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CAPÍTULO 1

CLASSICAL AND BIOTECHNOLOGICAL BREEDING OF TOMATO, CAPSICUM, AND LETTUCE FOR RESISTANCE TO ORTHOTOSPOVIRUSES IN BRAZIL

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1. Introduction

As molecular parasites, curative methods are not effective for the control of plant viruses such as the orthotospoviruses. In addition, there is an overall lack of efficiency in cultural methods as well as chemical control strategies of the orthotospovirus vectors. The unique biological properties of these plant viruses demand the implementation of preventive strategies to avoid pathogen establishment as well as to minimize disease onset and development. In this scenario, genetic resistance to orthotospoviruses and/or their vectors emerges as the most practical, efficient, and sustainable method of control. A list of terms employed in the study of plant-virus interactions and plant resistance to viruses is presented in **Box 1**.

Box 1. Concepts and terminology associated with plant-virus interactions and plant resistance to viruses
(Cooper & Jones, 1983; Fraser, 1992; Gómez et al., 2009; Choi & Klessig, 2016; Peng, van Wersch & Zhang, 2018).

Avirulent/avirulence: Viral isolate, strain, or species that is unable to cause disease in a given accession of the host plant carrying a specific resistance gene due to the presence of an avirulence (*Avr*) factor. Avirulence is a property of a genetic variant of the pathogen.

Damage-Associated Molecular Patterns (DAMPs): Host cell-derived molecules resulting from mechanical disruption or degradation due to the attempt of invasion of a potential pathogen. These molecules can be perceived by plant proteins named as PRRs (Pattern Recognition Receptors) and activate defense responses.

Effectors: Pathogen molecules that alter host cell structure and function, enabling colonization, infection, and systemic invasion via suppression of host immunity component(s).

Effector-Triggered Immunity (ETI): The defense response based on the recognition of the pathogen effectors, frequently culminating in a hypersensitivity response.

Gene introgression: Transfer of genetic factors via either regular hybridization or via cell biology techniques across close taxonomically related host species/genera.

Gene silencing: Systems of host plant defense against viruses, which involves the silencing of viral genes that are crucial to the infection process. These mechanisms have been used in biotech strategies aiming to develop transgenic plants with virus resistance.

Hypersensitive Responses (HR): Histological and biochemical modifications of a given plant tissue, resulting in localization and/or spatial restriction of the challenging pathogen. HR is a type of programmed cell death. Phenotypic expression of HR is usually characterized by local lesions and it is dependent on multiple factors (e.g., cultivar, viral isolate, and environmental conditions).

Immunity: An absolute state of suppression of the viral infection. The virus cannot be detected in the plant cells after successive inoculations and/or exposure to viruliferous vectors. Viral replication and/or translocation are not detected, even in protoplast cells.

Isolate: A sample of the viral population taken and purified from either the host plant or directly from the vector using biological and/or molecular strategies for pathogen characterization and purification.

PAMP (Pathogen-Associated Molecular Pattern): Conserved molecular patterns representing structural components of different etiological agents (bacteria, fungi, viruses, and nematodes) which may be identified as elicitors by the defense system of a given plant species.

PAMP-Triggered Immunity (PTI): System that plays a pivotal role as the first layer of basal defense by preventing the colonization/invasion of a potential plant host by yet non-adapted pathogens.

Pathotype: A subdivision of viral variants in terms of their ability to infect a given group of hosts (cultivars or related species), including host cultivars (= differential cultivars) with well-characterized resistance factors.

Pattern-recognition receptors (PRRs): Cellular components able to detect potential invasion and injuries induced by pathogens. Plant PRRs are either surface-localized receptor kinases (RKs) or receptor-like proteins (RLPs) containing various ligand-binding ectodomains that perceive PAMPs and DAMPs.

Resistance: The ability of accessions of a given host plant species to suppress, reduce, or delay the injuries and damages caused by viruses. There are degrees and/or levels of resistance, with immunity representing the strongest phenotypic expression of this trait.

Resistance breakdown: Occurs when a variant of a virus can attack/infect either a cultivar considered resistant or a host plant accession that carries a well-characterized resistance genes/locus.

Tolerance: The plant is locally or systematically infected (with viral replication and/or systemic movement), but the expression of symptoms is either absent or mild.

Viral suppressors of gene silencing: Some viruses can disable the defense systems and/or induce defense-related gene silencing in their hosts, leading to the inactivation of resistance mechanisms and/or facilitating the process of infection. These virus counter-defense mechanisms include the presence of RNA silencing suppressors and the adoption of silencing-tolerant RNA conformations.

Virulent/virulence: Ability of a pathogen to cause disease in a group of accessions of a plant recognized as a host. Virulence is a property of isolates/variants of a pathogen.

Virus strain: A variant that shares biological, serological, or molecular characteristics with a type species. It can also be defined by its virulence profile concerning to different host resistance genes (differential cultivars) as well as by its genomic variability.

