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Initial development of a set of introgression lines from *Solanum peruvianum* PI 126944 into tomato. Exploitation of resistance to viruses

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Abstract A set of introgression lines from *S. peruvianum* PI 126944 into the genetic background of cultivated tomato (*S. lycopersicum*) is being developed. Several generations were derived from three interspecific hybrids previously obtained. A lot of crosses and embryo rescue were required to obtain until the third backcross, due to the high incompatibility existing between tomato and PI 126944. Crosses between F₁ plants allowed the obtaining of a pseudo-F₂ generation. The same procedure was followed until pseudo-F₆ generation. Additional crosses between plants of different generations were made in order to increase progeny. Among a total number of 263 molecular markers tested, 105 resulted polymorphic between tomato and PI 126944. This set of polymorphic markers consisted in 90 Simple Sequence Repeats and 15 Cleaved Amplified Polymorphic Sequences. Generations available were genotyped with these markers, observing a progressive reduction in the *S. peruvianum* genome in the most advanced. A reduction of incompatibility was achieved as a consequence of the *S. peruvianum* genome reduction. In addition, *S. peruvianum* genome was almost completely represented considering the different plants of the most advanced generations, so the set of ILs will be basically developed from them. *Tomato yellow leaf curl virus* (TYLCV) and *Tomato spotted wilt virus* (TSWV) resistance was evaluated in some generations, having been successfully introgressed and expressed into tomato background.

Key words: embryo rescue, molecular markers, *Solanum lycopersicum*, TSWV, TYLCV

Introduction

Tomato breeding has been focused for decades on the exploitation of its wild relatives due to its narrow genetic basis (Miller and Tanksley 1990). The use of wild relatives has allowed the identification and introgression of many genes of interest as well as the construction of mapping populations with sufficient polymorphism at the DNA level. Populations initially used for mapping in self-pollinated crops were F₂/F₃, backcrosses or recombinant inbreds. However, this type of populations has several limitations in the accurate identification and fine mapping of quantitative trait loci (QTLs). The limitations include their low resolution power, the fail on the ability to identify QTLs with small effects and the possibility of interaction of two unlinked QTLs, which reduces the difference between the subgroups of the tested QTL. Additionally, in these populations each plant poses a large fraction of the wild species genome, affecting their fertility and the expression of yield and some other characteristics (Eshed and Zamir 1995). To avoid these problems other types of population have been derived such as backcross recombinant inbred lines (BCRILs, Ramsay et al. 1996) or introgression lines (ILs, Eshed and Zamir 1995). These populations circumvent also the problem of self-incompatibility of the interspecific hybrids, which occurs in crosses with some wild relatives and that prevent obtaining progeny by selfing and consequently the construction of populations like recombinant inbred lines.

In tomato several interspecific breeding populations have been developed: ILs from *S. pennellii* LA716 (Eshed and Zamir 1995), a BC₃ populacion from *S. peruvianum* LA1708 (Fulton et al. 1997), ILs and backcross inbred lines (BILs) from *S. habrochaites* LA1777 (Monforte and Tanksley 2000), BILs from *S. pimpinellifolium* LA1589 (Doganlar et al. 2002) and RILs from *S. pimpinellifolium* LA2093 (Ashrafi et al. 2009), and ILs from *S. lycopersicoides* LA2951 (Canady et al. 2005). These populations have been used for the identification of a high number of QTLs (Foolad 2007).

