon Technologies Inc., Alameda, California, USA). Then, the polymorphic markers found were validated among individuals of the F_2 population to confirm the linkage with the characteristic of interest (see BSA strategy description in Boiteux et al. 2000). For DNA extraction the leaf samples were collected from the apex of each individual plant prior to spraying the herbicide. The total DNA of each plant was extracted according to the methodology of 2X CTAB (Boiteux et al. 1999). For the characterization with RAPD markers the PCR reagents were placed in a final volume of 12.5 µL using 5.95 µL Milli-Q water, 1.25 µL of 10X Buffer *Taq* Polymerase (100 mM Tris-HCl, pH 8.3 and 500 mM KCl, 0.6 µL of $MgCl_2$ (50 mM); 0.5 µL of dNTPs (2.5 mM); 2 µL of primer, 0.2 µL *Taq* DNA polymerase (Invitrogen, São Paulo, SP), and 2 µL of DNA template. The amplification conditions were an initial stage of denaturation at 94 ° C for 2 minutes, followed by 35 cycles of: denaturation at 94 ° C for 30 seconds, anneling at 36 ° C for 1 minute and extension at 72 ° C for 1.5 minutes; ending with an extension step at 68 ° C for 10 minutes. For the visualization of generated DNA fragments, 5 µL of bromophenol blue + glycerol was added to each sample and then applied on an agarose gel (1.5%) containing 1.5% of ethidium bromide. The DNA fragments were separated by electrophoresis in TBE buffer (45 mM Tris-Borate and 1 mM EDTA) at 120V for a period of 2.5 to 4 hours. The gels were photodocumented on ultraviolet light.

RAPD marker sequencing – RAPD amplicons associated with the trait of interest were removed from the agarose gel and purified using the PureLink kit (Invitrogen- Thermo Fischer Scientific, California, USA). The fragment was cloned using TA Cloning Kit (Invitrogen- Thermo Fischer Scientific, California, USA), following manufacturer's instructions. Ten white colonies were selected and screened by colony PCR using the OP-Z11 RAPD primer. Plasmid DNA was purified from positive colonies and digested using *EcoRI* endonuclease to confirm insert size. Plasmid DNA from positive colonies was sequenced by Sanger method using M13 primers (Macrogen Inc., Seoul, Korea). Sequence quality analysis, the removal of low-quality fragments and obtaining of consensus sequences were performed using the SeqMan program (Lasergene, Madison-WI, USA).

Chromosomal location of the tolerance factor - From the obtained sequence of the linked marker, the BLAST algorithm (BLASTn tool) was used to find homologies within the tomato genome based on sequence nucleotide similarity comparison, and then, localize the tolerance factor in a specific chromosome.

Data analysis - A Chi-squared test was applied to: (a) fit the segregation ratios of the F_2 population to a Mendelian segregation model, and (b) to confirm the linkage between the metribuzin tolerance factor and the identified molecular markers, searching for a statistical difference from a 3:1 segregation ratio excpecyed forn an independent gene in a sub-group of tolerant and sensitive F_2 genotypes. In all cases a probability level (P-value) is given as the value for the null hypothesis.

4.4. RESULTS

Genetic basis of metribuzin tolerance – Under our experimental conditions, resistant individuals with scale grade = 0 (total absence of foliar phytotoxicity symptoms) and sensitive individuals with scale grade = 1 (presence of foliar phytotoxicity symptoms) were discriminated without problems. Phytotoxicity

symptoms (necrosis and chlorosis) were visible as early as the second day after application. At five days after application, symptoms displayed in Fig 1 were already conspicous. From this scale the plants belonging to the P_1, P_2, F_1 and F_2 populations were evaluated at five days after metribuzin application (Table 2). For P_1 ('Viradoro') the 20 plants evaluated were assigned to category 1, while the 20 plants evaluated from P_2 ('CNPH0498') were assigned to category 0. This highlighs the expected contrast between the parental lines. For F_1 ('Viradoro' *x* 'CNPH-0498') the 20 plants evaluated were assigned to category 1, displaying identical levels of phytotoxicity symptoms as the sensitive parent (Fig 2). This indicates a nuclear recessive inheritance of the tolerance. The evaluation of 515 individuals F_2 shows a segregation pattern for individuals with scale 0 and 1 (Figure 4) fitting a segregation ratio of 1:3 ($X^2 = 0.078$; P=0,78), indicating that tolerance is determined by a single nuclear gene with recessive inheritance (Table 2, Fig 3).

To confirm this result, 32 F_3 families of 15 plants derived from 16 F_2 individuals with scale = 0 and 16 F_2 individuals with scale = 1 were evaluated (Table 3). The 16 F_3 families from asymptomatic F_2 individuals (scale = 0) had their 15 plants with scale=0 (no segregation observed). Five families F_3 from F_2 individuals with visible symptoms of phytotoxicity (scale=1) had their 15 plants with scale 1 (no segregation observed), while the remaining 11 families from F_2 individuals with scale=1 segregating, with individuals evaluated as 0 and individuals evaluated as 1. This segregation pattern confirms the results obtained from the analysis of parental populations, F_1 and F_2 populations, reinforcing the hypothesis that the metribuzin tolerance trait is controlled by a single nuclear recessive gene which we tentatively named as *mtz*.

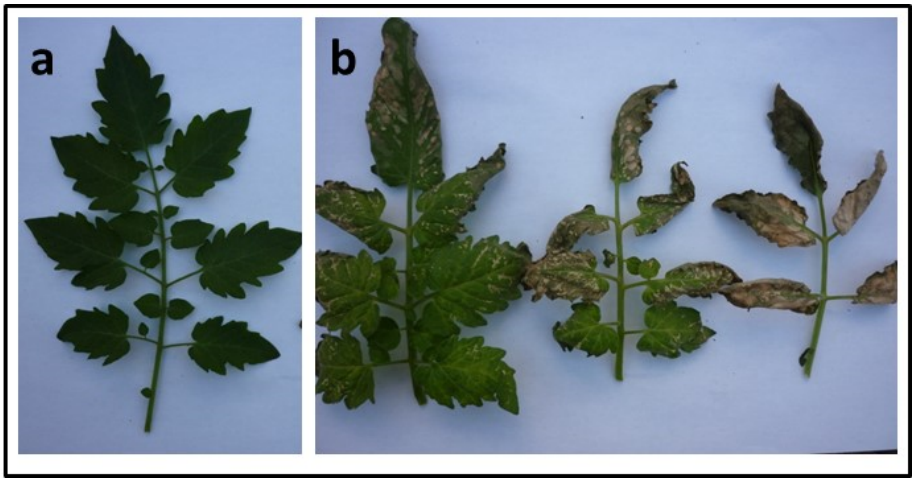

Fig 1 Visual scale of leaf phytotoxicity used at five days after a foliar spraying of 0.96 kg.ha-1 of metribuzin. (a) Absence of phytotoxicity symptoms. Visual assessment scale = 0. (b) Different phytotoxicity expressions on leaf. Visual assessment scale = 1.

Table 2 Observed frequencies for each foliar phytotoxicity to the herbicide metribuzin (0.96 kg.ha-1) within different tomato populations and their fit to a single recessive Mendelian segregation model.

		Foliar phytotoxicity scale				
Population	**n**	**0***	**1**	**Excpected ratio**	**X^2**	**P-value**
P_1 (Viradoro)	20	0	20	---	---	---
P_2(CNPH498)	20	20	0	---	---	---
F_1	20	0	20	---	---	---
F_2	515	126	389	1:3	0.078	0.78

*Visual assessment scale = 0 (no symptoms) and scale = 1 (with herbicide-induced symptoms).

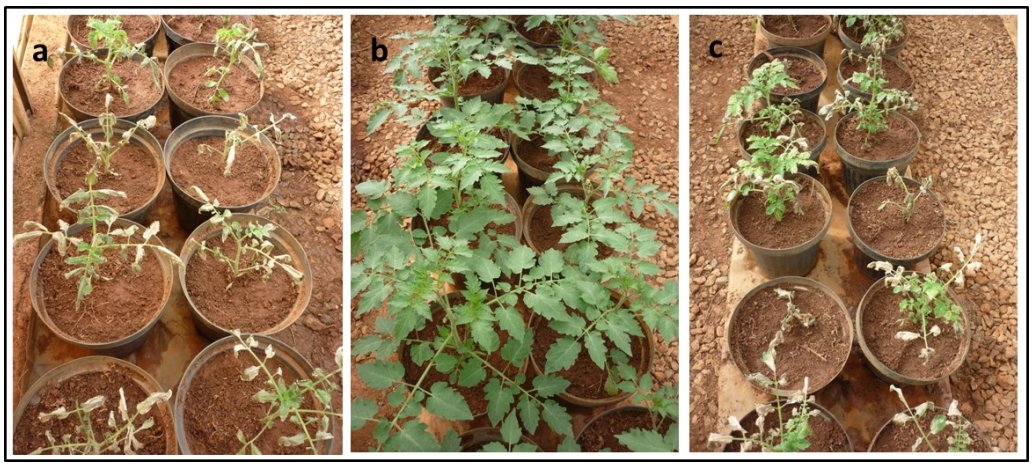

Fig 2 Response of contrasting parental lines and F_1 tomato plants five days after application of 0.96 kg.ha-1 of metribuzin. (a) Plants of the sensitive inbred line P_2 ('Viradoro'), displaying 100% of plants with severe foliar phytotoxicity; (b) symptom-free plants of P_1 ('CNPH-0498'), (c) F_1 plants, displaying 100% of the plants with severe foliar phytotoxicity, very similar to P_2.

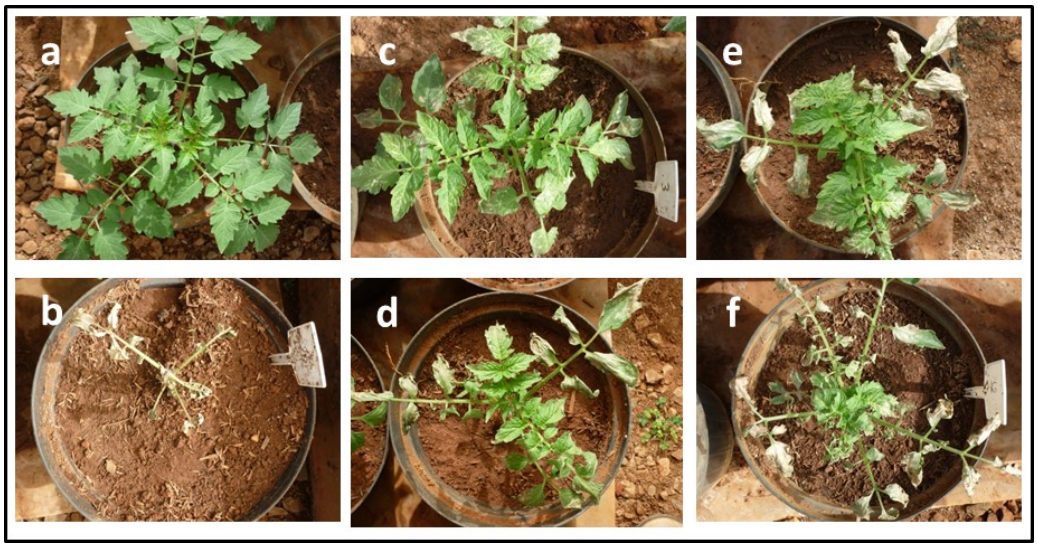

Fig 3 Responses of different genotypes of the F_2 tomato population five days after application of 0.96 kg.ha^{-1} of metribuzin (a) Complete absence of foliar phytotoxicity symptoms in a tolerant genotype. The distinct levels of foliar phytotoxicity in sensitive genotypes are illustrated in (b), (c), (d), (e) and (f).

Table 3 Response of 32 tomato F_3 families (16 derived from F_2 scale= 0 and 16 from F_2 scale=1) five days after application of 0.96 kg.ha-1 of metribuzin according to the segregation of evaluated 15 individuals of each family.

	Number of F_3 families with:		
	100% plants scale = 0*	100% plants scale = 1	segregating
F_3 families from F_2 plants with scale = 0 (n=16)	16	0	0
F_3 families from F_2 plants with scale = 1 (n=16)	0	5	11

*Visual assessment scale = 0 (no symptoms) and scale = 1 (with herbicide-induced symptoms).

Linked molecular markers to *mtz* – A first candidate marker (OPZ-11) that generated a polymorphic band of ≈ 930 bp associated with the sensitive parent was identified, that could be linked in repulsion phase with *mtz* (Fig 4). We call it OPZ11-930. The 11 sensitive F2 individuals analyzed had the marker OPZ11-930, while this marker was present only in 6 of 11 tolerant F2 individuals. To test the repulsion linkage between OPZ11-930 and *mtz*, 118 individuals from the F2 population were analyzed. The observed frequencies for each sub-sample of sensitive and tolerant F2 individuals were compared with frequencies expected for 3: 1 ratio, corresponding to two independent segregating loci (Table 4). The analysis shows a clear deviation to the expected independent segregation, so it was assumed that the marker OPZ11-930 is linked in repulsion phase with the locus *mtz*, both positioned on the same chromosome.

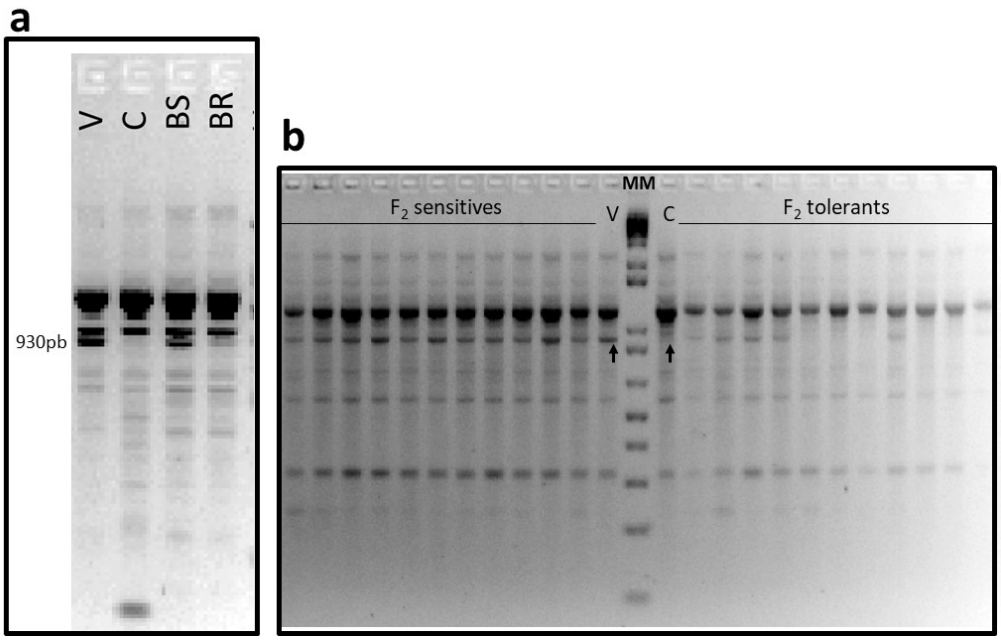

Fig 4 Agarose gels (1.5%) used for the detection of polymorphic band OPZ11-930. A) Detection of Z11-930 band by BSA: V= Sensitive parent 'Viradoro', C= tolerant parent 'CNPH468', BS= Bulk sensitive (an equimolar DNA mix of 7 F2 individuals with sensitive phenotype), BR=Bulk tolerant (an equimolar DNA mixture of 7 individuals F2 with tolerant phenotype). B) Detection of Z11-930 band on 11 sensitive F2 individuals, 11 tolerant individuals, V= Sensitive parent 'Viradoro', C= tolerant parent 'CNPH-0498'.

Table 4 Expected frequencies for independence (unlinked) loci and observed frequencies for the dominant marker OPZ11-950 in 118 phenotyped F_2 individuals of the 'Viradoro' *x* 'CNPH-0498' population.

	F_2 individuals (n=118)			
	Sensitive n=87		**Tolerant: n=31**	
	With marker	**Without marker**	**With marker**	**Without marker**
Expected (3:1)	65,3	21,8	23,25	7,75
Observed	76	11	15	16
X^2 (P-value)	7,10 (0,007)		11,71 (0,0006)	

Chromosomal location of *mtz* – All ten evaluated colonies confirmed the expected amplicon either after the PCR colony prove with primer OPZ-11 and after digestion of plasmid DNA with a specific insert-flanquing *EcoRI* endonuclease. Plasmid DNA of each colony was Sanger-sequenced using the primers pair M13. All sequences were identical and the sequence of the RAPD primer OPZ-11 was confirmed flanking 928 bp (near the OPZ11-930 expected size). (Supplementary material B1) The BLASTn analysis using the sequence of the amplicon match (E value = 0.0, cover = 99% and identity = 99.57%) on a specific region of chromosome 1 of *S. lycopersicum* reference genome (total length of 90,311,507 bp), at position 73,776,989-73,777,912.

4.5. DISCUSION

The inheritance study performed in the present work, based upon the phenotype and segregation pattern of two populations (F_2 and F_3), F1 and its contrasting parents, confirms that the high tolerance to metribuzin of the genotype 'CNPH0498' ('UGA1113-MT') is determined by a single nuclear recessive allele that we tentatively named as *mtz*. To date, a wide array of genetic mechanisms with distinct genetic control have been reported for metribuzin tolerance in different crops ranging from a single dominant gene for cultivated soybeans (Edwards et al. 1976), wild soybeans (Kilen and He 1992), and maize (Grogan et al. 1963), through simple co-dominant inheritance genes with additive effect in lupin (Si et al. 2011), up to multiple genes with additive effects in durum wheat (Villarroya et al. 2000).

In tomato, it has been more difficult to determine with precision the genetic basis of metribuzin tolerance. Firstly, Souzamachado et al. (1982) in studies with the tolerant variety 'Fireball', indicated the presence of a major gene and possible effects of modifiers. Phatak and Emmatty (1987) carried out assays employing nutrient solutions with the addition of metribuzin, concluded that the presence of a recessive gene in

high-tolerant genotype ‘UGA1113-MT’. However, few years later Liu and Phatak (1993) determined the presence of an incomplete recessive gene with modifiers.