2. Natural plant defense mechanisms against viruses

During co-evolution, plants have developed multiple defense mechanisms against viruses and their vectors, involving passive resistance (due to anatomical and/or biochemical components) as well as active resistance based upon recognition of structural and/or effector proteins of the pathogens/pests, triggering the production of reactive oxygen species (ROs), hormone-mediated resistance pathways, synthesis of secondary compounds, immune receptor signaling, and protein degradation pathways, among others (Calil & Fontes, 2016; Liu et al., 2017). The system for virus recognition can activate two layers of host plant defense, PTI (PAMP-triggered immunity) and ETI (effector-triggered immunity). PTI is the first layer of plant resistance against viruses, being able to recognize conserved molecules of the invading pathogen (Zipfel, 2008; **Box 1**). Similarly, endogenous injuries associated with virus infection can also elicit defense responses via DAMPs (Hou et al., 2019; **Box 1**). PAMPs comprise extremely conserved molecules that frequently play key roles in pathogen survival and infection ability (Boller & Felix, 2009). PAMPs were expanded to also include virus-derived nucleic acids that may also activate recognition receptors. After the recognition of these molecules by the plant cell surface PRR receptors (**Box 1**), the PTI response is initiated, generating a broad-spectrum defense response (**Box 1**). The PRR receptors involved in the PAMP recognition are subdivided into two main groups: kinase-type

receptors and protein-like receptors (Dodds & Rathjen, 2010; **Box 1**). Virus-derived nucleic acid PAMPs may also elicit the Nuclear Shuttle Protein-Interacting Kinase 1 (NIK1)-mediated antiviral signaling pathway, suppressing global host translation (Teixeira et al., 2019). Beside this, during PTI, the production of a series of defense compounds can occur, which in synchronic combination with the expression of a wide array of other resistance-related genes contribute to very effective defense responses. However, throughout the co-evolutionary process, pathogens have developed an arsenal of strategies to circumvent and/or suppress PTI, including the synthesis of effector proteins (**Box 1**) that can alter the physiological state of the host plant in their favor. On the other hand, natural genetic mutations in the host plants have generated a specific set of effector recognition systems, activating ETI, which is the second layer of plant defense (**Box 1**). ETI is based upon specific cellular recognition of effector proteins of host-adapted pathogens. An effector-coding gene (or its corresponding gene product) when detected by the host ETI is named as Avr gene corresponding to the ones described in gene-for-gene systems (Jones & Dangl, 2006). Hormones are pivotal players in plant-virus interactions modulating viral movement, replication, and systemic infection. Abscisic acid, salicylic acid, jasmonic acid, ethylene, gibberellic acid, cytokinin, and brassinosteroids are examples of phytohormones associated with plant response to pathogens (Zhao & Li, 2021). **RNA interference (RNAi)** – Plants also developed a recognition system of nucleic acids of their pathogens. During infection by both RNA and DNA viruses, Dicer-like proteins detect double-stranded RNAs, cleaving them into fragments of 21-24 nucleotides. These fragments named microRNAs (miRNA) or small interfering RNAs (siRNA) are carried to argonaut proteins, forming the RNA-induced silencing complex (RISC) or RNA-induced transcriptional silencing complex (RITS). The RISC complex recognizes and cleaves the target sequence and triggers the post-transcriptional gene silencing process, while the RITS complex either compresses (via histones) or methylates DNA, triggering transcriptional gene silencing (Calil & Fontes, 2016). Counteracting this resistance, plant viruses may encode viral suppressors of RNAi (VSRs) that can disturb the plant RNA silencing pathway, overcoming this host defense. Some of these silencing suppressors have been extensively studied in different viral species (e.g., NSs protein in *Orthotospovirus*; see sections below). **Loss-of-susceptibility mutations** – As molecular parasites, all viruses require host factors for their infection (= “host susceptibility factors”). Natural mutations in this subset of pivotal genes for virus infection may also confer resistance to a wide range of viruses and their pathotypes. Such natural sources of resistance are very interesting from the breeding standpoint since they are often monogenic recessive and, in general, stable and durable (Pavan et al., 2010).

3. Marker-assisted selection (MAS), isolation, and structural features of virus resistance genes in plants

Several virus resistance genes were isolated using high-resolution genetic/physical mapping strategies (= map-based cloning or positional cloning). Indeed, a collection of genes (dominant and recessive) controlling resistance to diseases of viral etiology has been cloned, and this information has been used in the development of locus-specific or gene-specific (= functional) markers (Dianese et al., 2010). A subset of cloned virus resistance genes is structurally characterized as typical dominant R-genes (de Ronde et al., 2014). The largest class of dominant virus genes encodes proteins with nucleotide-binding (NB) and leucine-rich repeat (LRR) domains, which recognize avirulence factors encoded in the viral genome (de Ronde et al., 2014). The C-terminal regions of the LRR domains control recognition and specificity. The NB domain functions as the molecular switch regulating the signal transduction activation leading to the resistant phenotype (Oliveira et al., 2018). The second type of virus resistance gene involves recessive (loss-of-function) genes (Pavan et al., 2010). Thus far, the majority of them corresponds to loss-of-susceptibility mutations in genes encoding eukaryotic translation initiation factors and molecular markers can be designed for their precise detection (Garcia-Ruiz, 2018).