S. peruvianum is considered the most variable tomato wild relative. This species is self-incompatible and its use as female parent in crosses with tomato is prevented by the existence of unilateral incompatibility (Hogenboom 1972). However this species has been extensively used in breeding due to the identification of accessions with resistance to abiotic and biotic stresses. Many genes have been introgressed into tomato, i.e. the *Sw-5* gene that confers resistance to *Tomato spotted wilt virus* (TSWV) (Stevens et al. 1992), the *Pyl* gene conferring resistance to the fungus *Pyrenochaeta lycopersici* (Laterrot 1978), the *Tm-2* (Laterrot and Pecaat 1969) and *Tm-2²* genes (Hall 1980) that confer resistance to *Tomato mosaic virus* (ToMV), the *Mi* gene associated to resistance to the main species of nematodes of the genus *Meloidogyne* (Gilbert 1958) and the *Ty-5* gene conferring resistance to the Tomato yellow leaf curl disease (TYLCD) (Anbinder et al. 2009). In particular, the accession *S. peruvianum* PI 126944 has been described as resistant to TSWV (Paterson et al. 1989), *Tobacco mosaic virus* (TMV) (Yamakawa and Nagata 1975), *Tomato leaf curl virus* (ToLCV) (Muniyappa et al. 1991), to some species belonging to the virus complex responsible of TYLCD (Picó et al. 1998; Pilowsky and Cohen 2000) and to *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Rowe and Farley 1981). Our group constructed some interspecific hybrids between this accession and tomato, which behaved as resistant to TSWV and TYLCD (Picó et al. 2002).

Diseases caused by TSWV and the complex of TYLCD-like viruses are two of the most devastating diseases that affect tomato cultivation in all tropical and subtropical

areas worldwide (Picó et al. 1996; Roselló et al. 1996; Hanssen et al. 2010). Genetic resistance has been identified for both viruses and transferred to tomato. The most used TSWV resistance gene is *Sw-5*, which was identified in the species *S. peruvianum* (Stevens et al. 1992). Some TYLCD resistance genes have been identified from different wild tomato relatives. *Ty-1* (Zamir et al. 1994), *Ty-3* (Ji et al. 2007) and *Ty-4* (Ji et al. 2009) come from *S. chilense*, *Ty-2* was identified in *S. habrochaites* (Hanson et al. 2006), and *Ty-5* in *S. peruvianum* (Anbinder et al. 2009). Quantitative resistance has also been reported derived from *S. peruvianum* (Pilowsky and Cohen, 1990; Vidavsky et al. 1998).

However, these genes are not a definitive solution for both diseases. On one hand, the high variability found in the pathogens often results in the appearance of new isolates able to overcome the existing resistance. This is the case of TSWV, for which isolates overcoming the resistance conferred by the *Sw-5* gene have been reported (Aramburu and Martí, 2003). On the other hand, the TYLCD resistance conferred by the genes available is not completely effective. Moreover, the great variability reported for TYLCV-like species from different geographical areas threatens the durability of TYLCV resistance genes. The use of different resistance genes contributes to prevent the development of epidemics and allows their pyramidalization, which has proved to be useful to increase the level of resistance to TYLCD (Vidavsky et al. 1998; 2008).

In order to better exploit the potential in breeding for resistance of PI 126944 we have initiated the construction of a set of introgression lines. Advanced generations obtained from the available interspecific hybrids have been tested to TWSV and TYLCD, confirming the expression of the resistance in the genetic background of tomato. We describe in this paper the current state of the set of introgression lines and the resistance to TWSV and TYLCV of some generations.

Material and Methods

Populations development

Plant material

Plant material consisted of a collection of generations derived from *S. peruvianum* PI 126944 (Fig. 1). In a previous work developed by our group, the tomato line NE-1 was crossed as female parent to some plants of *S. peruvianum* PI 126944 and three interspecific and self-incompatible hybrids (F₁-A, F₁-B and F₁-E) were obtained by embryo rescue (Picó et al. 2002). Three backcross generations to the tomato old variety Fortuna C (FC) were obtained. Due to the strong incompatibility between tomato and *S. peruvianum*, the number of plants generated by backcrossing was reduced and did not represent the whole *S. peruvianum* genome, being necessary to produce additional crosses. Self-incompatibility did not allow progenies by selfing to be obtained from the interspecific hybrids, so crosses between the hybrids F₁-B and F₁-E were made, obtaining a pseudo-F₂ generation (given that does not come from a selfed F₁ plant). The same procedure was carried out until the pseudo-F₆ generation, because of the persistence of self-incompatibility. From the pseudo-F₂ generation, a pseudo-F₂-BC₁ generation was obtained. One pseudo-F₃ generation was also backcrossed twice to tomato, obtaining five pseudo-F₃-BC₁ generations and one pseudo-F₃-BC₂ by embryo rescue. Six pseudo-F₃-BC₁ plants were intercrossed and abundant progeny was obtained. This progeny was backcrossed once to tomato and 4 plants were obtained from mature seeds. Several crosses between one BC₁ and one pseudo-F₃-BC₁ were also carried out.