In our case, we think that the good fit obtained for the single recessive gene model was achieved due to the methodological approach involving very high dose of foliar metribuzin. This stragegy was found to be a more precise, efficient, and simple to descrimate the contrasting phenotypes as demonstrated by the initial assays using the contrasting parental genotypes. Thus, by adjusting a visual binomial scale based upon phytotoxicity symptoms, it was possible to group individuals with different degrees of sensitivity within the category 1. These varying degrees of sensitivity probably caused some problem in classifying the phenotype could cause “noises” in the data analysis and interfered with the conclusion of the genetic control of this trait reported in previous inheritance studies. It is well-known that the sensitivity to metribuzin can be affected by different external and internal factors of plants (Phatak and Stephenson 1973, Fortino and Splittstoesser 1974). Therefore, by applying a high dose of metribuzin and grouping apparently distinct degrees of sensitivity under one category, was possible to determine with higher levels of precision that a single recessive allele (*mtz*) is responsible for the high-level of metribuzin tolerance.

Appling the BSA methodology, it was possible to identify a molecular marker (OPZ11-930) which shows linkage in repulsion phase with *mtz* at a distance calculated according to the number of recombinants observed (26 in 118) of 22 cM. The sequence of the corresponding 928 bp segment of the OPZ11-930 marker allowed to perform a nucleotide similarity analysis with the BLASTn tool. The result matched (99.57% of identity) with a segment of tomato chromosome 1. Therefore, most possibly the *mtz* allele co-localizes with OPZ11-930 on this chromosome. This result must be confirmed, for example, from the genome re-sequencing of the contrasting parents (‘Viradoro’ and ‘CNPH498’), either looking for a variant that explain the polymorphism of OPZ11-930 or designing new polymorphic molecular markers for chromosome 1 to confirm co-segragation. This last option would also allow to improve the positioning of the mtz allele by a specific genetic map. In addition, the genomes of ‘CNPH-0498’ and ‘Viradoro’ will be available for direct genomic comparison and for the search for candidate genes. In principle, variants with proven deleterious effect

(recessive genes) will be prioritized (see application in González-Arcos et al. 2019). If necessary, with the available genomic information, it would be possible to design a new set of molecular markers in order to carry out a fine mapping of the candidate genes.

Finally, any available information on the function of the protein encoding by the candidate gene would be useful. Until now, the implicated mechanisms studied in tomato metribuzin tolerance are indicating the foliar metabolization process of the herbicide. The conjugated molecules found, at higher levels in tolerant genotypes, are N-glycosides. Therefore, increased activity of the N-glucosyltransferase enzyme or a higher level of its substrate (UDP-glucose) are postulated as the potential mechanisms involved with the tolerant reaction (Frear et al. 1983, Phatak and Jaworski 1985, Davis et al. 1991).

N-glucoside conjugates have been reported as metabolites of several herbicides, such as chloraben, dinoben, metribuzin (Sencor), picloram (Tordon), propanil, and pyrazon (Lamoureux et al. 1991). It would be important to evaluate the effectiveness of the *mtz* gene in determining tolerance to other active principles. As an antecedent of interest, a genotype of the hybrid population PI 204976 (i.e. the same population that generates our tolerant line 'CNPH-0498') showed a high-level tolerance to acifluorfen (Ricotta and Masiunas 1992).

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5. AN INTROGRESSION ASSOCIATED WITH THE *Sw*-5 LOCUS IN TOMATO CHROMOSOME 9 PRODUCE AN INCREASE OF ASCORBIC ACID CONTENT IN MATURE FRUITS

5.1. ABSTRACT

In tomato (*Solanum lycopersicum* L) several quantitative trait loci (QTLs) for AA content have been mapped and some have co-localized with different enzymes related to the metabolic routes of AA. Alleles of breeding interest have been identified in *S. pennelli*, *S. habrochaites* and *S. lycopersicon var cerasiforme*. In preliminary works, we observed a significant increase in the AA content in red fruit when comparing *Tospovirus* resistant near-isogenic lines (carrying the *Sw*-5 on chromosome 9) with the susceptible lines. To confirm this hypothesis, we generated three groups of near-isogenic lines (NILs), incorporating by backcrossing the *Sw*-5 locus derived from a cultivar 'Stevens'-derived elite line in the breeding lines LAM147, CNPH1247 and LAM186. The presence of *Sw*-5 locus was confirmed with a functional molecular marker. The AA of red tomato fruit was determined by high performance liquid chromatography (HPLC) using an ion exchange column. For three independent seasons, we demonstrate that the presence of the *Sw*-5 locus was associated with increases in AA of 1.46, 1.24 and 1.63-fold (fresh weight) in the resistant lines LAM147, CNPH1247 and LAM186, respectively. This increase was inherited in a dominant way in the F_1 hybrid resulting from the crossing of both isolines. Fifty F_2 plants derived from the cross of two contrasting NILs displayed a strong relationship between the presence of *Sw*-5 and higher AA contents, with a percentage of recombination ≤12%. We postulate the name *Vtc* for the allele linked to *Sw*-5 locus that is associated with this phenotypic effect. At least five different genes coding for enzymes involved in the metabolic pathway of production and recycling of AA are co-localized with the *Sw-5* locus on chromosome 9. Among these, only the genes *GME2* (GDP-mannose 3', 5'-epimerase) and *LOC101249491* (Rho GDP-dissociation inhibitor 1) are in a position that would explain the results obtained here, and therefore, were postulated as candidates for the phenotypic effect associated with the *Vtc* locus. Sequencing the region wich comprise these candidate genes in both NILs will allow to discover polymorphisms that can explain these differences and thus be able to validate which gene corresponds to the *Vtc* locus.

Keywords: vitamin C, tomato breeding, *Solanum lycopersicum*, *Solanum peruvianum*

5.2. INTRODUCTION

Vitamin C or L-ascorbic acid (AA) is a six-carbon sugar that fulfills essential functions in the organism of animals. It participates as a co-factor in several enzymatic reactions associated to the biosynthesis of collagen, L-carnitine and the conversion of dopamine to norepinephrine (Rebouche 1991) and directly acts as a potent antioxidant (reducing agent) that efficiently prevents the potential oxidative damages of free radicals generated in the normal process of respiration (Arrigoni and De Tullio 2002). Therefore, the presence of adequate levels of AA is related to the prevention of several processes related to cellular oxidative stresses and degenerative disorders such as cancer and cardiovascular disease (Li et al. 2007a). On the other hand, low levels of AA are related to several human deficiencies, disorders and metabolic diseases (Mandl et al. 2009).

The metabolic synthesis of AA is not operative in humans and other mammals and therefore this compound must be obtained from the diet (Chatterjee et al. 1960). AA absorption into the organism is given by a substrate-saturable mechanism, which involves a specific low-expression transporter with negative regulation induced by the substrate (Li et al. 2007b). For this reason, the effective levels of AA are regulated at very low concentrations. The recommended daily intake values are very variable and range from 15 to 125 mg (National Institute of Health, 2018). AA is a water-soluble molecule and the excess of this molecule is easily eliminated by the organism and has no cumulative power.

Tomato (*Solanum lycopersicum* L.) and other vegetables have the ability to synthesize AA and they are the main sources of AA for humans. In plants, AA also functions as an antioxidant and as an enzymatic co-factor, playing important roles in several processes related to photoprotection, biosynthesis of essential compounds and various aspects related to organ development (Gest et al. 2013, Akram et al. 2017). In several crops of commercial interest, higher AA contents have been related with superior reactions to biotic and abiotic stresses (Davey et al. 2000, Conklin and Barth 2004, Kuzniak and Sklodowska 2005), as well as with the improvement of the post-

harvest quality of different products (Davey et al. 2007, Stevens et al. 2008, Landi et al. 2015).

The levels of AA are affected by the environmental conditions (Dumas et al. 2003, Roselló et al. 2011). The regulation of AA levels in the plant cells is controlled by the synthesis (Akram et al. 2017), recycling, degradation (Gallie 2013), and transport of this molecule across cells and organs (Horemans et al. 2000). The synthesis of AA can be achieved by four alternative routes (Akram et al. 2017), being the most studied and probably the most important the Smirnoff-Wheeler pathway, also called as the D-mannose/L-galactose pathway (Wheeler et al. 1998). In this pathway, six enzymes are involved in nine steps guiding the conversion of D-glucose to AA (Smirnoff et al. 2001). On the other hand, the recycling of AA is considered very important during the processes of response and adaptation to stress (Quin et al. 2011). The production and accumulation of reactive oxygen species (ROS) at the cellular level can generate oxidation of proteins, unsaturated fatty acids and nucleic acids, resulting in damage to cellular functions (Caverzan et al. 2016). In its role as an antioxidant, AA uses ROS to, in a reaction mediated by the enzyme ascorbate peroxidase (AP), generate the unstable radical monodehydroascorbate (MDHA), which is partly dissociated again in AA and dehydroascorbate (DHA) by non-enzymatic reactions. DHA is also unstable and degrades rapidly, so in this process the total amount of AA can be depleted if the oxidized forms of MDHA and DHA are not rapidly converted to AA by the action of two reductases: monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Smirnoff and Wheeler 2000).

Breeding programs aiming to improve the tomato contents of AA began in the middle of the last century. Lincoln et al. (1950) worked with the species *S. lycopersicum*, *S. habrochaites*, *S. pimpinellifollium*, identifying some advanced selections. In particular, they identified very high contents in accessions of *S. peruvianum* (*sensu lato*). The complex and additive control of the high AA content was demonstrated in tomatoes (Stevens and Rick 1986) as well as its high correlation with fruits of small size (derived from the same wild sources) and low fruit yield (Lincoln et al. 1950; Stevens and Rick 1986). More recent works identified about 30

QTLs located in chromosomes 2, 8, 9, 10 and 12 regulating the AA content in three interspecific populations of *S. lycopersicum* with *S. pennellii*, *S. habrochaites*, and *S. lycopersicum* var *cerasiforme* (Rousseaux et al. 2005, Stevens et al. 2008). These results indicated that AA content is a typical quantitatively-inheritance trait. However, across populations it was possible to identify common regions with higher level of control over the variation of this trait. One important genomic region for this trait is the tomato chromosome 9 where genes associated with the synthesis (*GME2* and *GMP2*) and recycling (*MDHAR3*) of AA are located (Rousseaux et al. 2005; Zou et al, 2006; Stevens et al. 2007). These enzymes positively regulate both the synthesis and the recycling of AA (Smirnoff and Wheeler 2000) so that the over-expression of these genes results in the increase of AA contents (Cronje et al., 2012, Li et al. 2012, Zhang et al., 2011). On the other hand, the loss of function of these genes would cause a significant reduction of AA contents.

The average value of AA content in mature fruits is 13.7 mg per 100 g of fresh weight [US Department of Agriculture, http://www.nal.usda.gov/fnic/foodcomp/search/. (Last accessed 12 December, 2018)]. In the case of genes related to the synthesis of AA, the overexpression of the GME2 gene has been shown to increase the AA contents in various organs of the plant, including increases raging from 1.21 to 1.24-fold in mature fruits and improving the plant response to oxidative stress, cold, and salinity (Zhang et al. 2011). Likewise, the over-expression of the GMP gene increased the AA content by 1.50-fold in green fruits and by 1.35-fold in mature fruits (Cronje et al. 2012). In genes related to the recycling pathway, it has been shown that the *MDHAR3* gene is related to the maintenance of AA levels in mature fruit exposed to cold stress, and that one allelic variant derived from *S. pennellii* displayed higher activity levels (Stevens et al. 2008).

These results show that genes encoding enzymes involved in the synthesis and recycling pathways in tomato can explain important increases in AA contents. It is likely that the phenotypic diversity of AA contents observed in accessions of *Solanum* (section *Lycopersicon*) germplasm is controlled by allelic variants capable of generating more efficient pathways.

In a preliminary characterization of tomato germplasm by AA content we observed a significant increase in the AA content in red fruit in *Tospovirus* resistant near isogenic lines carrying the *Sw-5* (Spotted Wilt Resistance-5) locus derived from *S. peruvianum* (Van Zijl et al. 1985, Stevens et al. 1992), located in the telomeric region of the long arm of chromosome 9 (Brommonschenkel and Tanksley 1997). The objective of the present work is to confirm the association between the *Sw*-5 locus and the increase of AA content in red fruit and to postulate a preliminary know set of candidate genes potentially associated to this trait.

5.3. MATERIALS AND METHODS

Plant material – Three tomato (*S. lycopersicum*) near-isogenic lines with different genetic backgrounds were used, in which the *Sw*-5 locus that confers resistance to *Tospovirus* species derived from *S. peruvianum* (Van Zijl et al. 1985, Stevens et al. 1992, Boiteux and Giordano 1993) was incorporated via backcrossing (Table 1). The inbred line 'TSW-10' (Boiteux et al. 1993) derived from the cross between the variety 'Stevens' (donor of the *Sw*-5 locus) and the cultivar 'Rodade' (Stevens et al. 1992) was employed as the resistant source. The incorporation of the *Sw*-5 locus in each line was made through four successive back-crosses to the original line, followed by seven generations of self-pollination and selection for *Tospovirus* resistance. At each backcrossing step the presence of the *Sw*-5 locus was determined after inoculation with the Tospovirus *Groundnut ringspot virus* (GRSV) following the protocol described in Boiteux and Giordano (1992). After the series of self-pollinations, the presence/absent of the *Sw-5* locus in the near-isolines was confirmed by using a functional SCAR molecular marker (Dianese et al. 2010). The three original (GRSV-susceptible) inbred lines used were 'CNPH-1496' (indeterminate, 'Santa Clara' type), 'CNPH-1306' (indeterminate, Italian type) and 'CNPH-1409' (determined, processing tomato type). The crosses between contrasting near-isogenic lines to obtain the F_1 (heterozygous for the *Sw*-5 locus) were performed and the segregating F_2 populations were obtained selfing the respective F_1 plants.

Table 1 Groups of contrasting near-isogenic lines and their genotypes at the *Sw*-5 (*Tospovirus* resistance) locus located in the tomato chromosome 9.

Near-isoline group	Line	Description	Genotype at Sw-5 locus
	CNPH-1496	Original 'Santa Clara' type line	-/-
1	LAM-147	CNPH-1496 + *Sw*-5	+/+
	CNPH-1409	Original processing type line	-/-
2	CNPH-1247	CNPH-1409 + *Sw*-5	+/+
	CMPH-1306	Original Italian type line	-/-
3	LAM-186	CNPH-1306 + *Sw*-5	+/+

Trial design and sampling – Three groups of near-isogenic lines were evaluated for AA content in three independent trials conducted in 2014, 2015, and 2016. The F_1 hybrids between pairs of near-isogenic lines (Table 1) were evaluated in two independent trials conducted in 2015 and 2016. The segregating F_2 populations (derived from the self-pollination of each F_1 hybrid) were evaluated in 2016. The corresponding contrasting parental inbred lines were always used as reference during the genetic studies employing the F_1 and F_2 generations. Trials were carried out under greenhouse in Brasilia-DF between April and August (= dry season). The parental lines, hybrids and segregating populations were sown in polystyrene trays and 30 days later transplanted to 5L-pots filled with a mixture of commercial peat and disinfected soil as substarte in an insect-proof glasshouse. The plants of the indeterminate materials were conducted with one stem while those of the determined material had no additional manipulation. For the evaluation of near-isogenic lines and hybrids (i.e. genetically pure populations) 20 individuals of each genotype were sown. Fifty fruits of each population were simultaneously labelled in order to standardize the fruit development stage and ripening conditions. Only 20 red fruits per genotype were chosen at the harvest time. These fruits were immediately frozen at -30 °C until the evaluation. At this time, 250 grams of fruit were set apart from the total sample of each

population and homogenized using a rotor and three sub-samples of approximately 4 grams were put aside and used for the evaluation of the AA content as will be described below.

The evaluation of the F_2 segregating populations was carried out using individual plants. For that, 50 individuals were planted together with 10 individual plants of their parents, which were used as a reference to calculate an ordinal scale of AA content. In each plant used for the evaluation, several fruits from the same set time were labelled at the first and second clusters. Three red fruits per plant were harvested and immediately frozen at -30 °C until the evaluation. At this time, the frozen fruit were homogenized using a rotor and three sub-samples of approximately 4 grams were set aside and used for the evaluation of the AA content as will be described below.

Evaluation of AA contents in tomato fruit tissues – The AA content of mature tomato fruits was determined by high performance liquid chromatography (HPLC) using an ion exchange column, according to the procedure described by Rosa et al. (2007). We started with fruit samples of approximately 4 grams (see above) extracted with a solution of EDTA 0.05% (mv^{-1}) in 0.05 M sulfuric acid (PA grade) in ultrasound for 10 minutes, taken to known volume, filtered in disposable filtering units of hydrophilic Teflon and placed in amber bottle with screw cap and silicone septum. The same extracting solution of EDTA 0.05% ($m.v^{-1}$) in 0.05 M sulfuric acid (PA grade) was used as a mobile phase. The BIORAD Aminex HPX87H column was selected as a stationary phase. The flow of the mobile phase was 0.8 mL / minute, the injection volume of 20uL and the wavelength of 242.6 nm. The ascorbic acid standard was prepared fresh. The external standard method of calibration was used. The original solution of ascorbic acid was diluted in at least five different concentratons in 20 uL, allowing the generation of a standard absorption curve with values between 0 and 1.

Characterization for the *Sw*-5 locus - Ten representative plants of the pure genotypes (contrasting lines and their F_1) and 50 individual plants of the F_2 populations were characterized by the presence of the *Sw*-5 locus using the functional SCAR

marker ‘Sw-5-2’ (Dianese et al. 2010) which generates an amplicon of 574 bp for the resistant allele and amplicons of 510 bp or 464 bp for the susceptible alleles. Genomic DNA of tomato plants was purified as described in Boiteux et al. (1999) using a modified 2X CTAB buffer and additional purification steps with organic solvents. PCR reactions were as follows: 0.1 µg of the template DNA, 1.25 µL of 10X PCR buffer, 0.75 µL of 50 mM $MgCl_2$, 1.25 µL of 2.5 mM dNTP, 100 ng of each primer, 0.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and water in a final volume of 12.5 µL. The thermocycler program steps were: denaturation at 95 °C for 1 minute, followed by 29 cycles of 95 °C for 30 seconds, 58 °C for 30 s and 72 °C for 1 minute and final extension at 72 °C for 7 minutes. PCR products were separated using 1% agarose gel in TBE buffer.