4. The genus *Orthotospovirus*

This group of thrips-transmitted pathogens is one of the most economically and biologically important plant-infecting viral genera, causing substantial losses on crop production worldwide (Pappu et al., 2009). *Tomato spotted wilt orthotospovirus* was included in a select group of the ten most relevant plant viruses worldwide from both scientific and economic perspectives (Rybicki, 2015). Until the early 1990s, the genus *Orthotospovirus* (former *Tospovirus*) was considered monotypic, having *Tomato spotted wilt orthotospovirus* as the type species. Since then, molecular and biological characterization of a wide range of isolates revealed novel species within the genus. Orthotospoviruses were initially classified within the former family *Bunyaviridae* (together with animal-infecting viruses) due to their similar virion shape, genomic organization, and phylogenetic relationships. Currently this genus belongs to the order *Bunyavirales*, composed of members with segmented, single-stranded, negative-sense or ambisense RNA genomes (Adams et al., 2017). The orthotospoviruses have enveloped and spherical particles (**Figure 1**) with tri-segmented genomes. These genomic segments are classified according to their size as small (S) (≈ 2.9kb), medium (M) (≈ 4.8kb), and large (L) (≈ 9kb) segments. The ambisense S RNA encompasses the coding region of the nucleocapsid protein (N) in the viral complementary sense and a non-structural silencing suppressor protein (NSs) in the viral sense. The ambisense M segment encompasses the coding regions corresponding to the non-structural movement protein (NSm), in the negative sense, and the glycoproteins (Gn/Gc),

in the positive sense. The L segment encodes an RNA-dependent RNA polymerase (RdRp) with negative polarity (Kormelink et al., 2021)

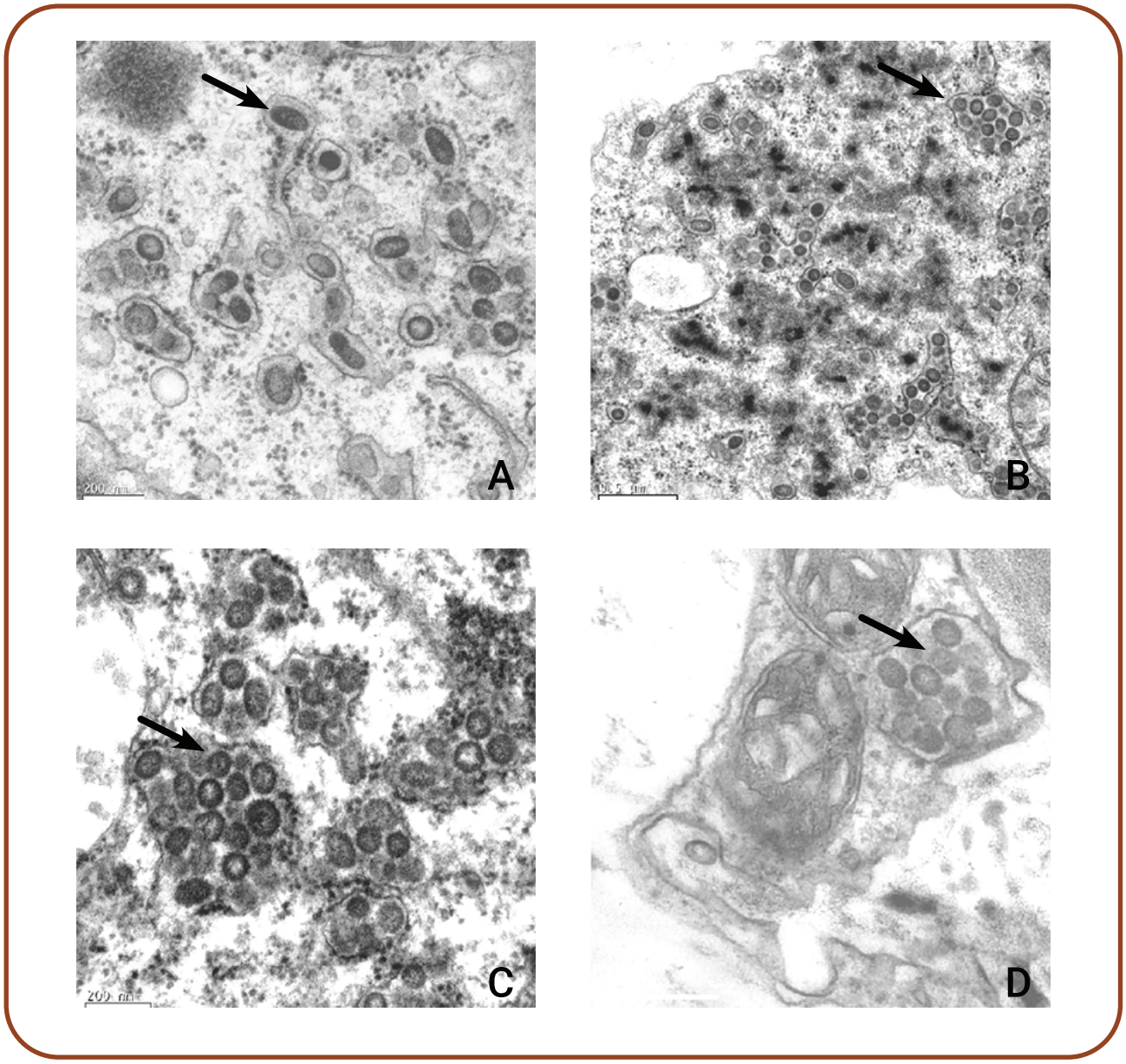


Figure 1. Transmission electron micrographs of foliar tissues of different host plants infected with orthotospoviruses. A = tomato (*Solanum lycopersicum* L.) infected by tomato spotted wilt orthotospovirus (TSWV); B = Leaf chicory (*Cichorium intybus* L., Asteraceae) infected with groundnut ringspot orthotospovirus – GRSV (Jorge et al., 2022); C = Chicory (*Cichorium endivia* L., Asteraceae) infected by GRSV (Jorge et al., 2021), and D = bell-pepper (*Capsicum annuum* L.) infected with chrysanthemum stem necrosis orthotospovirus – CSNV. Arrows indicate orthotospoviruses particles. (Source: Elliot Watanabe Kitajima).

5. Taxonomic criteria and diversity of the orthotospoviruses

The demarcation criteria for novel *Orthotospovirus* species are defined by combining information about vector specificity, host range, N protein serology, and N protein amino acid identity (which must be smaller than 90% in comparison with other previously described species) (King et al., 2011). The initial subdivision of the *Orthotospovirus* species was based upon phylogenetic analysis of partial segments of the N protein, resulting in two geogroups: Old World (Asia) and New World (Americas) (Gilbertson et al., 2015). However, recent studies based on complete sequences of 22 *Orthotospovirus* species showed that the division into geogroups is not consistent (Butković, González & Elena, 2021). These viruses were subsequently grouped into four phylogroups, with the open possibility to create new genera within each phylogroup, splitting the former monotypic *Orthotospovirus* genus (Butković, González & Elena, 2021). Nowadays, 26 *Orthotospovirus* species are recognized, and thus far seven of them were reported in Brazil (Oliveira et al., 2012). Groundnut ringspot orthotospovirus (GRSV), tomato spotted wilt orthotospovirus (TSWV) and tomato chlorotic spot orthotospovirus (TCSV) are the main orthotospoviruses infecting vegetable crops in Neotropical areas. Sporadic infections of tomato and *Capsicum* by chrysanthemum stem necrosis orthotospovirus – CSNV (**Figure 1**; Bezerra et al., 1999) have also been observed (Dianese et al., 2011).

6. Symptoms induced by orthotospoviruses

Plants infected by orthotospoviruses display a typical set of symptoms especially on leaves, stems, and fruits (**Figure 2**). Leaf and fruit symptoms usually consist of chlorotic (yellow) or necrotic (brown) ringspots. Tomato and sweet-pepper (*Capsicum annuum* L.) fruits may be deformed, due to production of necrotic sunken areas. Necrotic lesions may also appear on stems. On young leaves, numerous small lesions may develop, given an appearance of bronzing and leaf curling (Lima et al., 2016). On lettuce, brown spots develop on leaves, followed by necrotic areas. Lettuce plants may become stunted and deformed, showing asymmetric leaf growth. Symptoms induced by TSWV, GRSV or TCSV are indistinguishable, giving no clear indication of which virus is involved. *Orthotospovirus* species can be precisely identified only by using serological and/or molecular methods.

Figure 2. Foliar and fruit symptoms induced by orthotospoviruses in tomatoes (left panel), in *Capsicum* species (central panel), and in lettuce (right panel). (Sources: Mirtes F. Lima, Tiago S. Jorge and Leonardo S. Boiteux).

7. Vectors and epidemiology of the orthotospoviruses

Orthotospovirus infect more than 1,000 monocotyledonous and dicotyledonous plants belonging to more than 82 botanical families. The orthotospoviruses are transmitted mechanically (under experimental conditions) and, in natural conditions, by a limited number of thrips species (order *Thysanoptera*), in a circulative-propagative manner, replicating in both plant and invertebrate hosts (Rotenberg & Whitefield, 2018). Globally, *Frankliniella occidentalis* is the major orthotospovirus vector species, but *F. schultzei* is also found in tomatoes in Brazil (Gilbertson et al., 2015). The life cycle of the vectors is composed of four stages, but only instars L1 and L2 are competent in virus acquisition. The viral particles are passed transstadially across life stages and the adult insect becomes a competent virus vector throughout its entire life. Seed transmission in many hosts remains uncertain (Rotenberg & Whitefield, 2018).

8. Advances in horticultural plant breeding for resistance to orthotospovirus-induced diseases

Genetic resistance is the most promising control measure, being obtained by either conventional breeding methods or via biotechnology. Natural virus resistance is predominantly a qualitative (monogenic) trait in which expression is usually not affected by the environment (i.e., displays high heritability), even though some thermal instabilities have been detected. The incorporation/introgression of resistance factors depends upon the selection of individual plants via direct evaluation (resistant or susceptible) with and without controlled inoculations. In this regard, classical breeding methods such as mass selection, stratified mass selection, and backcrossing are usually very effective. The employment of MAS in the early stages is useful to accelerate and intensify the selection gains. Here, we will review the pathosystems, resistance sources and, breeding strategies employed in the classical and biotech control of orthotospovirus-induced diseases in tomato, *Capsicum* species, and lettuce. The sections are organized according to the advances made in resistance breeding against orthotospovirus in each pathosystem.