Candidate gene search and mapping – The best candidate genes controlling AA content were chosen considering information generated in previous works as well as in the database of the Solgenomic network (https://solgenomics.net) and of the the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov). Searches were carried out for genes located within chromosome 9 related with enzymes involved in the metabolic pathway of ascorbic acid. The identified genes were positioned on a physical map using the NCBI genome data viewer tool (https://www.ncbi.nlm.nih.gov/genome/gdv/) assembly SL3.0, annotation release 103.

Statistical analysis – For the comparison of AA content in mature fruits in near-isogenic lines and hybrids, analyses of variance followed by comparison of means (Fisher LSD) were employed. To classify the individuals of the F_2 family according to a high/low AA content scale, AA values (mg/100 grams) of 10 different plants of ‘LAM-186’ and ‘CNPH-1306’ that were growing together with the F_2 population were used to estimate a confidence interval (95%), which was used as a range interval to classify the value of each F_2 plant as high (value within the interval generated with plants of LAM-186) and low (value within the interval generated with the plants of CNPH-1306). The evaluation of plants that were recombinant or those that gave values

between 15-17 mg / 100 grams (intermediate between the two interval ranges) was repeated to confirm their classification. To determine if the presence of the *Sw*-5 gene is related to the AA content, a contingency table and Pearson's CHI-squared statistic were used.

5.4. RESULTS

AA content in near-isogenic lines and its hybrids – The three groups of near-isogenic lines showed significant differences ($p < 0.05$) in AA content, confirming an increase in AA content for the line with incorporation of the *Sw*-5 locus of 1.46 in LAM-147, 1.24 in CNPH-1247 and 1.62-fold in LAM-186, respect to the contrasting lines (i.e. without *Sw-5* locus) CNPH-1409, CNPH-1406, and CNPH-1306, respectively (Fig 1, a). When the near-isogenic lines were compared with their corresponding hybrids, it was found that there were no significant differences between the hybrid and the line with *Sw*-5 (Fig 1, b). This agrees with a dominant inheritance control model for the high AA content trait.

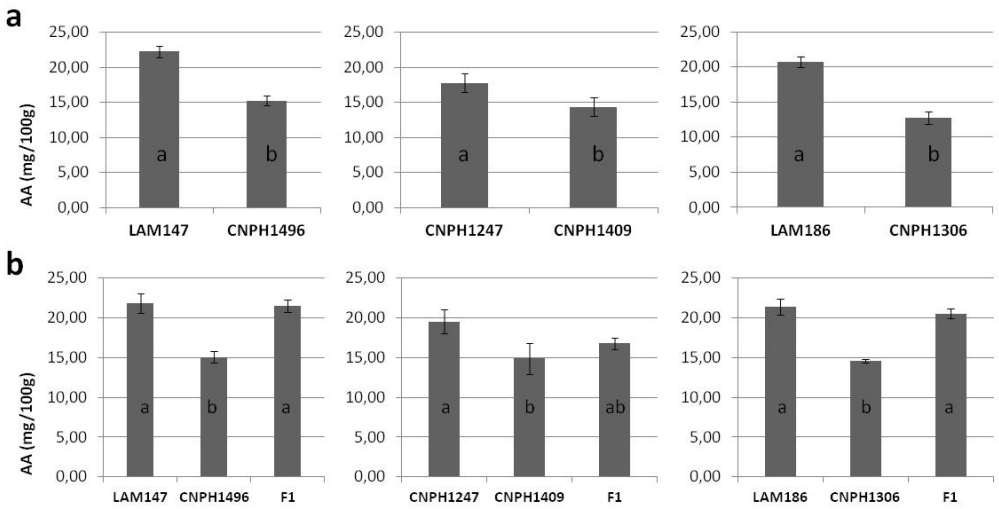

Fig 1 AA content (mg/100g) in mature fruits from three groups of contrasting tomato near-isogenic lines (with and without the Sw-5 locus) and their corresponding hybrids. For the comparison of near-isogenic lines (a) data from three independent trials conducted in 2014, 2015, and 2016 (in Brasília-DF) were used. For the comparison of the contrasting near-isogenic lines and their corresponding hybrids (b) data from two independent trials conducted in 2015 and 2016 (in Brasília-DF) were used. Values are averages of replications per year and are presented with their standard error. Within the same graphic, different letters indicate values with significant differences according to the Fisher LSD test ($p < 0.05$).

***Sw*-5 locus association with higher AA contents –** From the previous results it was decided to work with the near-isogenic lines from group 3, composed by the original *Tospovirus*-susceptible line 'CNPH-1306' and the resistant counterpart (with the *Sw*-5 locus) line 'LAM186' (Table 1). This 'CNPH-1306' and 'LAM186' near-isogenic pair was chosen since it displayed the greatest differences in AA contents (62.49% of increment for the *Sw-5* presence) when compared with the other two pairs of near-isogenic lines. Fifty plants of the F_2 population were individually characterized by genotype at the *Sw*-5 locus and by AA content, together with 10 plants of both parental near-isogenic lines and the F_1 resulting from their crossing (Fig 2). Of the ten

plants evaluated for each isoline, the highest CV was for LAM-186 (24.7%) respect to CNPH-1306 (10.8%). In fact, some plants of the line LAM-186 displayed lower levels of AA (example plant #10 with 15.2 mg / 100 g), similar to the average value of ten plants of CNPH-1306. All ten F_1 hybrid plants evaluated had the lowest CV (8.0 %), similar to LAM-1306. To evaluate the F_2 population, we used a binomial ordinal scale (H = high / L = low) using the range confidence interval generated from the individual values of 10 plants of both near-isogenic lines (Table 2). A contingency table was constructed using the *Sw-5* locus genotype in the columns and the AA contents in the rows (Table 3). The Pearson Chi-square statistics shows a significant relationship between both variables (p=0.0048), reinforcing the hypothesis that for this segregating population the presence of the *Sw*-5 locus is associated with higher AA contents. Of the 50 evaluated F_2 plants, nine recombinants (i.e. absence of *Sw*-5 and high AA content / presence of *Sw*-5 and low AA content) were found.

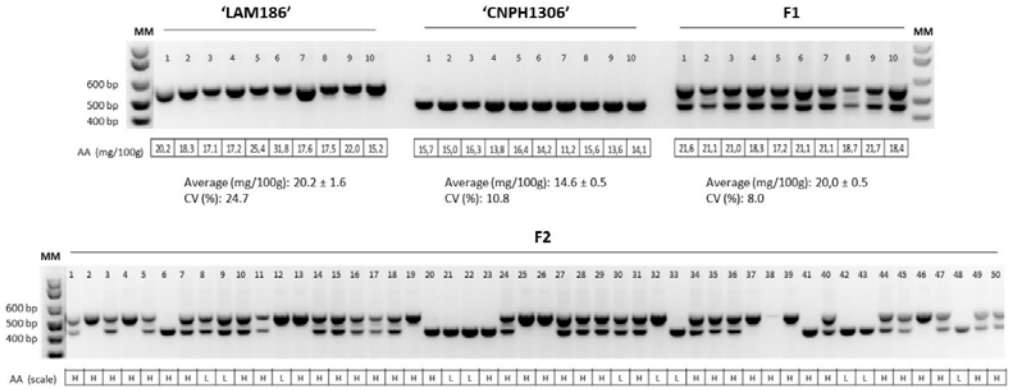

Fig 2 Agarose gel visualization (1.5%) of the 'Sw-5-2' SCAR marker in 10 plants of the near-isogenic line 'LAM-186', 10 plants of the near-isogenic line 'CNPH-1306', 10 F_1 hybrid plants and 50 plants of the corresponding F_2 population. Upper band of 574 bp indicates presence of the resistance locus *Sw*-5, while lower band of 464 bp indicates the presence of the susceptible locus. For the ascorbic acid (AA) content (mg/100 g) of 'LAM-186', 'CNPH-1306' and their F_1 plants, the coefficient of variation and the average +/- the standard error of 10 plants are shown. For each individual F_2 plant, the classification of the AA content (mg/100 g) value in an ordinal scale of H = high and L = low (see Table 2) are shown.

Table 2 Confidence interval (95%) used as a rank to classify the value of each F_2 plant derived from the cross between two contrasting near-isogenic lines ['LAM-186' (with the *Tospovirus* resistance locus *Sw*-5) and 'CNPH-1306' (without the *Sw*-5 locus)] as high (value within the interval generated with 10 plants of LAM-186) and low (value within the interval generated with 10 plants of CNPH-1306).

AA content scale	AA content (mg/100g)		
	Media	Ll[1] (95%)	Ul[1] (95%)
High	20.23	16.64	23.82
Low	14.6	13.47	15.72

[1] Ll: lower limit of the confidence interval, Ul: upper limit of the confidence interval

Table 3 Contingency table with F_2 individuals derived from the cross between two contrasting near-isogenic lines ['LAM-186' (with the *Tospovirus* resistance locus *Sw*-5) and 'CNPH-1306' (without the *Sw*-5 locus)]. Columns correspond to the genotype in the *Sw*-5 locus. Rows correspond to the ascorbic acid (AA) content.

	Genotype at the *Sw-5* locus			
AA content	+/+	-/+	-/-	**X^2 Pearson**
High	10	25	4	0.0048
Low	2	3	6	

Candidate gene search and positioning – We propose as most likely candidates some annotated genes related to enzymes involved in the metabolic pathway of production and/or recycling of AA (Table 4). The genes *MDHAR*, *GME2* and *LOC101257102* (possible *GMP2* according to Stevens et al. 2007) were already reported as associated with QTLs determining increase in AA contents in tomato red fruit. The genes *LOC101258987* and *LOC101249491* have not yet been related to AA content. The Fig 3 shows the relative position of these genes in chromosome 9 together with *LOC101248920* corresponding to the protein "Spotted Wilt Resistance-5" (= the functional *Sw*-5 allele).

Table 4 Candidate genes for the tentatively named *Vtc* locus (controlling higher ascorbic acid content) in tomato chromosome 9.

Gene Symbol	Description [1]	Position [2]	Map ref. [3]	Reference
LOC101258987	L-ascorbate peroxidase 2, cytosolic	864,904 - 869,559	1	---
AFRR / MDHAR	Monodehydroascorbate reductase	2,834,932 - 2,841,023	2	Stevens et al. 2007; Stevens et al. 2008
LOC101257102	Mannose-1-phosphate guanylyltransferase 1-like	4,557,932 - 4,562,666	3	Stevens et al. 2007
LOC101249491	Rho GDP-dissociation inhibitor 1	65,704,334 - 65,718,542	4	---
GME2	GDP-mannose 3',5'-epimerase	69,110,963 - 69,116,724	5	Zou et al. 2006; Zhang et al. 2011

[1] NCBI *Solanum lycopersicum* Annotation Release 103
[2] SL3.0 assembly.
[3] Reference number in Fig 3.

Fig 3. Phisical map of tomato chormosome 9. Black vertical lines indicate the position of different genes. Numbers 1 to 5 are candidate genes for the *Vtc* locus (described in Table 4). Number 6 is the position of *LOC101248920* (protein "Spotted wilt resistance-5" = functional *Tospovirus* resistance *Sw-5* allele) (Brommonschenkel et al. 2000).

5.5. DISCUSSION

This work reports the advances in the characterization of a genetic factor in *S. lycopersicum* that increases the AA content in tomato red fruit, which was found to be associated with the introgressed segment from *S. peruvianum* that harbors the functional *Tospovirus* resistance *Sw*-5 allele (Fig 1, Fig 2). Classical genetics studies indicated that a locus with dominant effects is associated with the higher AA levels (Fig 1b, Table 3). Therefore, we tentatively suggested the name *Vtc* (Vitamine-c) for the locus associated with this phenotypic effect. This dominant inheritance may be explained by a possible loss of function of the corresponding allele in domesticated tomato germplasm, which is yet functional in accessions of the wild species *S. peruvianum*. Our studies also reinforced the notion that the *Vtc* locus is linked with the *Sw*-5 locus on chromosome 9 (Fig 2, Table 3).

We proposed as most likely candidates for the *Vtc* locus a sub-group of annotated genes related to enzymes involved in the biosynthetic and recycling AA pathways with genomic localization also in chromosome 9 (Table 4). Fig 3 shows the physical location of these genes and the *LOC101248920* corresponding to the protein "Spotted Wilt Resistance-5" (functional *Sw*-5 allele) (Brommonschenkel et al. 2000). The putative presence of recombinants in the progeny of 50 F_2 plants derived from the resistant near-isogenic line LAM-186 (Fig 2), indicates an estimated genetic distance of 18 cM (Table 3). Since it is possible that recombinant plants derived from LAM-186 could have been used in the F_1 hybrid seed production, and subsequent in the F_2 population, it is possible that some recombinants identified in the F_2 population were, in fact, a result of the backcross program that produced this *Tospovirus* resistant near-isogenic line. It is important to highlight that the development of the contrasting near-isogenic lines 'LAM-186' (with the *Tospovirus* resistance locus *Sw*-5) and 'CNPH-1306' (without the *Sw*-5 locus) was carried out solely to incorporate the resistance locus. In this way, the genetic distance calculated between *Sw*-5 and *Vtc* loci could be even smaller.

Therefore, taking into consideration only a genetic distance of ≈18 cM we propose as the best candidates for *Vtc* locus the genes *GME2* (GDP-mannose 3',5'-

epimerase) and *LOC101249491* (Rho GDP-dissociation inhibitor 1). GDP-mannose 3',5'-epimerase is a key enzyme in the biosynthesis of Vitamin C, catalysing the conversion of GDP-D-mannose to GDP-L-galactose in the D-mannose/L-galactose pathway. Stevens et al. (2007), Gilbert et al. (2009) and Zhang et al. 2011 confirmed that GME plays a key role in the regulation of ascorbate biosynthesis in tomato. The cytosolic regulatory protein GDP dissociation inhibitors is important for the dynamics of Rho GTPases, short G proteins wich exhibit GDP/GTP binding properties and bind to various proteins which modulate their GTP/GDP state. GDP dissociation inhibitors are thought to regulate the GTPase 'switch' negatively by maintaining the GTPase in a GDP-bound 'inactive' state, so can prevent GTP hydrolysis or inhibit GDP dissociation and maintain Rho GTPases in cytosolic location (Hancock and Hall, 1993). The physical proximity of these candidates and the *Sw*-5 locus (*LOC101248920*) (Fig 3) suggests that it is quite likely that these genes have been selected together in the interspecific crossing program in South Africa (Van Zijl et al. 1985) that resulted in the introgression of *Tospovirus* resistance locus from *S. peruvianum* into *S. lycopersicum* cultivar Stevens.

Until now, genetic studies of sources of alleles on chromosome 9 that provide increases in AA contents were carried out in *S. pennellii* (QTL co-localizing with *MDHAR3* and *GME2* alleles), in *S. habrochaites* (QTL co-localized with *GME2* allele) and in *S. lycopersicum* var. *ceraciforme* (QTL co-localized with *MDHAR3*, *GME2* and *GMP2* alleles) (Stevens et al. 2007) populations. Although *S. peruvianum* had already been highlighted as an important genetic source to increase AA content (Lincoln et al. 1950; Stevens and Rick 1986), our report is the first that relates this species to a particular genetic factor for this trait on chromosome 9.

In our work, the presence of *Sw*-5 locus is associated with increases in AA of 1.46, 1.24 and 1.62-fold (fresh weight) as indicated by the comparison of the resistant near-isogenic lines (viz. LAM-147, CNPH-1247 and LAM-186, respectively) when compared with their susceptible counterpart lines. In addition, we must keep in mind that these values could be underestimated if we consider the potential presence of recombinants in the near-isogenic lines harboring the *Sw*-5 locus. Despite that, these values are similar to those reported by other authors. For example, Stevens et al. (2007)

observed increases in AA contents in red fruits (relative to fresh weight) of up to 1.19-fold, which was explained by the presence of a QTL (co-localized with the *GME2* gene) derived from *S. pennellii* LA0716. In addition, transgenic plants over-expressing *GME2* exhibited a significant increase of 1.24-fold in total AA content in red fruits compared with wild-type plants (Zhang et al. 2011).

From the tomato breeding standpoint, the *Vtc* locus characterized here has two important advantages. First, it is already available in distinct *S. lycopersicum* backgrounds. Our original source of the *Sw*-5 allele, the inbred line 'TSW-10' (Boiteux et al 1993), is a fresh-market, semi-determinate, round fruit line derived from the cross between the cultivar 'Stevens' (donor of the *Sw*-5 allele of *S. peruvianum*) and the variety 'Rodade' employed for genetic characterization of the Sw-5 locus (Stevens et al. 1992). In any event, it is possible that the *Vtc* locus be available in a wide array of *S. lycopersicum* backgrounds since the *Sw*-5 locus was incorporated in virtually all breeding program around the world (Oliveira et al. 2018). Second, its dominant inheritance facilitates the use of *Vtc* in hybrid cultivars. The heterozygous F_1 hybrids (with only a single copy of the *Sw*-5 locus) displayed similar AA contents when compared with their parental lines with homozygous presence of the gene (Fig 1b). This could also be seen in the analysis of the F_2 family (Fig 2).

The identification of the gene on chromosome 9 that is responsible for *Vtc* will allow to advance in the development of functional molecular markers capable of assisting selection processes with high efficiency. In addition, it would help to understand the molecular bases of the AA increase. For that, there are some possible ways that we proposed to continue this work: (i) Search for additional polymorphic molecular markers between the isolines, beginning to filter molecular markers located close to the *Sw-5* locus. With other polymorphic markers available it would be possible to carry out a fine mapping of the region and improve the *Vtc* position. (ii) Sequencing the most likely candidate genes *GME2* and *LOC101249491* of both near-isogenic lines and compare their sequences. If polymorphisms can be detected, molecular markers can be designed to validate their effect in a F_2 population. (iii) the re-sequencing the genomes of the contrasting isolines and directly compare the sequences of

chromosome 9 in search of polymorphisms. With this information, we can design a novel set of markers and validate them in segregating F_2 populations.