8.1. Tomato breeding for resistance to *Orthotospovirus*

8.1.1 Resistance to isolates of *Orthotospovirus* species in tomato plants

The syndrome known as “spotted wilt” is one of the most destructive viral diseases of tomatoes and major outbreaks have been reported since 1930s in Brazil (Kitajima, 2020). The predominant species causing “spotted wilt” are GRSV, TSWV, and TCSV. Several sources of genetic resistance have been found in *Solanum* (*Lycopersicon* section), especially in the germplasm of the wild tomato species *S. peruvianum* (Gordillo et al., 2008; Dianese et al., 2011). The major resistance source thus far is the dominant

Sw-5b gene (introgressed from a *S. peruvianum* accession), which confers broad-spectrum resistance to different orthotospoviruses and has been widely used in commercial breeding programs (Van Zijl et al., 1985; Stevens et al., 1992; Boiteux & Giordano, 1993; Roselló, Díez & Nuez, 1998). The *Sw*-5b gene restricts systemic infection, and the inoculated leaves show a hypersensitivity response (Brommonschenkel, Frary & Tanksley, 2000). A novel resistance gene (named *Sw*-7) has been characterized (Qi et al., 2021), but it was not yet properly evaluated under Brazilian conditions.

8.1.2. Viral outbreaks, germplasm screening, and classical tomato breeding in Brazil

The initial breeding efforts for orthotospovirus resistance in tomatoes were carried out by the Instituto Agronômico de Campinas (IAC) in the 1970s with a focus on the development of resistant fresh-market cultivars (Nagai, 1975; Lourenção et al., 1999). In the early 1990s, a series of orthotospovirus outbreaks caused a great economic and infrastructural crisis in the tomato processing industry sector in the Brazilian Northeast. In an attempt to minimize this crisis, Embrapa Vegetable Crops (CNPH) and the Instituto Agronômico de Pernambuco (IPA) established a joint breeding program, which resulted in the release of 'Viradoro', the first *Orthotospovirus* resistant cultivar for processing in the country (Giordano et al., 2000). Despite its unquestionable utility, the *Sw*-5b gene displays some significant limitations viz.: (**1**) resistance expression may be adversely affected under high virus pressure or it does not express fully in regions with drastic fluctuations between day and night temperatures; (**2**) phenotypic expression does not show complete penetrance; and (**3**) some virus isolates may breakdown this resistance. For these reasons, a search for new sources of broad-spectrum resistance genes has been carried out (Dianese et al., 2011). Alternatively, strategies of artificial evolution used in the *Sw*-5b gene lead to an improved resistance response to TSWV in tomatoes. The identification of key amino acid residues in the NSm protein followed by specific mutations improved defense against resistance-breaking TSWV isolates (Huang et al., 2021).

8.1.3. Mechanisms of GRSV pathogenesis in tomatoes

Thus far, only the dominant *Sw*-5b gene confers resistance to GRSV isolates. A comprehensive set of analyses of differentially expressed genes (DEGs) was carried out during a compatible interaction between S. *lycopersicum* 'Santa Clara' and GRSV aiming to elucidate the mechanisms of GRSV pathogenesis. These DEGs were grouped into three major categories: "biological processes", "molecular functions", and "cellular components". DEGs involved in RNA silencing pathway as well as in association with cell signaling, photosynthetic processes (chloroplasts, thylakoids, and photosystems), endoplasmic reticulum, Golgi complex, plasmodesmata, elongation factors, and cellular detoxification were identified (Fontes, 2017).

8.1.4. MAS for the *Sw*-5b gene in tomato breeding

The locus encompassing the *Sw*-5b gene on chromosome 9 was map-based cloned and it was found to reside within a cluster of five analogous copies of the same ancestral

gene, named from *Sw*-5a to *Sw*-5e (Brommonschenkel, Frary & Tanksley, 2000; Spassova et al., 2001). The *Sw*-5b gene belongs to the class of resistance genes termed CC-(NB--ARC)-LRR (Brommonschenkel, Frary & Tanksley, 2000). A comprehensive set of analyses was also carried out demonstrating that *Sw*-5b is the single copy responsible for the resistance phenotype (Spassova et al., 2001; Oliveira et al., 2018). Studies carried out to elucidate the interaction of *Sw*-5b with viral proteins have shown that NSm (movement protein) is the *Avr*-determinant of *Sw*-5b gene (Hallwass et al., 2014; Peiro et al., 2014). The genomic information encompassing the *Sw*-5b locus enabled the development of a functional, locus-specific, and codominant molecular marker system that has been used in MAS worldwide (**Figure 3**; Dianese et al., 2010).

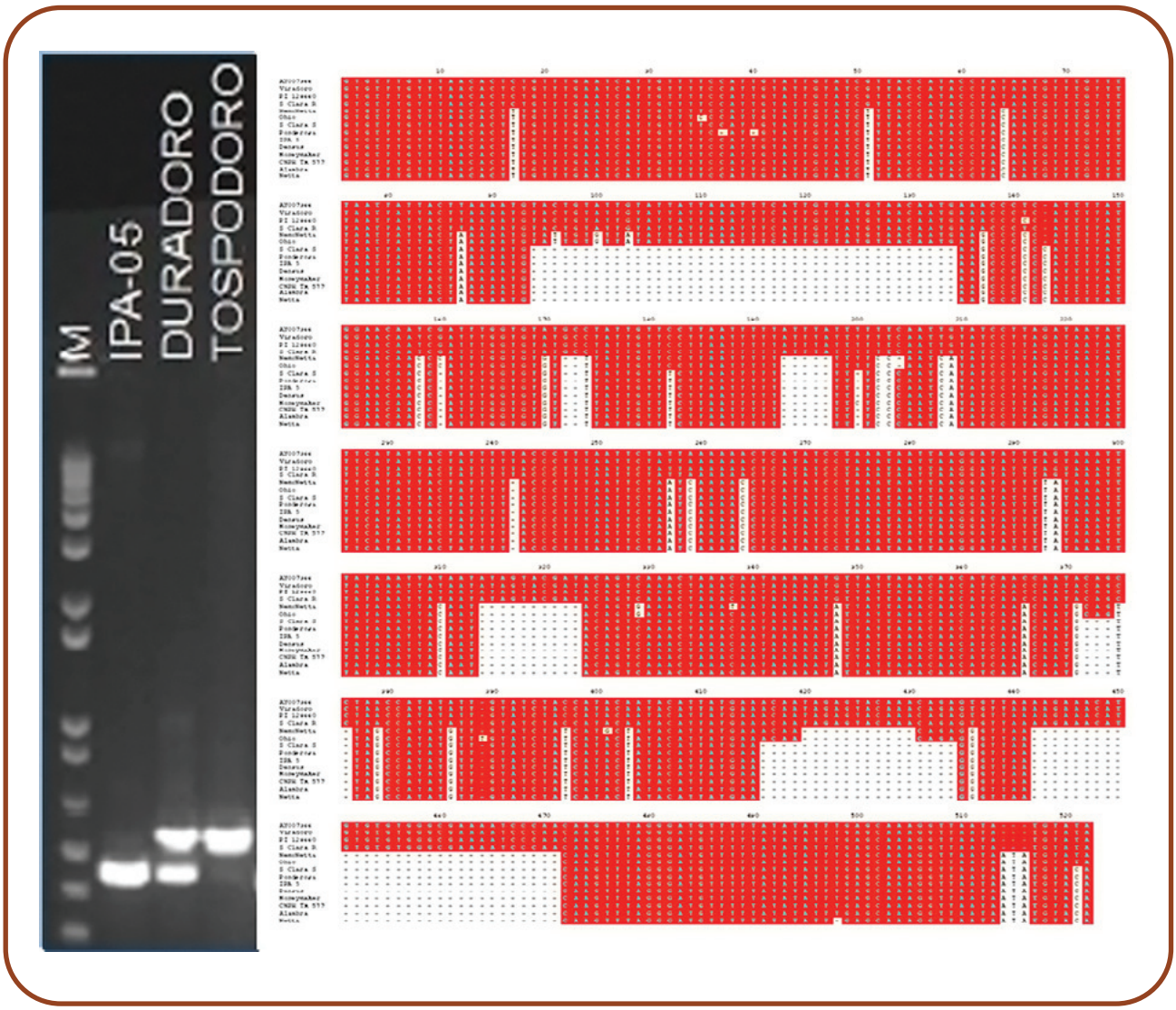
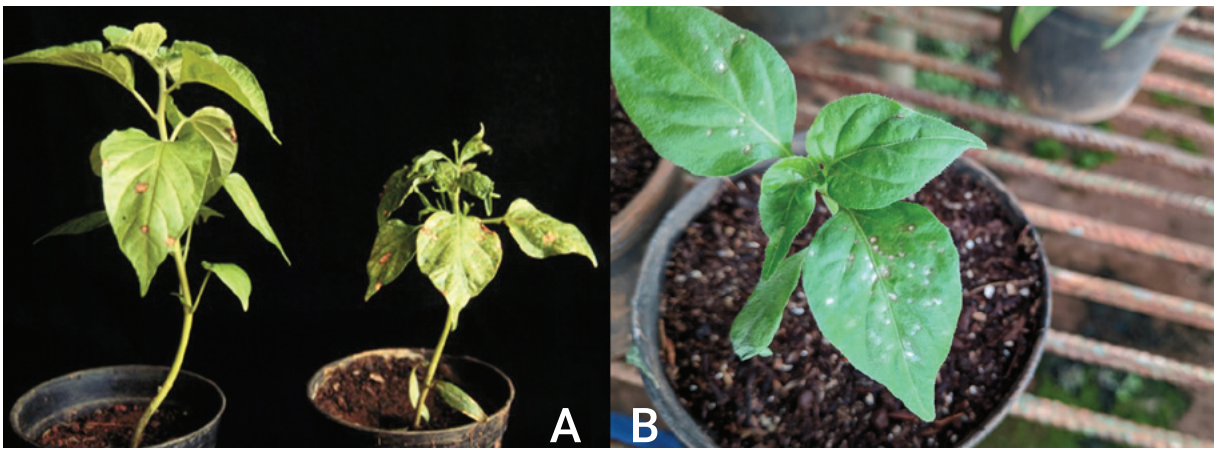


Figure 3. Left Panel: Agarose gel displaying the amplicon profiles of a codominant marker system for the *Sw*-5b locus in tomato; IPA-5 (susceptible cultivar; *sw*-5b/*sw*-5b), 'Duradoro' (heterozygous hybrid; *Sw*-5b/*sw*-5b) and 'Tospodoro' (homozygous dominant; *Sw*-5b/*Sw*-5b); M = 100 bp size marker. Right Panel: Sequence alignment encompassing the region of *Sw*-5b locus amplified by the PCR primers reported by Dianese et al. (2010). Nucleotides in red are conserved across 14 distinct tomato accessions; whereas nucleotides in the white boxes are mutations and (-) signals are indicating nucleotide deletions. The susceptible allele displays a large number of INDELs in comparison with the genomic region of the resistant accessions (the first four lines in the top of the panel).