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6. A LOSS-OF-FUNCTION ALLELE OF A *TAC1*-LIKE GENE (*SLTAC1*) LOCATED ON TOMATO CHROMOSOME 10 IS A CANDIDATE FOR THE *ERECTOID LEAF* (*Erl*) MUTATION*

6.1. ABSTRACT

The genetic basis of an erectoid leaf phenotype was investigated in distinct tomato breeding populations, including one derived from *Solanum lycopersicum* 'LT05' (with the erectoid leaf phenotype and uniform ripening, genotype *uu*) × *S. pimpinelifollium* 'TO-937' (with the wild-type leaf phenotype and green fruit shoulder, genotype *UU*). The erectoid leaf phenotype was inherited as a semi-dominant trait and it co-segregated with the *u* allele of gene *SlGLK2* (*Solyc10g008160*). This genomic location coincides with a previously described semi-dominant mutation named as *Erectoid leaf* (*Erl*). The genomes of 'LT05', 'TO-937', and three other unrelated accessions (with the wild-type Erl^{+} allele) were resequenced with the aim of identifying candidate genes. Comparative genomic analyses, including the reference genome 'Heinz 1706' (Erl^{+} allele), identified an *Erectoid leaf*-specific single nucleotide polymorphism (SNP) in the gene *Solyc10g009320*. This SNP caused a change of a glutamine (CAA) codon (present in all the wild-type genomes) to a TAA (= ochre stop-codon) in the *Erl* allele, resulting in a smaller version of the predicted mutant protein (221 versus 279 amino acids). *Solyc10g009320*, previously annotated as an 'unknown protein', was identified as a *TILLER ANGLE CONTROL1* (*TAC1*)-like gene. Linkage between the *Erl* and *Solyc10g009320* was confirmed via Sanger sequencing of the PCR amplicons of the two variant alleles. No recombinants were detected in 265 F_2 individuals. Contrasting S_7 near-isogenic lines were also homozygous for each of the alternate alleles, reinforcing *Solyc10g009320* as a strong *Erl* candidate gene and opening the possibility for fine-tuning manipulation of tomato architecture in breeding programs.

Key words: *Solanum lycopersicum*; resequencing; comparative genomic analysis; plant architecture; breeding

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6.2. INTRODUCTION

Genetic factors are the major determinants of plant architecture, even though the spatial structure of a plant might also be influenced by various environmental stimuli such as light, temperature, humidity, mineral and organic nutrition (Wang et al. 2018). For this reason, breeding programs have placed great emphasis in exploiting the genetic diversity associated with plant architecture as a strategy to develop high-yielding cultivars with greater adaptation to wide array of environmental conditions and cropping systems (Coyne 1980; Huyghe 1998; Jiao et al. 2010).

In tomato (*Solanum lycopersicum* L.), plant growth habit (i.e. determinate, semi-determinate, and indeterminate), foliar insertion angle, leaf size and internode length are important plant architectural traits for breeding, especially because of their impact on the vertical distribution of light through the crop canopy and the consequent effects on the efficiency of light interception (Sarlikioti et al. 2011a, 2011b; Silva et al. 2018). Currently, tomato breeding for indeterminate growth habit is focused on reducing the length of internodes to increase the number of trusses per stem length (Zsögön et al. 2017). However, this can increase self-shading and reduce the efficiency of light absorption (Sarlikioti et al. 2011b). At a given light intensity, the leaf insertion angle has direct implications on the amount of light received per leaf surface unit (Ehleringer and Werk 1986; Ezcurra et al. 1991) whereby a smaller leaf insertion angle (i.e. more erect leaves) can lead to more uniform light intensities vertically through the canopy, reducing light stress at the upper levels and increasing photosynthesis in the lower levels. More erect leaves may also improve the efficacy of contact pesticide applications by improving penetration through the crop canopy and reaching more efficiently abaxial leaf surfaces.

Although the control of lateral growth has been extensively studied at the molecular level in some species, the genetic and physiological mechanisms that define the insertion angle of distinct organs (including leaves) have not been properly characterized in many taxa, including Solanaceae (for general review see Wang and Li 2008; Teichmann and Muhr 2015; Roychoudhry and Kepinski 2015). So far, all physiological models converge to the central role of auxin content and distribution in

the response to the continuous growth of shoots and roots (Firml et al. 2003; Roychoudhry and Kepinski 2015). Polar transport mediated by PIN and AUX/LAX proteins is a major mechanism that regulates auxin distribution in plants. These gene products control cellular auxin efflux and influx, respectively, through their subcellular localization at the plasma membrane (Wiśniewska et al. 2006; Vanneste and Friml 2009).

In tomato, characterization of genes that control auxin fluxes and directional growth of organs is still quite limited. Pattison and Catalá (2012) studied the function of the genes in tomato homologous to *PIN* and *AUX*/*LAX* and they verified that *SlPIN4* and *SlPIN3* have specific roles in the regulation of vegetative shoot architecture. Recently, Shi et al. (2017b) proposed a model to explain how the auxin polar transport mediated by *PIN1* is critical in tomato leaf polarity formation. In other plant species, a distinct set of genes associated with plant architecture has been identified. In monocotyledons, *TILLER ANGLE CONTROL1* (*TAC1*) (Yu et al. 2007) and *LAZY1*, a gravitropism-related gene (Li et al. 2007), are the main genetic factors identified as being involved in the regulation of shoot angle in rice (*Oryza sativa* L.). In the case of *TAC1*, a mutation that reduces gene expression is responsible for a lower insertion angle of the lateral shoots (i.e. more erect). The *LAZY1* gene has sequence motifs similar to *TAC1* with the addition of an Ethylene-responsive element binding factor–associated Amphiphilic Repression (EAR) domain. *LAZY1* loss-of-function mutants are associated with larger insertion angle of lateral shoots. It has been demonstrated that the recessive *lazy1* mutant increases the polar (apical–basal) auxin transport and decreases lateral transport, generating an abnormal auxin flow/distribution. The associated phenotype is due to the loss of gravitropism, leading to larger leaf insertion angles (Roychoudhry and Kepinski, 2015). In peach [*Prunus persicae* (L.) Batsch], the semi-dominant allele "*broomy*" (*br* – which was later designated as "*pillar*") is responsible for a more vertical growth of the branches (Scorza et al. 1989, 2002). Subsequent genetic/genomic characterization determined that the recessive *br* allele is a non-functional mutation of a homologue of the monocotyledonous gene *TAC1*, which they denominated *PpeTAC1* (Dardick et al. 2013). Plants with the homozygous *br* allele showed higher auxin concentration in shoots when compared with wild-type

plants with a horizontal branch pattern (Tworkoski et al. 2006). The auxin content in peach was found to be inversely proportional to the expression of the *TAC1* gene (Tworkoski et al. 2015). The silencing of *PpeTAC1* orthologue gene in *Arabidopsis thaliana* generated a "more erect plant", suggesting that this class of genes works universally in promoting vertical growth. *TAC1* and *LAZY1* belong to a superfamily of genes defined by an IGT (GφL(A/T)GT) domain, which is present in a wide array of plant genomes and is related to the vertical growth of the shoots through the regulation of auxin polar transport as demonstrated in rice, maize, *A. thaliana*, and peach (Dardick et al 2013, Roychoudhry and Kepinski 2015). The understanding of the genetic control of shoot architecture could provide breeding tools for selection of cultivars with improved utilization of light, more adapted to high planting densities (Testa et al., 2016), and that also increases the efficacy of pesticide applications.

We have observed in some segregating tomato breeding lines a peculiar erect leaf phenotype, which apparently affects all aerial organs, especially young shoots. A highly endogamic breeding line (named 'LT05') was recovered and it showed a genetically stable erect leaf phenotype. Two apparently similar phenotypes have already been described in tomato: a radiation-induced recessive mutant *erecta* (*er*) (Tomato Genetics Resource Center: http://tgrc.ucdavis.edu/, last accessed 12 March, 2018), present in the accession *S. lycopersicum* LA600 (derived from the 'Codine Red' cultivar) and a spontaneous semi-dominant mutation named *Erectoid leaf* (*Erl*) (Georgiev and Kraptchev 1992; Tomato Genetics Resource Center: http://tgrc.ucdavis.edu/, last accessed 12 March, 2018). Additional observations indicated that the *Erl* mutation co-segregated with the *uniform ripening* (*u*) mutation (Georgiev and Kraptchev 1992). The *u* mutation was identified as a loss-of-function allele of the *GOLDEN2-LIKE* (*GLK2*) gene located on tomato chromosome 10 (Kinzer et al. 1990; Powell et al. 2012). Here, we investigate the genetic basis and chromosomal location of the erect leaf phenotype observed in the inbred line 'LT05'. Based on a further genomic analysis, we report a strong candidate gene at the *Erl* locus containing a loss-of-function mutation. The identification of genetic variability associated with leaf angle in tomato opens the possibility for fine-tuning manipulation of plant architecture in breeding programs.

6.3. MATERIALS AND METHODS

Accessions employed as parental lines and development of segregating populations - The genetic basis of the erectoid leaf mutation was investigated using three distinct segregating F_2 populations. The first segregating F_2 population was generated from a cross between the *S. lycopersicum* inbred line 'LT05' (with the erectoid leaf phenotype and uniform ripening, genotype *uu*) and *S. lycopersicum* 'LT17' (an inbred line with the wild-type horizontal leaf phenotype). Analyses were conducted with the contrasting parental lines (13 plants each) and five crossing generations: F_1 (n=13), reciprocal F_1' (n=13); backcross (BC) to 'LT05' (n=32), BC to 'LT17' (n=37), and F_2 (n=138). The second segregating F_2 population (n=274) was obtained from the cross 'LT05' × *S. pimpinelifollium* 'TO-937' (with green fruit shoulder, genotype *UU*) (Powell et al. 2012). A third F_2 population was produced by first generating a pair of near-isogenic lines (NILs) and then crossing them. These NILs were created as follows: from the *S. lycopersicum* 'LT05' × *S. lycopersicum* 'LT17' cross, three putative heterozygous F_2 plants (with intermediate leaf phenotype) were visually selected and selfed to generate three segregating F_3 families of 20 plants each. Individual F_3 plants with intermediate leaf phenotype (i.e. putative heterozygous) were then chosen to continue a consecutive progeny testing-based process of selection and subsequent selfing. This process was repeated until obtaining segregating F_5 families. In this step, three individual F_5:F_6 plants with erect leaf phenotype and three with normal leaf phenotype were visually selected within the same segregating progeny and then selfed. Single F_6 plants able to generate progenies with stability for each of the opposing traits (i.e. the erect leaf versus normal leaf) were chosen as the contrasting near-isogenic lines and named as 'IsoL-EL' (with stable erectoid leaf phenotype) and 'IsoL-WTL' (with stable wild-type leaf phenotype). The NILs were then crossed ('IsoL-EL' × 'IsoL-WTL') and the F_1 plants were selfed to generate an F_2 population (IsolF_2) composed by 127 plants. The IsolF_2 population was, therefore, segregating mainly for the erect/normal leaf phenotype, whereas outside this locus the genetic background was predominantly a homozygous non-segregating mosaic of the

genomes of the two parental lines ‘LT05’ and ‘LT17’. The IsolF$_2$ population was also used in candidate gene validation analyses (see section below).

Evaluation of the leaf growth pattern (erectoid versus wild-type) - All evaluated plants were cultivated under greenhouse conditions in 5L pots filled with a mixture of soil and commercial peat. Individual plants were pruned to a single main stem. For inheritance studies, the leaf insertion angle (α) between the leaf petiole and the main stem (Supplementary Material C1) was measured in fully developed leaves and employed as a phenotypic indicator of each individual plant. In the case of the F$_2$ population derived from the cross *S. lycopersicum* ‘LT05’ × *S. lycopersicum* ‘LT17’, two measurements were made at 80 days after sowing. One measurement was done in the lower leaf (immediately below the first floral truss) and other in the first fully developed leaf (counting from the apex). For analyses, both measures were averaged to generate a mean α angle value that was converted to an ordinal scale according to the following criteria: mean α angle < 100° = erectoid leaf, mean α angle between 100-125° = intermediate, mean α angle > 125° = wild-type with standard leaf phenotype. For all segregating F$_2$ populations used in trait chromosomal location and candidate gene validation studies, the classification of the leaf growth trait was done by directly assessing the general aspect of the plants under greenhouse conditions. The plants were classified as either erect leaf or non-erect leaf. This last category involved intermediate as well as wild-type leaf phenotypes.

Chromosome mapping of the erectoid leaf trait - The F$_2$ population from the cross *S. lycopersicum* ‘LT05’ × *S. pimpinelifollium* ‘TO-937’ was employed to verify the linkage of the erectoid leaf phenotype with the uniform ripening *SlGLK*2 gene (*u, Solyc10g008160*) (Powell et al. 2012). The green fruit shoulder phenotype (presence of the *U* allele) or its absence (due to the homozygous presence of the *u* allele) was used as a phenotypic marker to evaluate co-segregation with the erectoid leaf trait observed in our populations. Evaluation was carried out visually employing a simple scale of presence/absence for both traits.

Genetic and statistical analyses - For leaf insertion angle (α), the standard error and ANOVA were calculated using the software InfoStat/L free version 2008 (Di Rienzo et al. 2008). For ANOVA, significant differences were claimed for $P < 0.01$ in a Tukey and Dunn's post-hoc test. A chi-squared test was applied to: (a) verify across the F_2 populations the goodness-of-fit of the erectoid vs. wild-type segregation ratios to Mendelian segregation models, and (b) to confirm the linkage between the erectoid leaf trait and the phenotypic marker green fruit shoulder, searching for a statistical difference from a 3:1 segregation ratio (i.e. independence) in a sub-group of erectoid and wild-type F_2 genotypes. In all cases a probability level (*P*-value) is given as the value for the null hypothesis.

Comparative genomic analyses of the chromosome 10 and variant screening - Resequencing information was obtained using genomic DNA from the erectoid leaf line 'LT05' and from four genetically diverse wild-type leaf accessions: 'TO-937' (Powell et al. 2012), 'CNPH498' (data not shown), 'Santa Clara' (Carmo et al. 2017), and 'Viradoro' (Giordano et al. 2000). Genomic DNA was extracted from leaf tissue of these accessions using DNeasy PowerPlant Pro Kit (QIAGEN Hilden, Germany). Whole genome sequencing of individual samples was sequenced on one lane of a HiSeq 2500 (Illumina Inc., San Diego, CA), at the Centro de Biotecnologia Animal (ESALQ/USP). Sequencing was performed with 100 bp paired-end reads. Quality control was done using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). Sequencing data from each of the five accessions was assembled separately against the tomato reference genome (version SL2.50) using SeqMan NGen version 14 software with default parameters (Lasergene, DNASTAR, Madison, WI, USA). An in-line Bayesian modeled variant detector based on the MAQ caller (Li, 2008) was used to tabulate SNPs and small indels relative to the reference genome in each accession. Variant calls from the five assemblies were then combined for further analysis in ArrayStar 14 (Lasergene Suite 14). Based on the chromosome mapping studies of the erect leaf trait, we carried out a search for gene variants only within chromosome 10 using variant calls from the five accessions. Since we also found the reference genome, 'Heinz 1706'

(Tomato Genome Consortium, 2012), which have a standard, wild-type (non-erect) leaf pattern, we focused on identifying non-synonymous variants that were exclusively found in the erect leaf line 'LT05'. To do so, the following series of filtering criteria was used in ArrayStar: (1) non-synonymous variants occurring in 'LT05' but none of four non-erect leaf accessions (and by definition, not in the reference sequence); (2) variant positions with a minimum depth of coverage of 10; (3) SNP% of 100 and (4) Qcall ≥ 7. Summary variant information was exported for individual accessions and then imported into Microsoft Excel (Office 2016; Microsoft Corp., Redmond, WA) for further filtering and analyses. These final variants were manually verified by inspection of the corresponding sequence assemblies to eliminate artifacts arising from assembly differences. Finally, the best candidate genes were chosen considering: the physical/genetic position respect to *SlGLK2*, the protein annotation (ITAG 2.40; http://solgenomics.net/, last accessed 18 April, 2018), the predicted effects of amino acid substitutions on protein function using the PROVEAN tool (Choi et al. 2012) (http://provean.jcvi.org/index.php, last accessed 18 April, 2018) and the expression pattern according to TomExpress RNAseq database (Zouine et al. 2017) (http://gbf.toulouse.inra.fr/tomexpress/, last accessed 12 March, 2018).

Multiple alignments of the predicted protein sequences from *TAC1*-like genes of tomato and other plant species - An erectoid leaf locus-specific non-synonymous, single nucleotide polymorphism (SNP) was found in the putative tomato *TAC1*-like gene located on the chromosome 10 (XP_004248091 = *Solyc10g009320*). For this reason, multiple alignments of the predicted TAC1-like amino acid sequences from the wild-type and erectoid leaf tomato lines as well as from different plant species were carried out. TAC1-like amino acid sequences of peach (XP_020413395), soybean (KRH06413), *A. thaliana* (OAP08981.1), maize (NP_001170644), and rice (BAF25656.2) were retrieved from GenBank database and aligned employing the Muscle algorithm with the default parameters on Geneious software v7.1.5.