8.2. *Capsicum* breeding for resistance to *Orthotospovirus*

8.2.1. Breeding programs for *Capsicum* resistance to orthotospoviruses in Brazil

TSWV and GRSV also induce severe symptoms in all *Capsicum* species, causing serious economic losses. The first formal breeding initiative to develop *Capsicum* lines/cultivars with resistance to *Orthotospovirus* species in Brazil was initiated in the 1980s at Embrapa (Beek, 1987). Sources of resistance were identified in field and greenhouse screenings (Beek, 1987; Boiteux et al., 1993). *Capsicum chinense* 'PI 159236' displayed a typical HR with large local lesions upon mechanical inoculation with TSWV isolates (**Figure 4**). Inheritance studies indicate that a dominant allele (named *Tsw*) controls this HR only against TSWV isolates (Boiteux & Avila, 1994). However, the *Tsw*-mediated resistance was not effective to GRSV and TCSV isolates upon mechanical inoculation assays (Boiteux, 1995). In addition, the *Tsw* gene has its effectiveness compromised when continuously exposed to temperatures above 32° C and plants heterozygous for the *Tsw* locus have lower levels of resistance in comparison with homozygous lines (Moury et al., 1998).



Fonte: Leonardo S. Boiteux

Figure 4. Mechanical inoculation of the accession *Capsicum chinense* 'PI 159236' with tomato spotted wilt orthotospovirus (TSWV) and groundnut ringspot orthotospovirus (GRSV) isolates. The accession 'PI 159236' is the source of the dominant allele *Tsw*, which controls a typical hypersensitive response with large local lesions upon mechanical inoculation with TSWV isolates (left plant in the Panel A and Panel B). However, the *Tsw*-mediated resistance is not effective against GRSV and tomato chlorotic spot orthotospovirus (TCSV) isolates, displaying systemic infection (right plant in the Panel A).

8.2.2. Isolation and marker-assisted selection (MAS) of the *Tsw* gene in *Capsicum*

The *Tsw* allele was mapped to the distal portion of chromosome 10 and closely linked molecular markers were identified (Jahn et al., 2000). Later, the *Tsw* gene was map-based cloned and structural analyses indicated that it belongs to the class CC-

-NB-ARC-LRR of typical R-genes (Kim et al., 2017). Despite the similar action against orthotospoviruses (especially TSWV), the *Tsw* and *Sw*-5b genes display substantial evolutionary differences with a very low amino acid identity (<15%). Moreover, *Tsw* can detect a distinct *Avr* factor, which is the NSs protein (de Ronde et al., 2013).

8.2.3. *Tsw*-resistance breaking isolates and viral species

As mentioned, the *Tsw* gene is not effective against GRSV and TCSV isolates, being highly effective only against Brazilian isolates of TSWV (**Figure 4**; Boiteux, 1995). However, many TSWV isolates that are able to overcome the *Tsw*-mediated resistance have been reported around the world (Kwon et al., 2021). The emergence of pathotypes that suppress *Tsw*-mediated resistance might be explained by positive selection in NSs protein as well as reassortment of one or more genomic segments (Kwon et al., 2021).

8.3. Lettuce breeding for resistance to *Orthotospovirus*

8.3.1. Classical breeding for *Orthotospovirus* resistance in lettuce

Lettuce is one of the most important vegetables worldwide (Reyes-Chin-Wo et al., 2017). TSWV, GRSV and TCSV isolates have been reported infecting lettuce in Brazil (Colariccio et al., 2004; Fontes et al., 2019). Resistance to *Orthotospovirus* species in lettuce was reported initially by O'Malley & Hartmann (1989) in two accessions: 'PI 342517' (= 'Ancora') and 'PI 342444' (= 'Tinto'). These sources displayed partially dominant inheritance, and the resistance factors are more likely allelic (O'Malley & Hartmann, 1989). Additional sources of resistance to TSWV and impatiens necrotic spot virus (INSV) were detected in accessions of the wild lettuce species, such as *L. saligna, L. serriola, L. virosa,* and *L. inermis* (Cho et al., 1996; Simko, Richardson & Wintermantel, 2018). In the cultivated lettuce germplasm, promising levels of partial resistance to INSV were found in 'Amazona', 'Antigua', 'Commodore', 'Eruption', 'Iceberg', 'La Brillante', 'Merlot', and 'Telluride' (Simko, Richardson & Wintermantel, 2018). However, the highest levels of resistance to INSV reported thus far were detected in the accessions 'PI 342444' and 'PI 342517' (Simko, Richardson & Wintermantel, 2018).

8.3.2. Lettuce breeding for *Orthotospovirus* resistance in Brazil

The pioneering initiative to develop lettuce cultivars with resistance to *Orthotospovirus* species in Brazil was initiated in 1987 also in the IAC, São Paulo State (Nagai, 1989). The primary goal was to introduce resistance into cultivars of the 'Butterhead' morphotype by developing progenies from the cross 'Regina' x 'PI 342517' (Nagai, 1989). The accessions 'PI 342444' and 'PI 342517' were also identified as the most promising breeding sources of resistance in field assays conducted under natural inoculum conditions in Brazil (Pavan et al., 2008). These accessions displayed mild symptom expression, being consistently less severe than that of susceptible standards (Nagai, 1989). However, there was no consistent indication to which *Orthotospovirus*

species the accessions were exposed in those assays. Fontes et al. (2019) mechanically inoculated lettuce seedlings of 'PI 342444' with seven isolates (three TSWV and four GRSV isolates). Severe symptoms and positive serological results were obtained for the susceptible lettuce control (cultivar 'Vanda'), whereas 'PI 342444' plants did not exhibit symptoms after inoculation with TSWV isolates. In contrast, all GRSV-inoculated 'PI 342444' plants displayed early onset of severe symptoms, indicating that this resistance is not effective against this viral species. Therefore, an additional search for sources of genetic resistance in lettuce germplasm effective against GRSV isolates is a major breeding priority under Brazilian conditions since it is currently the predominant lettuce-infecting orthotospovirus in the country.

8.3.3. Potential application of transcriptomic analysis to study the interactions of GRSV with lettuce cultivars

Natural sources of genetic resistance to GRSV were not identified in lettuce germplasm thus far. Therefore, innovative approaches are necessary to solve this problem. One promising genetic strategy is identifying and editing pivotal "host susceptibility genes" utilized by this virus during infection. These host genes acting as facilitators during the infection process can be recognized by their altered levels of expression in RNAseq analyses (Puyam & Kaur, 2020), and can be targets of gene editing strategies (see below). A similar set of analyses is now underway in the lettuce-GRSV pathosystem.

9. Biotech breeding strategies for controlling *Orthotospovirus*-induced diseases

9.1. Transgenic approaches

Viral genes have been widely used in the development of resistant transgenic plants (= "pathogen-derived resistance") and have been effective in different pathosystems (Goldbach, Bucher & Prins, 2003). Constructs with viral genes encoding the RNA-dependent RNA polymerase, protease, movement protein, satellite, and defective RNAs in addition to non-coding regions of the viral genome have also been used with varying degrees of success. Initially, viral gene expression was presumed to be necessary for resistance to be effective. However, several results in recent years have shown that post-transcriptional gene silencing (PTGS) may occur, which does not require full gene expression.

9.2. RNA gene silencing or RNA interference (RNAi)

Defense responses of plants triggered by viral pathogens generally uses RNA-mediated resistance. RNAi is a major basal defense against viral invasion in plants. This mechanism is triggered by double-stranded RNA molecules (dsRNA), which are recognized and cleaved by a host Dicer-like ribonuclease (DCL) into 21–24-nucleotide short interfering RNAs (siRNAs). The siRNAs are recruited to a functional RNA-induced silencing complex (RISC) and then act as guides to direct them to their target viral RNA molecules, which have complementary sequences. As consequence, viral RNAs are degraded by the core components of RISC, which are members of the Argonaut (AGO) protein family (Ghildiyal & Zamore, 2009). This strategy has been used to develop transgenic plants resistant to begomoviruses (Aragão & Faria, 2009). Likewise, transgenic lettuce lines with potential GRSV resistance are under evaluation employing the RNA interference (RNAi) strategy, paving the way towards the biotech breeding development of novel orthotospoviruses resistant cultivars of this vegetable crop.

9.3. Cisgenic strategy

The mobilization of genes using genetic engineering techniques, both between different accessions/cultivars from a single species and across related species (= 'cisgenesis'), may also be an interesting approach for controlling viral diseases (Jacobsen & Schouten, 2007). Resistance genes against pathogens with a wide host range (such as orthotospoviruses) can be transferred across host varieties, wild species, or even from phylogenetically more distant taxonomic groups. For example, Picoli et al. (2006) used this strategy with success to transfer *Sw*-5b gene from tomato to the eggplant, generating plants with resistance to TCSV.

9.4. Generation of loss-of-susceptibility genes for control of orthotospoviruses

An example of susceptibility factors for genetic management of orthotospoviruses is eukaryotic translation initiation factor EF1A that was able to inhibit TSWV replication, providing good levels of resistance via gene editing strategies in tomato (Hadidi et al., 2016). We have employed transcriptomic analysis as a strategy for the discovery of loss-of-susceptibility genes to GRSV in tomatoes. DEGs were detected during a compatible interaction between the highly susceptible *S. lycopersicum* cultivar 'Santa Clara' and GRSV using Illumina technology (HiSeq 2500). Approximately 396 million reads were mapped to the tomato reference genome. A total of 2,344 DEGs were found in the Santa Clara-GRSV interaction when compared with the mock-inoculated controls. A total of 1,911; 268; and 416 DEGs were identified at the periods 0, 3–5, and 7–10 days after inoculation, respectively. This subset of DEGs may represent potential targets for biotech manipulation aiming to obtain cultivars with resistance to GRSV (Fontes, 2017).

10. Final considerations

Resistance to orthotospoviruses and/or *Thysanoptera* vector species, when available, is the most feasible disease control strategy. Therefore, significant research effort has been devoted to this field. Great advances have been achieved using classical and molecular breeding, however, the biggest obstacle for the breeding programs is yet to identify and incorporate, on a large scale, multiple and/or large-spectrum resistance factors into elite lines of these vegetable crops and in order to anticipate potential problems with a novel, emerging orthotospovirus-induced diseases.

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