Validation of *Solyc10g009320* as a candidate gene of the erectoid leaf phenotype - Individuals of two F_2 populations were used: the 127 individuals of the IsolF_2 and 71 erect and 67 non-erect individuals of the ('LT05' × 'TO-937') F_2, comprising a total of 265 F_2 individual plants. Genomic DNA was extracted from leaf samples collected at the apex of each individual plants according to a modified methodology employing 2X CTAB and organic solvents (Boiteux et al. 1999). From the sequence variants of *Solyc10g009320*, a primer pair TAC-Tom (F/R) flanking the identified SNP site was designed using PrimerSelect (Lasergene, DNASTAR, Madison, WI, USA). PCR with this primer pair amplified a 400 bp DNA fragment encompassing the SNP site detected within allelic variants of *Solyc10g009320*. Primer sequences for TAC-Tom (F/R) were: 5′-GAG-TTC-AGT-AAG-TGG-TCA-AGA-3′ / 5′-AAA-GAA-AGG-ATC-ACT-CTA-GCA-GA-3′. PCR reagents were adjusted to a final volume of 12.5 µL using 5.95 µL Milli-Q water, 1.25 µL of 10X Buffer *Taq* polymerase (100 mM Tris- HCl, pH 8.3 and 500 mM KCl), 0.6 µL of $MgCl_2$ (50 mM); 0.5 µL of dNTPs (2.5 mM); 1 µL of each primer, 0.1 U/µl *Taq* DNA polymerase (Invitrogen, Gaithersburg, MD, USA), and 2 µL of DNA template (30 ng μL^{-1}). The amplification conditions were an initial stage of denaturation at 94 °C for 2 minutes, followed by 30 cycles of: denaturation at 94 °C for 30 seconds, annealing at 60 °C for 1 minute and extension at 72 ° C for 1.5 minutes; ending with an extension step at 68 °C for 10 minutes. After purification of PCR products using Wizard kit (Promega, Madison, WI, USA), the amplicons were subjected to Sanger sequencing using an ABI Prism 3130 sequencer of the Genomic Analysis Laboratory (Embrapa Vegetable Crops, Brasília–DF, Brazil) employing the ABI Prism BigDye version 3.1 Kit (Applied Biosystems Division, Foster City, CA, USA) and the primer pair TAC-Tom (F/R). Sequence quality analysis, the removal of low quality fragments, and the identification of consensus sequences were performed using the SeqMan program (Lasergene, Madison, WI, USA). Alignment of multiple protein sequences was performed using the Clustal W method of the MegAlign software package (Lasergene, Madison, WI, USA).

6.4. RESULTS

Phenotypic characterization and genetic basis of the erectoid leaf growth trait - The most remarkable effect of erectoid leaf phenotype present in the inbred line 'LT05' (our original source of this mutation) consisted of a significantly more vertical growth of leaves, leaflets, and lateral shoots when compared to the control wild-type phenotype 'LT17' (Fig. 1). This trait was also easily identifiable by visual analysis in all segregating F_2 populations. In fact, this mutation expressed its phenotypic effects on all aerial structures (i.e. leaflets, leaves, shoots, and trusses) with more striking manifestation in young shoots. However, due to a former description in the literature limited to the leaf phenotype (Georgiev and Kraptchev 1992; Tomato Genetics Resource Center: http://tgrc.ucdavis.edu/), we decided to keep the nomenclature of this mutation as "*erectoid leaf*". The analysis of the phenotypic results of the cross 'LT05' × 'LT17' population showed a clear contrast between the parental lines ($P < 0.0001$). The mean insertion angle α was 77 ° ± 2 ° for 'LT05' (n=13) and 142 ° ± 1 ° for 'LT17' (n=13). The reciprocal hybrids (obtained using each parent as either male or female) displayed similar results.. The reciprocal hybrids displayed an intermediate leaf insertion angle between both contrasting lines ($p < 0.0001$). The average insertion angle α was 120 ° ± 1 ° and 119 ° ± 1 ° for F_1 (n=13) and F_1' (n=13), respectively (Fig. 2 a). These results suggested the absence of maternal inheritance effects. For the segregating families the distribution of their individuals in three different categories of leaf insertion angle (erectoid, intermediate, and wild-type) was analyzed (Fig. 2 b). The backcross family 'LT05' × F_1 segregated very close to a 1:1 (intermediate : erectoid) ratio. The same was observed with the backcross family 'LT17' × F1 with a 1:1 (intermediate : wild-type) ratio. The F_2 family segregated very closely to the expected 1:2:1 (erectoid : intermediate : wild-type) ratio. Finally, the F_3 families derived from the self-pollinating of F_2 plants with wild-type leaf insertion (Fig. 2 c right) displayed 100% individuals with wild-type leaf insertion, while in F_3 families derived from F_2 individuals with erectoid leaf insertion (Fig. 2 c left) had 100% of individuals with erectoid leaf insertion. These segregation patterns indicate a strong fit for a single gene model with semi-dominant inheritance of the erectoid leaf phenotype.

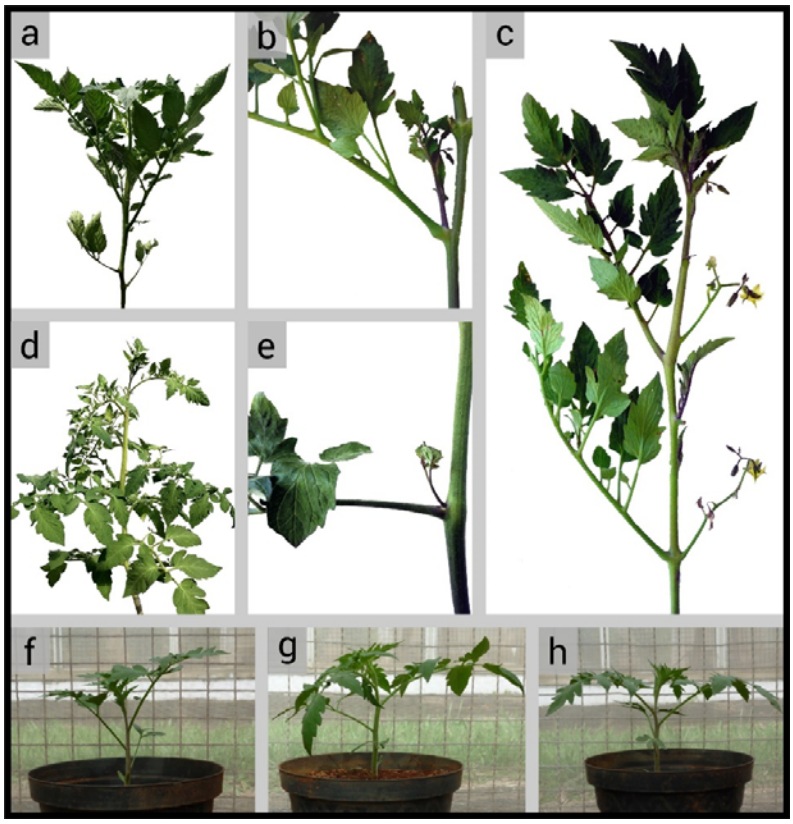

Fig. 1 Vegetative effects of the contrasting erectoid leaf alleles (*Erl*$^{+}$ versus *Erl*) in distinct developmental stages of tomato (*Solanum lycopersicum*) plants. (**a)** Plant of the inbred line 'LT05', the original homozygous (*Erl*/*Erl*) source of the erectoid leaf phenotype; (**b)** Detail of the foliar insertion angle with respect to the main stem in the 'LT05' line. (**c)** Detail of an apical section of a main stem of 'LT05' line, showing the effects of the erectoid leaf mutation on the growth of leaflets, leaves, shoots, and trusses. (**d)** Plant of the inbred line 'LT17', used as a reference for the standard wild-type phenotype (*Erl*$^{+}$/*Erl*$^{+}$). (**e)** Detail of the foliar insertion angle with respect to the main stem in 'LT17' line. (**f)** Plant of 'IsoL-EL' isoline (*Erl*/*Erl*). (**g)** Plant of 'IsoL-WTL' isoline (*Erl*$^{+}$/*Erl*$^{+}$). (**h)** Heterozygous (*Erl*$^{+}$/*Erl*) F_1 plant derived from the cross of 'IsoL-EL' × 'IsoL-WTL' with intermediate leaf angle phenotype.

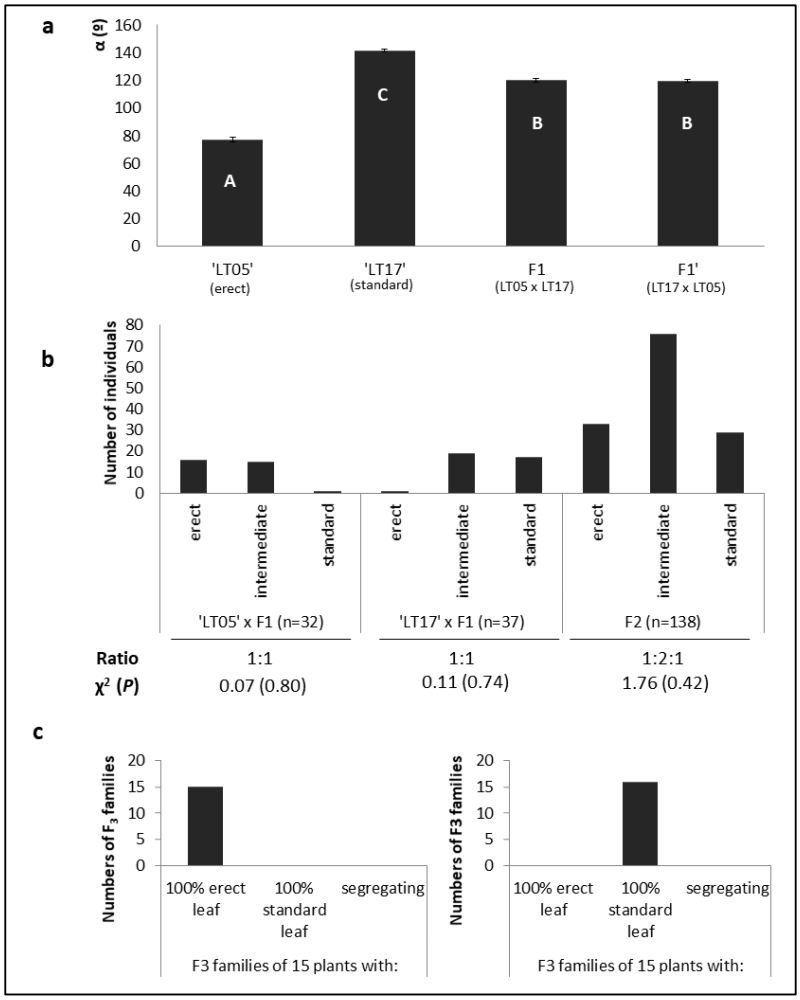

Fig. 2 Leaf insertion angles in 'LT05' × 'LT17' in segregating populations. (**a)** Value of the α angle in 'LT05' (erectoid) and 'LT17' (wild-type with standard leaf growth) and the reciprocal hybrids generated in both crossing directions F_1 ('LT05' × 'LT17') and F_1' ('LT17' × 'LT05'). Values shown as mean ± standard error (n=13). Treatments with different letters in the bars are significantly different (P<0.0001). (**b)** Phenotypic frequency (mean α angle of erectoid < 100°, mean α angle of intermediate between 100-125° and mean α angle of wild-type >125°) in three segregating families: ('LT05' × F_1), ('LT17' × F_1) and F_2 (F_1 × F_1). The Chi-squared test for Mendelian segregation models is shown. (**c**) **Left**: F_3 families derived from self-pollinating of F_2 plants with erectoid leaf growth phenotype. **Right**: F_3 families derived from self-pollinating of F_2 plants with the standard, wild-type (=non-erectoid) leaf growth phenotype.

Chromosome location of the gene controlling the erectoid leaf phenotype - Each plant of the F_2 family derived from the cross between 'LT05' and 'TO-937' was simultaneously evaluated for the erectoid leaf and the uniform fruit ripening phenotype. A clear-cut 1:3 segregation (monogenic) was observed for both erectoid leaf growth (75:199) and uniform fruit ripening (68:206) (Table 1). Within the subgroup of "erectoid" leaf growth (n=75) and the subgroup of "intermediate or wild-type" leaf growth (n=199), it was possible to observe a strong deviation of the expected ratio 3:1 for green fruit shoulder (dominant) and uniform fruit ripening (recessive), indicating that the two loci were not segregating independently, thus confirming their co-location on the tomato chromosome 10. A frequency of 12% of recombinants (33/274) indicated a relatively close genetic distance between these two loci.

Table 1 Phenotypic evaluation of an F_2 population (n=274) derived from the cross between 'LT05' (erectoid leaf growth; uniform fruit ripening) and 'TO-937' with standard (wild-type) leaf growth and green fruit shoulder trait.

	Erectoid leaf growth	**No-erectoid leaf growth (intermediate or standard)**
Uniform ripening	20	186
Green shoulder	55	13
Fit for expected 3:1 ratio	χ^2>20 (P<0.0001)	χ^2>20 (P<0.0001)

Comparative analyses of chromosome 10 in the genomes of contrasting (erectoid vs. wild-type) lines - To identify potential polymorphisms associated with the erectoid leaf trait, a strategy based upon the comparative genome analyses of the original erectoid leaf source (= *S. lycopersicum* 'LT05') with the genomes of genetically unrelated accessions displaying standard (wild-type) leaf angle phenotypes (viz. 'Viradoro', 'TO-937', 'Santa Clara', 'CNPH498' and 'Heinz 1706') was employed. A total of 27,259 variants were found only on chromosome 10. After

filtering for variants exclusively present in 'LT05', a total of 1,702 variants were obtained (Fig. 3 a). When filtering those variants for non-synonymous variants only five SNPs remained (Fig. 3 b, Table 2). One of these variants (corresponding to the alkaloid biosynthesis gene *Solyc10g086620*) was discarded because it was located far from the *SlGLK2* gene (*Solyc10g008160* located at position 2,293,088 in SL2.50), which was not in agreement with our data of 12% recombination (Table 1). The PROVEAN tool predicted that from the four remaining gene variants only one (*Solyc10g009320*) contained an amino acid change capable of inducing deleterious effect: an SNP that creates a new stop codon (Fig. 3 c and d). This *Erectoid leaf*-specific single nucleotide polymorphism (SNP) was found in the position 3,394,715 of chromosome 10, where a DNA substitution of a cytosine (C) for a thymine (T) (C>T) was observed. This SNP resulted in a change in the CAA codon (=coding for a glutamine) of the wild-type genomes to a TAA (= ochre stop-codon) in the genome of the line 'LT05' (with erectoid leaf trait). *Solyc10g009320* is about 1.1 Mbp from *SlGLK2*, in the euchromatin, consistent with the observed recombination frequency of 12%. The TomExpress RNASeq database reported a similar expression pattern in seeds, meristems, stem, leaves, and fruits for *Solyc10g009320*, but with higher expression in flowers and lower in roots (Supplementary Material C2). Thus, *Solyc10g009320* became our single candidate gene for controlling the erectoid leaf growth phenotype.

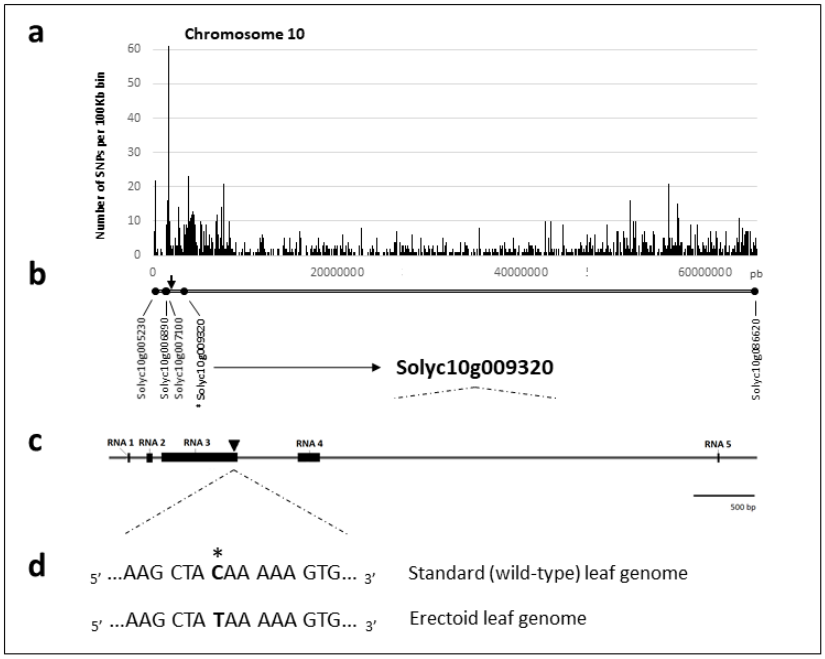

Fig. 3 (a) Variant density plot of the tomato (*Solanum lycopersicum* L.) chromosome 10. The figure depicts synonymous and non-synonymous variants that were exclusive for the genome of 'LT05' (an inbred line with the erectoid leaf growth) in comparison with the genomes of four accessions with standard (wild-type) leaf (viz. 'Viradoro', 'TO-937', 'Santa Clara', and 'CNPH498') as well as the reference genome 'Heinz 1706'; (**b)** Positioning of the single nucleotide polymorphisms – SNPs (black circles) that were selected after applying a filter for non-synonymous variants. The black arrow indicates the position of the linked *SlGLK*2 gene (controlling uniform fruit ripening) that was used as phenotypic marker to confirm the chromosome location of the erectoid leaf growth trait; (**c)** Structural features of the *Solyc10g009320* gene, identified as a strong candidate for control of the erectoid growth trait in the tomato line 'LT05'; (**d)** Genomic sequences of the accessions with standard (= wild-type) and with the erectoid leaf growth. The asterisk marks the position 3,394,715 of chromosome 10, where the 'LT05' genome (with the erectoid leaf growth trait) displayed a substitution of a cytosine (C) for a thymine (T) (C>T). This SNP resulted in a change in the CAA codon (=coding for a glutamine) of the wild-type leaf growth to a TAA (= ochre stop-codon) in the 'LT05' genome and in F_2 individuals carrying the erectoid leaf growth trait.

Table 2 Candidate genes located in the genomic region associated with the erectoid leaf growth trait (located at chromosome 10), containing *Solanum lycopersicum* 'LT05'-specific non-synonymous, single nucleotide polymorphisms (SNPs).

Gene identity	Nucleotide position (SL2.50)	Protein annotation (ITAG 2.40)	SNP position (SL2.50)	Non-conservative amino acid substitution	PROVEAN Prediction Score[1]
Solyc10g005230	184314-188942	Unknown protein	184523	V43M	Neutral (-1.068)
Solyc10g006890	1323124-1333653	WD-repeat protein	1323966	R183C	Neutral (-1.514)
Solyc10g007100	1487998-1492135	Protein detoxification 18	1491582	C56S	Neutral (-0.057)
Solyc10g009320	3390388-3395779	Unknown protein	3394715	Q222*	Deleterious (-61.263)
Solyc10g086620	65405159-65407893	Tropinone reductase homolog At5g06060 isoform X1	65392038	G170*	Deleterious (-248.543)

[1]PROVEAN (Protein Variation Effect Analyzer) is a tool which predicts impact of an amino acid substitution or indel on the biological function of a protein. Variants with a score equal to or below –2.6 are considered 'deleterious' and variants with a score above –2.6 are considered 'neutral'.

Sequence analysis of *Solyc10g009320* and impact on the predicted proteins - The gene *Solyc10g009320* was found to encode a predicted protein of 279 amino acids. When translated, the *Erl*-specific SNP caused a change of a glutamine (CAA) codon (present in all the wild-type genomes) to a TAA (= ochre stop-codon) at amino acid position 222 in exon 3 (Q222*) in the 'LT05' genome (with erectoid leaf trait), resulting in a smaller version of the predicted mutant protein (221 amino acids) (Fig. 3 c and d, Supplementary Material C3). BLAST analysis (tBLASTx tool) of the predicted protein encoded by *Solyc10g009320* gene indicated 36% amino acid identity with the gene *PpeTAC1*, belonging to the gene family IGT, described to have a major role in determining plant architecture in peach.

Multiple alignments of the predicted protein sequences from *TAC*1-like genes of tomato and other plant species - Multiple alignments of TAC1-like proteins from tomato and from a diverse group of plant species revealed the presence of the conserved domains described by Dardick et al. (2013), and specifically the presence of an IGT (GφL(A/T)GT) domain (Fig. 4), which is present in a wide array of plant genomes and is related to the vertical growth of the shoots (Dardick et al. 2013, Roychoudhry and Kepinski 2015). Due to the similarities between *TAC1* and *Solyc10g009320* we named this tomato ortholog as *S. lycopersicum TAC1* (*SlTAC1*) gene.

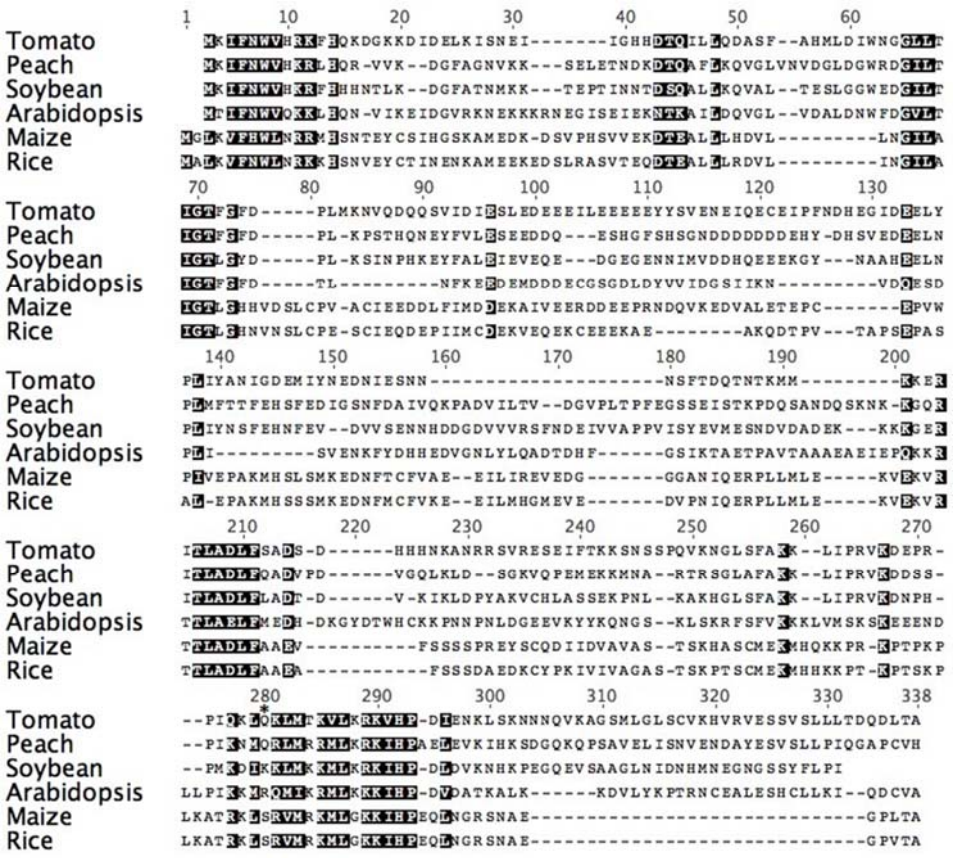

Fig. 4 Multiple alignments of the predicted protein sequences from *TAC1*-like genes of tomato (*Solyc10g009320*), peach (*PpTAC1*), soybean (KRH06413); *Arabidopsis thaliana* (*AtTAC1*), maize (*ZmTAC1*), and rice (*OsTAC1*). The plant species corresponding to each *TAC1* homolog are indicated in left column. Highly conserved residues are highlighted in black, including the conserved G□L(A/T)GT domain (at the position 70), which is characteristic of the IGT gene family. The gene *Solyc10g009320* was found to encode a predicted protein of 279 amino acids. The black asterisk highlights the position of the Q222* on the tomato homolog sequence (corresponding to the position 280 of the consensus alignment) where the stop-codon mutation was fond in the *Erectoid leaf* (*Erl*) allele.

Sanger sequencing validation of the SlTAC1-derived marker - PCR products obtained with the TAC-Tom (F/R) primers (designed to flank the identified SNP causing an early stop codon on *Solyc10g009320*) were Sanger sequenced in individual samples of two F_2 populations and the two contrasting near-isogenic lines. A total of 265 F_2 individuals were sampled from two F_2 populations. Sequence analyses of these individuals indicated the constant presence of the homozygous C>T substitution in all 108 F_2 plants with the erectoid leaf growth phenotype, in the parents, 'LT05' and in the near isogenic line IsoL-EL. On the other hand, the C>T substitution was found to be either absent or in heterozygous condition in all 157 F_2 with either intermediate or wild-type growth phenotype, in the parental lines 'TO-937' and IsoL-WTL (Supplementary Material C4).

6.5. DISCUSSION

The erectoid leaf phenotype is controlled by a semi-dominant locus on chromosome 10 - Our results indicated that the erectoid leaf phenotype observed in the line 'LT05' is controlled by a single semi-dominant gene/locus at the top of chromosome 10. This information was experimentally confirmed by the phenotypic analyses across distinct segregating populations and by linkage analysis with the uniform fruit ripening-coding *SlGLK2* gene, which is located on chromosome 10 (Kinzer et al. 1990; Powell et al. 2012). Two mutations with similar phenotypes were previously described in tomatoes: the *Erectoid leaf* (*Erl*) (Georgiev and Kraptchev 1992) and *erecta* (*er*) (LA0600). Given the similar characteristics in terms of location and pattern of inheritance, we assume that the gene that determines the erectoid leaf phenotype in the line 'LT05' is either the same gene or an allelic variant of the wild-type (Erl^+) gene as previously described by Georgiev and Kraptchev (1992) (Tomato Genetics Resource Center: http://tgrc.ucdavis.edu/, last accessed 12 March, 2018). Our results indicated that the Erl^+ allele is associated with the wild-type phenotype,

whereas the *Erl* allele is associated with the erectoid leaf trait. Heterozygous (*Erl*$^{+}$/*Erl*) plants displayed an intermediate leaf growth phenotype. Genes with similar phenotypic expression have been reported in other dicot and monocot plant species. In rice, the recessive loss-of-function allele of the *OzTAC1* gene (Yu et al. 2007) is one of the determinants of erect tiller growth and it has been used, along with other allelic variants, in several modern cultivars in order to generate more efficient crops (Dong et al., 2016). In peach, the "*broomy*" (*br*) allele of *PpeTAC1* defines the plant architecture by modifying the angle of insertion of the lateral branches and also displays a semi-dominant inheritance (Scorza et al. 1989, 2002). In this case, the possibility of manipulating the degree of branch inclination with distinct doses of the allele *br* (homozygous or heterozygous form) was suggested (Tworkoski and Scorza 2001).

A *TAC1*-like gene is the best candidate related to the erectoid leaf phenotype - Our genomic and genetic analyses allowed us to indicate *Solyc10g009320* (previously annotated as an 'unknown protein') as being the best candidate gene related to erectoid leaf growth observed in the 'LT05' line (zero recombinants in 265 F_2 plants analyzed, Supplementary Material C4). The methodology that allowed to identify *Solyc10g009320* as the more likely candidate gene was based on the genomic comparison of a line with phenotypically stable erectoid leaf phenotype ('LT05') with the genomic information obtained from the reference genome 'Heinz 1706' (Tomato Genome Consortium, 2012) and from four accessions with wild-type leaf growth (viz. 'Viradoro', 'TO-937', 'Santa Clara', and 'CNPH498'). The previous confirmation (by mapping) of the chromosomal location of the erectoid leaf phenotype was a key information that allowed us to apply genomic filters directly to chromosome 10, starting with a total number of 27,259 variants and reaching only five candidate genes according to additional genomic and putative gene function analyses (Table 2). This filtering allowed a more precise landing on potential candidate genes/loci. The estimated location on the chromosome 10 (close to the *SlGLK2* gene), and the identification of loss-of-function mutation altogether allowed selection of the *Solyc10g009320* as the most likely candidate gene associated with the erectoid leaf

growth phenotype. The validation using Sanger sequencing of the F_2 segregating population showed a 100% association of the erectoid leaf phenotype and *Solyc10g009320* mutation.

Due to the structural similarities of *Solyc10g009320* with an array of *TAC1*-like genes (see Fig. 4), we tentatively named this gene as *S. lycopersicum TAC1* (*SlTAC1*). Our annotation of *Solyc10g009320* as an ortholog of *TAC1* genes is consistent with a recent phylogenetic analysis (Guseman et al. 2017) where this tomato gene was placed into a small cluster along with other *TAC1* genes (e.g. *PpTAC1*, *OsTAC1*, *ZmTAC1*, and *AtTAC1*) within the IGT gene family. This work also identified a second tomato gene (*Solyc01g096260*) within the cluster of *TAC1*-like genes (Guseman et al. 2017). *Solyc01g096260* is located on tomato chromosome 1 and displayed ≈ 40% amino acid identity with *SlTAC1* (Supplementary Material C5). For this reason, we also examined polymorphisms within this gene across our resequenced accessions with contrasting leaf architecture. However, no variants were identified in comparative analyses of the proteins encoded by *Solyc01g096260* in the 'LT05' genome (with erectoid leaf growth phenotype) and the genomes of the four accessions with wild-type leaf phenotype (Supplementary Material C6). These analyses indicated that this evolutionary and functionally-related gene on chromosome 1 has no allelic variation in the coding sequence that could explain the phenotypic impact on the erectoid leaf growth in the germplasm employed in the present study, and indeed the genetic analysis showed that segregation of the locus on chromosome 10 was able to fully explain the occurrence of the erectoid leaf phenotype without the need to propose the involvement of a second locus. Moreover, the expression patterns reported in the TomExpress RNA Seq database (Supplementary Material C7) show differences to *Solyc01g096260* when compared with *Solyc10g009320*, with a notably greater increase of expression in flowers and fruits and a low or no expression in vegetative parts. Although these data should be confirmed with expression and functional tests, it is likely that these genes, even though belonging to the same family, could be under control of distinct expression mechanisms across distinct plant organs.

It is not yet known how the *TAC1* genes are involved in determining the direction of lateral growth, although some evidence suggests that it would be directly

or indirectly implicated in a negative regulation of *LAZY1* (Dardick et al. 2013) and this interaction, which varies depending on the position and aerial organ of the plant, would arise from a gravitropic response. In all cases described in the literature, the loss-of-function of *TAC1* genes (in both monocots and dicots plants) is associated with narrower insertion angles in tillers, leaves, and flowers (i.e. more erect posture) in comparison to the wild-type controls (Yu et al. 2007, Ku et al. 2011, Dardick et al. 2013). This fact is associated with higher auxin content in the affected organs, apparently resulting from a modification in the polar transport of this hormone (Li et al. 2007, Yoshihara and Iino 2007, Yoshihara et al. 2013). Since auxin transport/content is regulating a multiplicity of developmental processes (Reinhardt et al. 2003) mutants involved in this process might have pleotropic effects in several aspects of agronomic interest.

Auxin also controls many aspects of fruit development, including the sequential stages of fruit formation, expansion, ripening, and abscission (Gillaspy et al., 1993; Srivastava and Handa, 2005). In tomato, Pattison and Catalá (2012) showed a coordinated action of PIN and AUX/LAX proteins in the establishment of auxin gradients during fruit development. Artificially increasing the auxin levels in the ovary can bypass fertilization and lead to the development of parthenocarpic fruits (Lipari and Paratore 1988; Ficcadenti et al. 1999). This would have an interesting effect in extreme/hostile environments where fertilization is compromised by pollen viability problems. Additionally, regulation of auxin efflux by *SlPIN1* prevents flower abscission by maintaining a high auxin transport activity in the abscission zone (Shi et al. 2017a).

On the other hand, little is known about possible effects of the *TAC1*-like genes at the root system. The pioneering study of Tworkoski and Scorza (2001) in peach provided information that mutations in aerial architecture (including *tac1*) is also associated with changes in root growth pattern. In rice, it was also reported that *DEEPER ROOTING*1 (*DRO1*) controls the depth of the root system via the regulation of the insertion angle of lateral roots (Uga et al. 2013). An increase of the expression of this factor increases the depth of the root system. The deeper roots determined by *DRO1* allow a better performance and higher yield of the rice in water deficit

conditions (Uga et al. 2013). Later, Guseman et al. (2017) generated evidence and hypothesize that, since the functions of *LAZY1* and *DRO1* are homologous, in shoots and roots respectively, *TAC1* could be a negative controller of both genes, and this may explain why a reduced function of *TAC1* could influence the vertical growth both of the canopy and of the roots. In this context, it would be also of interest to investigate the potential effects of the *Erl* locus on the root system in tomatoes.

Phenotypic and agronomic effects of the *Erectoid leaf* (*Erl*) mutation - So far, no strategies have been proposed to genetically manipulate the inclination angles of lateral shoots in order to obtain tomato ideotypes with adaptation to a wide range of environmental conditions (Zsögön et al. 2017). The importance of leaf insertion angle in relation to efficiency and distribution of light absorption (and hence the photosynthetic crop capacity) has been established in theoretical models using tree/forest species (Pearcy and Yang 1998, Sinoquet et al. 2005). Most studies are in agreement that larger leaf insertion angles (i.e. more horizontal leaves) intercept a considerable greater amount of light when the sun is placed at high angle in relation to the horizon (e.g. midday, summer season, low latitude areas) while smaller leaf insertion angles (i.e. erectoid leaf) intercept a greater portion of light when the sun is at low angles in respect to the horizon (e.g. during early mornings and late afternoons, winter season, and high latitude areas). Falster and Westoby (2003) reinforced these observations by performing studies with several plant species under high radiation conditions, proposing that the cost of increased light interception of horizontal leaves can involve higher leaf temperatures, higher risk of sunscalds and photo-inhibition. Thus, under high radiation conditions, a smaller leaf insertion angle (i.e. more erect leaves) would allow greater protection against the damages caused by the excess of radiation, including the reduction of the heat levels at the leaf surface, increasing the efficiency of water use, minimizing leaf burn damage (Werner et al. 2001), decreasing photo-inhibition (Ryel et al. 1993, Valladares and Pugnaire 1999, Werner et al. 2001) and improving the water use in relation to the daily carbon gain (Cowan 1982). Our data also indicate that is possible to manage the dosage of the *Erl* allele in tomato hybrids, regulating, to a certain extent, the angle of inclination of leaves and leaflets

in relation to the horizontal plane, which in turn, could result in plants with wider adaptation. Higher levels of environmental adaptation would result not only in the increase of the light interception and temperature control, but also by improving the rates of ventilation renewal in the plant canopy. Better ventilation in the plant canopy can influence the CO_2 content as well as the relative humidity of the surrounding air. Another positive crop management consequence of the erectoid leaf trait is to facilitate the distribution of pesticides, especially those targeting the abaxial leaf surface. This leaf surface is the major site of oviposition of important pests such as whiteflies (Silva et al 2014), being also the place of sporulation of a wide array of fungal pathogens. In fact, the erectoid leaf trait may also provide micro environmental conditions that prevent fungal spore germination by maintaining lower humidity levels under the canopy, thus potentially reducing the frequency of fungicide applications. This possible increase in efficiency in pesticide application might reduce both the frequency of sprayings as well as the production costs.

In summary, we found that the genetic factor controlling a peculiar tomato erectoid leaf phenotype (that affects all the aerial organs, especially the young leaf shoots) is located at chromosome 10. We also found that this semi-dominant allele *Erl* co-segregated with a loss-of-function mutation of the gene *Solyc10g009320*. This gene is a strong candidate for the genetic identity of *Erl*. In fact, the protein coded by *Solyc10g009320* has structural features of TAC1-like proteins of the IGT gene family, which are distributed across a wide array of plant genomes, having a crucial role in the control the vertical growth of the shoots (Dardick et al 2013, Roychoudhry and Kepinski 2015). *TAC*1-like genes regulate auxin polar transport and content in shoots of several species, although it is not possible to discard that *TAC1*-like genes also control auxin transport/content in fruits and roots. From the breeding standpoint, this characterization of the mutant *Erl* will open the possibility for fine-tuning manipulation of tomato architecture and will allow development of more practical co-dominant molecular markers for employment in marker-assistance selection systems of this important trait. In addition, the genetic characteristics (loss-of-function mutation) of *Solyc10g009320* make it a potential target for gene editing strategies (Belhaj et al. 2015; Zsögön et al. 2017).

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7. CONCLUSIONES

A continuación, se enumeran las conslusiones de este trabajo de tesis:

1) La metodología aplicada permitió avanzar con éxito en la identificación de genes candidatos. La metodología aplicada puede dividirse en tres niveles. El primero corresponde al análisis de variables fenotípicas. En este paso es fundamental contar con las poblaciones de interés y las herramientas necesarias para la toma de datos. A partir del análisis de las variables dentro de las diferentes poblaciones fue posible determinar, comparando con modelos mendelianos básicos, las bases genéticas de los caracteres seleccionados. En primer lugar, determinar el número de genes involucrados o, en otras palabras, confirmar la presencia de un gen principal (con efecto principal en la determinación del fenotipo), es condicional para el planteao de las siguientes etapas. Caracteres de herencia compleja, determinados por la acción de varios *loci* de efecto compartido, requerirían una estrategia metodológica que tenga en cuenta algunas precisiones en la etapa de medición de variables fenotípicas (variables cuantitativas, efecto ambiental) y la etapa molecular (mapeo genético). En segundo lugar, conocer el tipo de herencia puede dar indicios del tipo de control génico (por ejemplo: ganancia de función relacionado a alelos dominantes y pérdida de función a alelos recesivos). Un desafío importante de esta etapa se plantea a la hora de disponer, en tiempo y forma, de las poblaciónes segregantes de interés. Luego será necesario contar con la capacidad de generar el ambiente necesario para la expresión del carácter y con una metodología sencilla y eficiente para su medición. Lograr una caracterización fenotípica de calidad, sencilla y representativa de la realidad, con el mínimo error experimental, condiciona el éxito de las siguientes etapas.

El segundo nivel fue el molecular. Siguiendo nuestros objetivos y habiendo confirmado la presencia de un gen principal, este paso se hace necesario para hacer una primera localización a nivel de cromosoma del factor genético, útil para poder facilitar/dirigir la búsqueda de polimorfismos asociados a nivel genómico. Se usaron diferentes estrategias, desde un marcador fenotípico conocido (maduración uniforme, gen *u*), marcadores RAPD dentro de la estrategia de Bulked Segregant Análisis (BSA)

y estudios de co-segregación con marcadores SCAR conocidos o diseñados especialmente a partir de información genómica (secuencia de parentales). Dependiendo de la información previa y de los recursos económicos o de infraestructura disponibles, otras estrategias han sido implementadas con éxito en esta etapa. Por ejemplo, trabajos recientes han realizado estudios de co-segregación con marcadores moleculares especialmente diseñados para cada brazo cromosómico a partir del resequenciamiento de ambos parentales contrastantes (Silva Ferreira et al. 2018). También pueden ser efectivas en este paso estrategias basadas en el secuenciamiento y comparación de conjuntos contrastantes de individuos F_2 (Dardick et al. 2013). Ambas estrategias no solo ubican el factor genético en un cromosoma sino que también generan alguna información más precisa sobre su posición, pudiendo a partir de aquí generar un filtro mayor para los genes candidatos.

Siguiendo con nuestra propuesta, el tercer paso se basó en el análisis comparativo de secuencias genómicas. Para eso es necesario re-secuenciar los genotipos portadores de los caracteres de interés y al mismo tiempo disponer de diferentes secuencias del "wild type" (en nuestro caso se utilizaron cuatro). Con esta información es posible filtrar de forma comparativa las variantes detectadas, incorporando la información previa generada: localización cromosómica, distancia aproximada a un marcador ligado, tipo de función génica (ganancia o pérdida de función). De esta forma, las varientes pueden asociarse con genes anotados, algunos de función conocida. Nuevos marcadores moleculares pueden diseñarse con el fin de seleccionar/validar el gen/variante dentro de una población segregante.

Los avances en tecnologías de secuenciamiento hacen cada vez más accesibles los estudios basados en comparaciones genómicas. Sin embargo, aún persisten (o pueden presentarse) limitantes relacionadas al costo o la capacidad estructural para manejar grandes volúmenes de información. Para compensar estas limitantes, en este trabajo se utilizó en forma práctica la combinación de herramientas metodológicas clásicas y modernas. Desde conceptos relacionados con el aterrizaje cromosómico o *chromosome landing* (Tanksley et al., 1995) hasta el uso del genotipado por secuenciamiento. Esta estrategia combinada, explotando al máximo el conocimiento

generado del carácter en cuestión, fue la que permitió resolver con éxito los problemas de investigación planteados.

2) La información generada facilita el uso en mejoramiento de nuevos caracteres de interés para la producción de tomate. Aplicando esta metodología, que integra conocimientos de medición y análisis fenotípicos, moleculares y genómicos, fue posible avanzar de forma significativa dentro de los cuatro caracteres seleccionados. El Cuadro 1 muestra el grado de avance logrado en este período de 4 años.

La información generada permite facilitar la utilización de estos caracteres en el mejoramiento genético de tomate. En primer lugar, fue hecha una descripción general de sus efectos fenotípicos, generando una idea del posible impacto en los sistemas productivos. Luego, fue determinado el tipo de herencia, lo que ayuda al planteo de estrategias de mejoramiento con el objetivo de incorporarlos en material elite. También, en algunos casos fue determinada la ubicación cromosómica, permitiendo conocer genes ligados (en acoplamiento y repulsión) con lo cual diseñar mejores estrategias de piramidación. Por último, se avanzó en conocer genes candidatos. De validarse con éxito, esto permitiría conocer otros efectos fenotípicos de interés asociados al mismo carácter. Tambien, generar marcadores moleculares funcionales para asistir procesos de selección asistida de precisión. Por último, abriría las puertas para posibles manipulaciones del gen tanto en tomate como en otras especies de interés

Cuadro 1 Avances realizados durante el trabajo en las cuatro características estudiadas.

Fenotipo de interes	Base genética	Marcador ligado	Cromosoma	Gen/es candidato/s	Gen validado geneticamente
Tolerancia a crinivirus	*cvt*: alelo recesivo	O14-450	---	---	---
Resistencia a metribuzin	*mtz*: alelo recesivo	Z11-930	1	---	---
Aumento de AA	*Vtc*: alelo dominante	*Sw-5-2*	9	GME2 LOC101249491	---
Crecimiento erecto	*Erl*: alelo dominante	*u*	10	Sl10g009320	*SlTAC1*

3) Se plantean nuevas hipótesis de trabajo para complementar y profundizar el conocimiento generado. Para el alelo *cvt* que determina tolerancia a *Crinivirus* aún resta por determinar si efectivamente la menor expresión de síntomas se relaciona con menores pérdidas a nivel productivo y en calidad de fruta. Además, como también quedó demostrado, el factor podría estar relacionado con una menor degradación de clorofila ante situaciones diversas de estrés.

El caso del alelo *Erl* que determina el crecimiento erecto, se identifican varios aspectos de interés relacionados a la adaptación al ambiente. En primer lugar, la posibilidad de manejar el ángulo de inclinación respecto a la horizontal de órganos vegetativos como las hojas, permite adaptarse a situaciones diversas respecto a la cantidad de luz, exceso de temperatura, ventilación y densidad de plantación, factores que tienen que ver con el concepto de eficiencia productiva. Pero además este gen tiene que ver con una regulación hormonal que, como se ha demostrado en otras plantas, puede alterar procesos relacionados con la adaptación ante otras situaciones de estrés abiótico relacionadas al ambiente del suelo. Al ser un gen cuya función está determinada por la pérdida de actividad génica, es posible que pueda ser explorado en otras especies de interés comercial.

El alelo *mtz*, relacionado con una alta resistencia al herbicida Metribuzin, podría generar un impacto considerable en la producción a campo, por ejemplo, en la

destinada a abastecer las industrias de procesamiento. Estos sistemas son los más extensivos dentro de la producción de tomate y buscan maximizar la ecuación calidad/costo. Este gen podría facilitar la etapa de control de malezas y hasta la implementación, hasta ahora limitada, de la siembra directa (sin trasplante), aportando a la mecanización del proceso productivo y a la eficiencia de la cadena producción-industria. Por el tipo de gen y su probable función detoxificadora, queda por explorar su involucramiento en la adaptación a otros grupos de herbicida e inclusive su respuesta ante otros estreses bióticos/abióticos. Al ser un gen cuya función está probablemente determinada por la pérdida de actividad génica, en la medida que se encuentre conservado en otras especies es factible de ser manipulado con por estrategias de edición genómica.

El alelo *Vtc*, relacionado con el aumento de ácido ascórbico (Vitamina C) en fruta madura marca un escalón considerable en la mejora del producto por calidad nutricional. Los incrementos aquí reportados, de hasta 60% del contenido en peso fresco de ácido ascórbico, no solo significan un aporte extra de la Vitamina C a nivel de consumidor, sino que estarían también relacionados con una mayor tolerancia de la fruta a estreses bióticos y abióticos.

4) Se fortalece el concepto de que integrar equipos multidisciplinarios es la mejor estrategia para potenciar la capacidad de innovación. El mejoramiento genético tiene como fin crear cultivares con al menos un carácter diferente o una combinación diferente de caracteres, con el objetivo solucionar un problema productivo. Existen dos niveles de acción: i) trabajar con caracteres conocidos para incorporarlos/combinarlos en un contexto genómico adecuado, y ii) trabajar en la identificación e incorporación de nuevos caracteres. Este segundo nivel potencia la capacidad de innovación de un programa de mejoramiento. Para abarcarlo, es necesario incorporar capacidad de trabajo y análisis en las tres etapas señaladas anteriormente: fenotípica, molecular y genómica.

La identificación de nuevos génes relacionados con efectos fenotípicos de interés no es solo un aporte directo al mejoramiento de un cultivo. La información generada puede ser utilizada como base para ampliar el conocimiento científico sobre aspectos

relacionados a la función génica, aportando por ejemplo a la genómica funcional y la proteómica. Desde ahí, la biotecnología aplicada puede generar procesos para controlar aspectos de interés en diferentes especies y aportar nueva diversidad a los procesos de mejoramiento. Por tanto, integrar equipos multidisciplinarios con capacidades y fortalezas en estos tres niveles permitiría no solo hacer aportes directos en tecnológía para la cadena producción-consumidor/industria a través del mejoramiento, sino también de base para el conocimiento científico y la industria biotecnológica.

Como reflexión final, es sabido que los reservorios de diversidad son un gran patrimonio del mejoramiento genético. Para especies relativamente estudiada y con gran trabajo acumulado desde el mejormiento genético, muchas veces se declara que la diversidad en el germoplasma cultivado es estrecha o limitada. Sin embargo, si consideramos que los sistemas productivos son exigidos continuamente a adaptarse a los cambios, ya sean impuestos por el ambiente (cambio climático, nuevas zonas de producción), por la disponibilidad de recursos para la producción (económicos, mano de obra), o las exigencias del consumidor final (calidad nutricional, inocuidad, impacto ambiental), podemos pensar que los mismos caracteres que antes pasaban desapercibidos hoy pueden ser de interés para resolver un nuevo problema. A modo de ejemplo, los alelos *cvt*, *Erl* y *Vtc* fueron observados desde sus efectos fenotípicos en campos de selección dentro del proceso de mejoramiento genético. El alelo *mtz* fue revalorizado a partir de datos bibliográficos de más de 30 años. Por tanto, queda comprobado que, aún dentro de material elite de *S. lycopersicum*, la diversidad no explorada es muy amplia, quedando muchos aportes por hacer en términos de descripción de nuevos caracteres de interés comercial.

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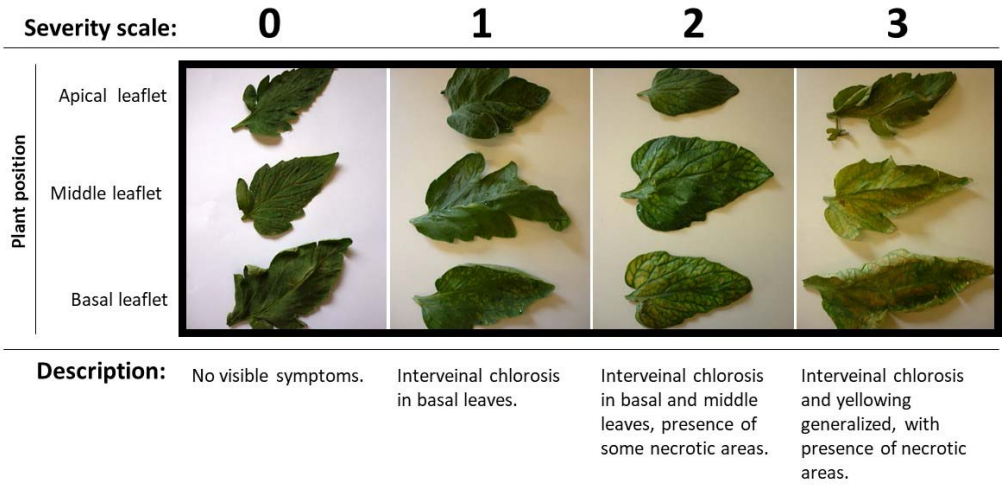
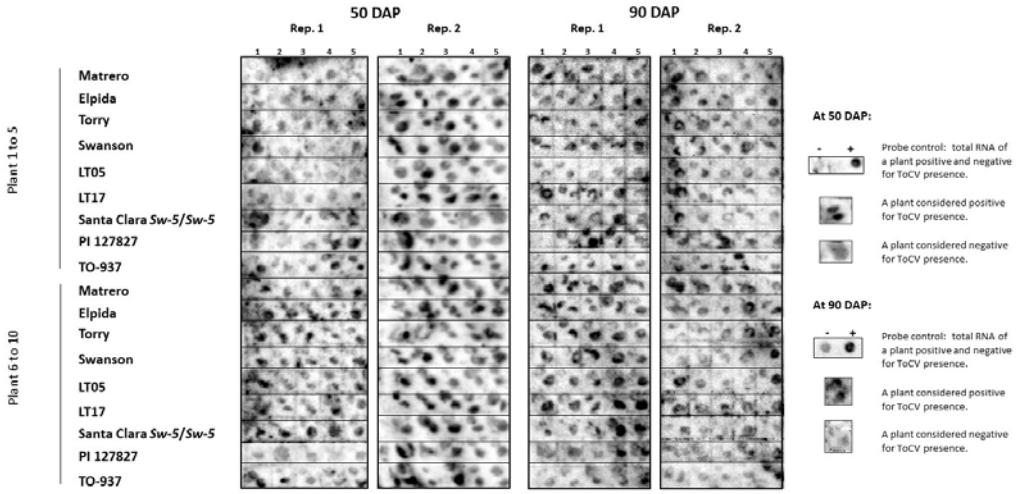
9. ANEXOS

A continuación, se aporta en idioma inglés el material suplementario referenciado en los capítulos 2, 4 y 6.

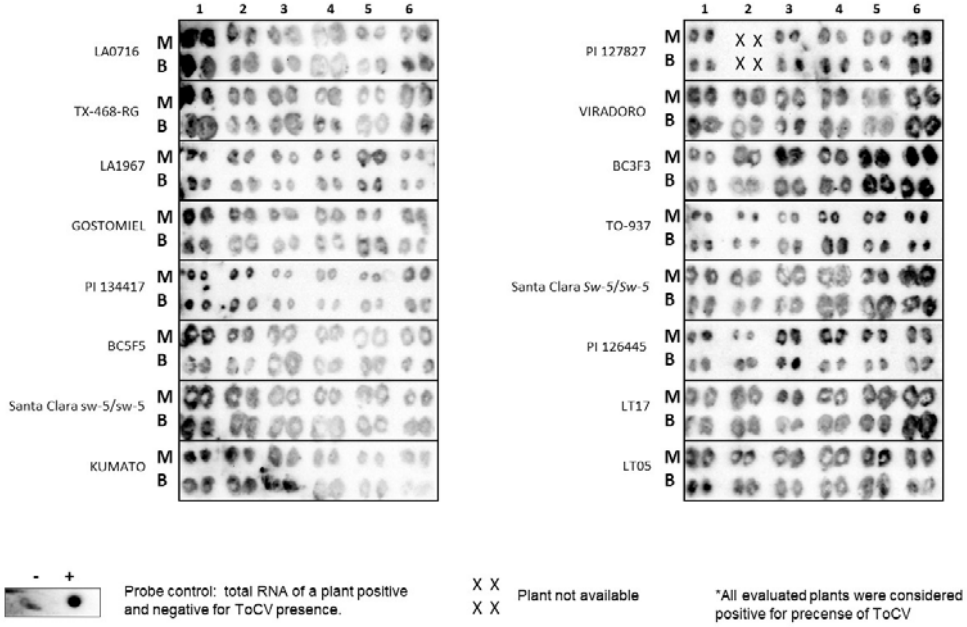
Supplementary Material A1 Visual assessment scale of ToCV-induced symptom severity.



Supplementary Material A2 For ToCV Trial #2, systemic presence of ToCV in all ten plants of each genotype. The evaluation was made for duplicate (Rep. 1 and Rep. 2) along with the symptoms evaluation at 50 and 90 days after plant (DAP). For tissue-print hybridization, fresh leaf petiole sections of basal leaves of each plant were directly squash-blotted (two squash blots per leaf petiole) on positively charged nylon membranes.



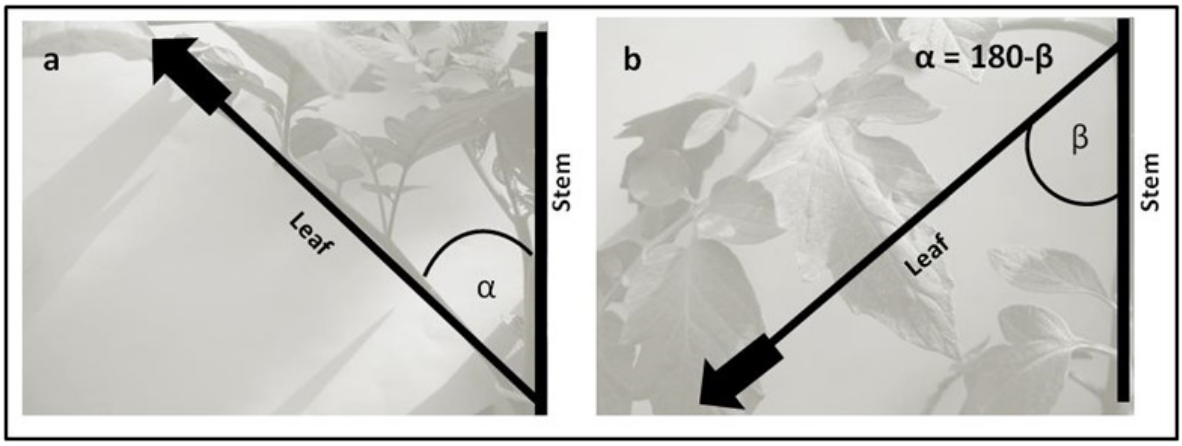
Supplementary Material A3 For ToCV Trial #3, systemic presence of ToCV in six plants of each genotype. The evaluation was made along with the symptoms evaluation at 70 days after plant (DAP). For tissue-print hybridization, fresh leaf petiole sections of basal (B) and middle (M) leaves of each plant were directly squash-blotted (two squash blots per leaf petiole) on positively charged nylon membranes.



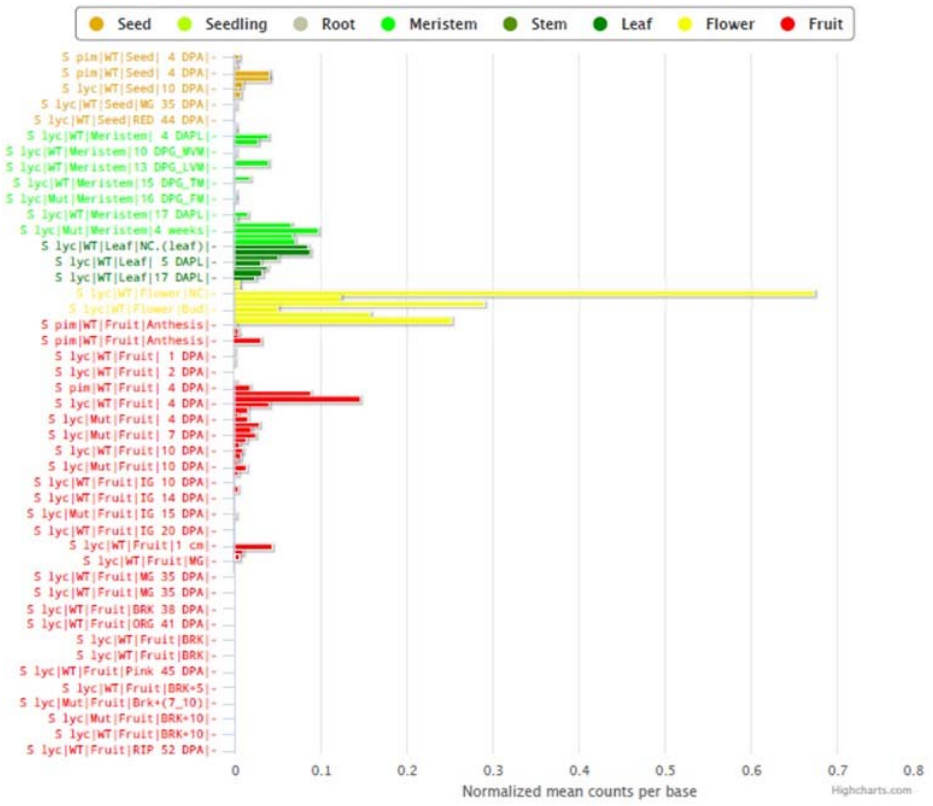
Supplementary Material C1 Sequence obtained for the polymorphic amplicon of OPZ11-930 marker. Plasmid DNA of each of ten colonies evaluated wer Sanger-sequenced using the primers pair M13. All sequences were identical. The sequence of the RAPD primer OPZ-11 (yellow highlighted) was confirmed flanking 928 bp (near the OPZ11-930 expected size).

GACTAGTCCTGCAGGTTTAAACGAATTCGCCCTTCTCAGTCGCAGCATTACCTGTAG
CGAACCAGTGAGGACAACCGGCTTCAAAGAATCATATAAACCAAAGTAGAGACCGCG
GTACACAATGATACCAACACATGAGATATTGAATCCACGGTAAAGTCCAGCAACTCC
ATCAGATGCTAATGTCTTCTTGTAAACATCAACCATACCATTGAACTGTCGATCTCC
TCCTCCTTTTGCAGCCTTAGCATCATTAGCTAGCCTCGTTCGGGCATAGTCAAGAGA
ATAGACAAAAAAAAGGGAaGATGCACCAGCTGCACCTCCTGATGCAAGATTACCAGC
AAACCATTTCCAGTAACCGTCTCTGTCCTTTTTGAAGTTAAAAAGCCTCTTGAAGTA
ATCCTTAAACGCAAAATTCAAgGGCCTGCACAAAAAAGTGAAAGAGATAAAATATAT
AGCACGAGATCAACGAAATGAACAAGGGATCTAAATTGTCTTGAGAAAGTAACGAAC
CTGAGTTGGGAAATAACGAATAACGTTTGCTGtGTTGCCTCTCCaCAAAGACaTAAC
TCCTTCcTCcTTcATtGGTCTGCTAAAACATTCAGCTATGCCGCCGTATGGTTTAGA
TAACCTCCCAGCCTTGATCATCTCACCCTGATTTTGGATCAGGAGTTTGACACGCTC
AATTGGAGCAGCTGCTGTCTTTGAAACAGCTGCTGAGACACCACCCATAAGAAAATC
AATTGCAAAGCTGGTAAAGCCTTTCTCTGAAGGAGCTTGGACGAAAACTGGCGATGA
GTAAGATGAAACCACGGGCATCCCTTCACATCTATGCAACCAGGGCTTAGTCTGACT
TCCATATTTATATTGCCTTTGGTATGCCATAAAATCTCGATTTTGCGTAGATCTACC
AAGGAAGTTTCCAGCTGCCTTGTGAGTAACAGAGGGGTATTGCGACTGAGAAGGGCG
AATTCGCGGCCGCTAAA

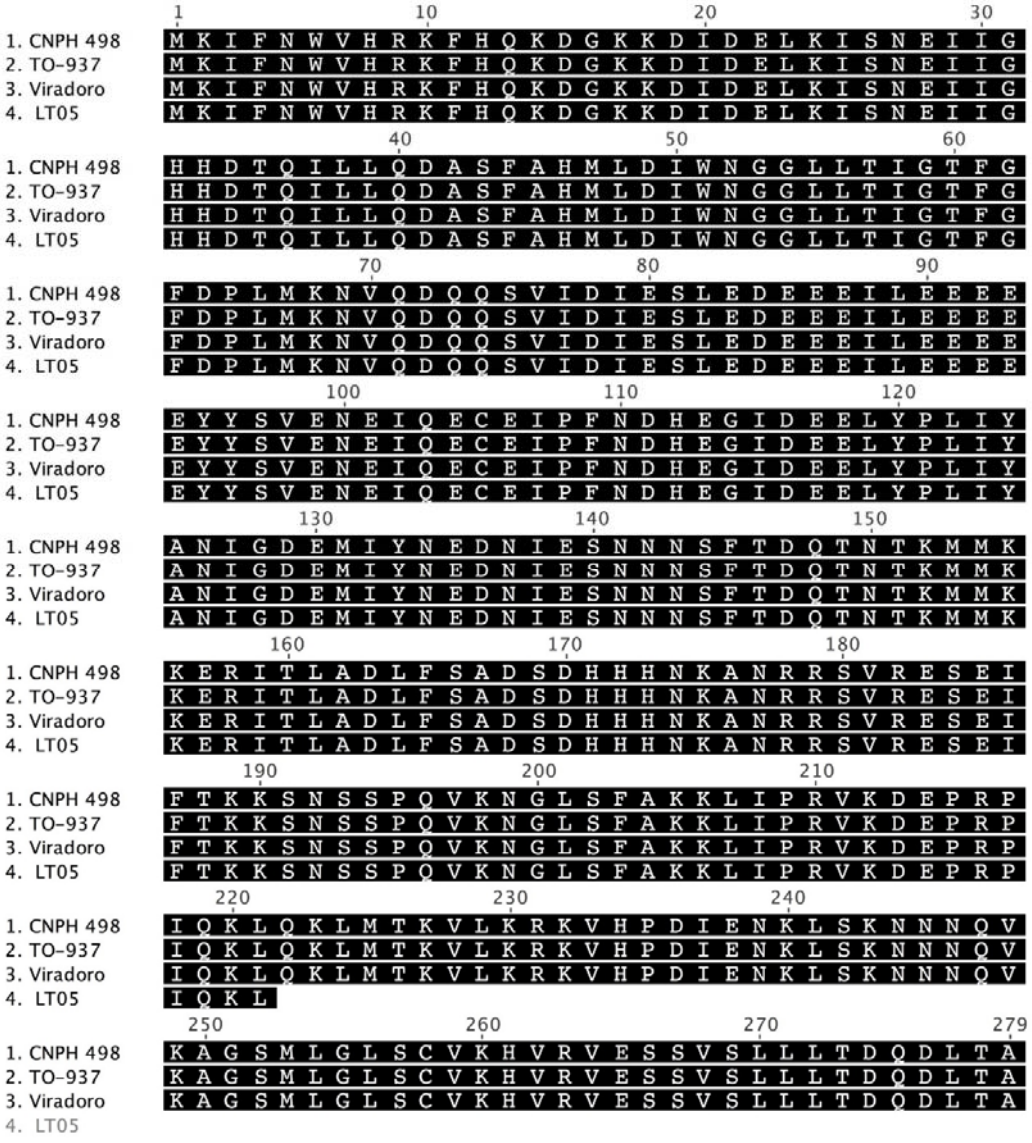
Supplementary Material C1 Representation of the α angle that was used to measure of leaf insertion type respect to the central stem (a) Erect leaf insertion of 'LT05' genotype at the first developed leaf from the apex (b) Standard leaf insertion of 'LT17' genotype at the first developed leaf from the apex.



Supplementary Material C2 Tissue-specific expression patterns of *Solyc10g009320*. Normalized mean counts per base are shown for each tissue sample. Data and downloads of plot images are from the TomExpress database and website (http://tomexpress.toulouse.inra.fr/) representing a collection of public RNAseq experiments. Different colors indicate distinct tissue types.



Supplementary Material C3 Multiple alignments of the predicted *Solyc10g009320* protein sequences of the tomato line 'LT05' (with erectoid leaf growth) and three wild-type (with standard leaf growth) accessions ('CNPH498', 'TO-937', and 'Viradoro'). The proteins are identical in their 279 amino acids, except for 'LT05' that presents a premature interruption at the amino acid residue 221.



Supplementary Material C4 Validation of the SNP within Solyc10g009320 gene in F2 individuals from different segregating populations characterized by growth type.

Population [1] IL-F2

	G>A substitution [3]		
Phenotype [2]	homozygous	absent	heterozygous
erect	38	0	0
non-erect	0	20	69

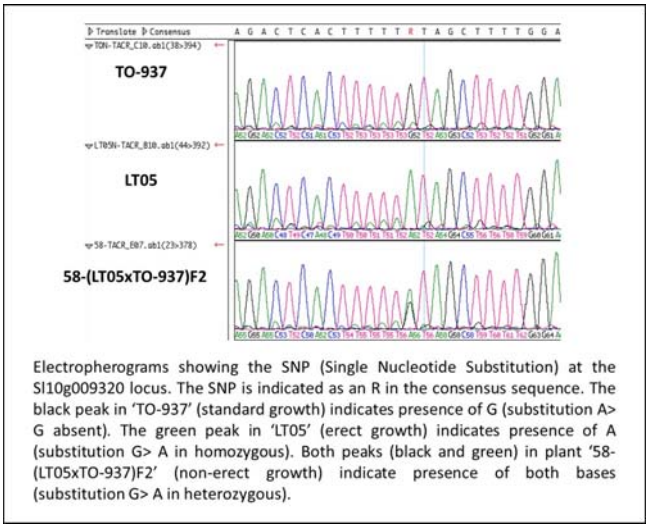
Population [1] (LTO5xTO-937)F2

	G>A substitution [3]		
Phenotype [2]	homozygous	absent	heterozygous
erect	71	0	47
non-erect	0	20	69

[1] **IL-F2**: F2 population derived from the cross between two near-isogenic lines (IL): IL-EL (erect growth) and IL-WTL (wild type standard growth). **(LTO5xTO-937)F2**: F2 population derived from the cross between 'LT05' (erect growth) and 'TO-937' (standard growth).

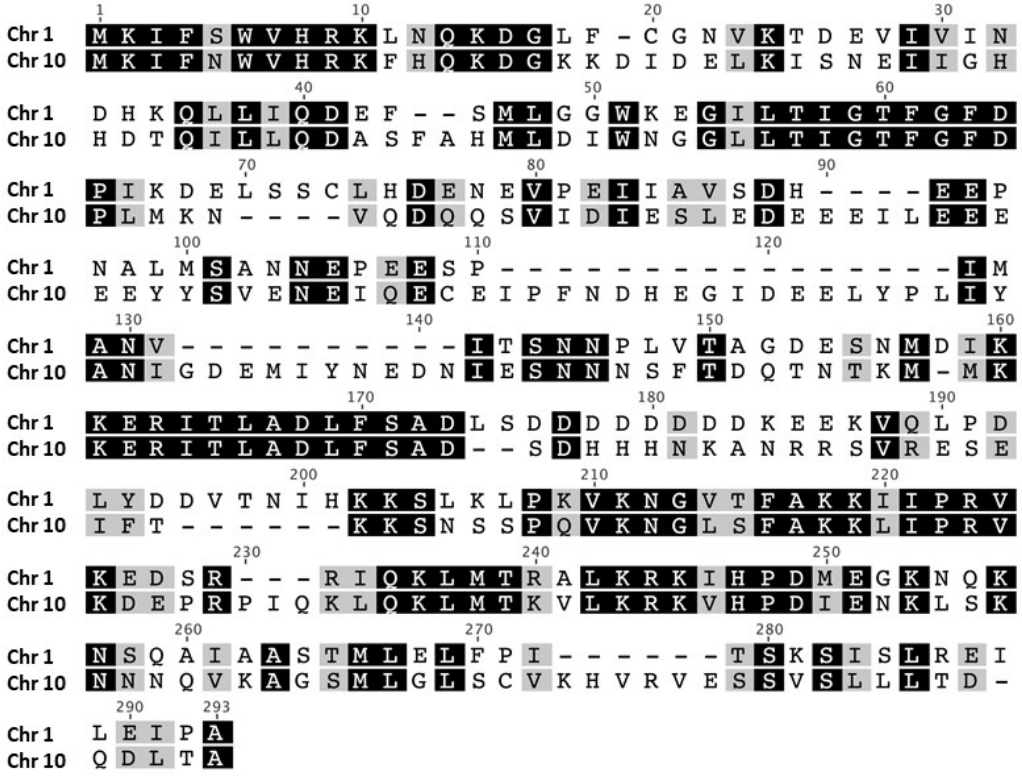
[2] **erect**: typical plant showing erect growth phenontype (first developed leaf with α angle ≤ 70 °). **non-erect**: plant showing intermediate or standard growth phenotype (first developed leaf with α angle > 70°)

[3] Presence of the G>A subtitution within the Sl10g009320.2 gene, determined using the primer pair TAC-Tom (specially designed for flanking the SNP site) followed by Sanger sequencing. See figure below.

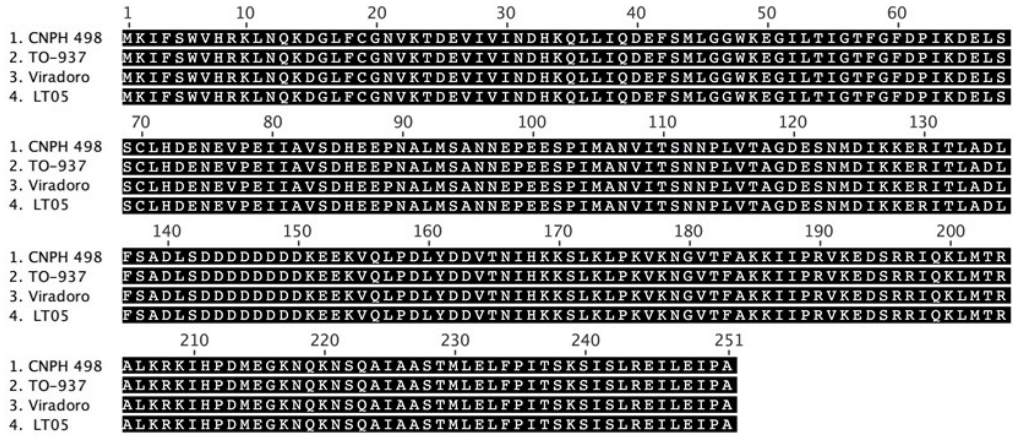


Electropherograms showing the SNP (Single Nucleotide Substitution) at the Sl10g009320 locus. The SNP is indicated as an R in the consensus sequence. The black peak in 'TO-937' (standard growth) indicates presence of G (substitution A> G absent). The green peak in 'LT05' (erect growth) indicates presence of A (substitution G> A in homozygous). Both peaks (black and green) in plant '58-(LT05xTO-937)F2' (non-erect growth) indicate presence of both bases (substitution G> A in heterozygous).

Supplementary Material C5 Multiple alignment of two predicted TAC1-like protein sequences from tomato: *Solyc01g096260* located on chromosome 1 (Chr 1) and *Solyc10g009320* located on chromosome 10 (Chr 10). Chromosome locations of each protein are indicated in the left column. Highly conserved residues are highlighted in black, included the universally conserved motif (GφL (A/T)IGT) at the position 58, which is characteristic of the IGT family.



Supplementary Material C6 Multiple alignment of the predicted *Solyc01g096260* protein sequences (located on chromosome 1) from the line 'LT05' (with erectoid leaf growth) and three wild-type (with standard leaf growth) accessions. All proteins were found to be identical.



Supplementary Material C7 Tissue-specific expression patterns of *Solyc01g096260* (located on tomato chromosome 1). Normalized mean counts per base are shown for each tissue sample. Data and downloads of plot images are from the TomExpress database and website (http://tomexpress.toulouse.inra.fr/) representing a collection of public RNAseq experiments. Different colors indicate distinct tissue types.