Immature embryo rescue

Immature embryo rescue was carried out from fruits three weeks after pollination. Different media were selected depending on the embryo developmental stage (Online Resource 1). For globular embryos medium 1 was used (4.414 g/L Murashige & Skoog Medium (MS) + Gamborg Vitamins B5, 30 g/L sucrose, 1 g/L yeast extract, 0.8% agar, 2 mg/L 2,4-dichlorophenoxyacetic acid, 1 mg/L 6-benzilaminopurine. The pH was adjusted to 5.7). Globular embryos were cultured inside the opened immature seeds to protect embryos from dehydration. This was performed by making a small cut in the chalazal region and placing the cut side in contact with medium 1. To induce organogenesis pathway, embryos were kept in the dark for seven days at 24-26°C. Once organogenesis pathway was induced, *calli* were transferred to medium 2 (4.414 g/L Murashige & Skoog Medium (MS) + Gamborg Vitamins B5, 20 g/L sucrose, 0.8% agar, 2 mg/L indolacetic acid, 1 mg/L N6-[2-isopentenyl]adenine. The pH was adjusted to 5.7) and were grown in a chamber with fluorescent light (50 μmol photons m⁻² s⁻¹) for 16 h per day. Heart and abnormal torpedo embryos were found mostly from crosses of pseudo-F₃-BC generations to FC (Fig. 2). For these embryos medium 3 was used (4.414 g/L Murashige & Skoog Medium (MS) + Gamborg Vitamins B5, 20 g/L sucrose, 0.8% agar, 0.1 mg/L indolacetic acid. The pH was adjusted to 5.7). Once the plants started to grow from the *callus*, they were transferred to a base medium without growth regulators, medium 4 (4.414 g/L Murashige & Skoog Medium (MS) + Gamborg Vitamins B5, 20 g/L sucrose, 0.8% agar. The pH was adjusted to 5.7).

Inoculation and disease assessment

Inoculation trials were conducted to test resistance to TSWV and TYLCV-like viruses.

Plant material

Clonal replicates of the three hybrids developed by Picó et al. (2002) were employed in a first inoculation trial (Inoculation trial 1, IT1, Table 1). Inoculation of pseudo-F₂, pseudo-F₃-BC₁ and intercrosses between pseudo-F₃-BC₁ plants (Inoculation trial 2, IT2) was also carried out (Table 1). In inoculation trials I and II, the tomato lines NE-1 and FC were used as susceptible controls and accession PI 126944 as resistant control for both viruses. The TY-197 line, with resistance to TYLCV derived from *S. peruvianum*, was also employed as resistant control. In inoculation trial II, the RDD line, homozygous for the *Sw-5* gene, was also used as susceptible control for TSWV isolate GRAU, which overcomes the resistance conferred by *Sw-5* gene (Aramburu and Martí, 2003).

Inoculation and assessment for TSWV

Clonal replicates of each hybrid, pseudo-F₃-BC₁, pseudo-F₃-BC₁ x pseudo-F₃-BC₁ generations and controls were inoculated (Table 1). Two TSWV isolates were used, one not overcoming the resistance conferred by the *Sw-5* gene and the other one overcoming this resistance: HA-931100 (provided by Dr. C. Jordá, Universidad Politécnica de Valencia) in IT1, and GRAU (provided by Dr. J. Aramburu, Recerca i Tecnologia Agroalimentàries, IRTA, Barcelona) in IT2, respectively. Mechanical inoculation was carried out in a climatic chamber with environmental conditions of 25°C/18°C (day/night) temperature, 65%/95% (day/night) relative humidity and 65 to 85 $\mu\text{mol s}^{-1} \text{m}^{-2}$ of irradiance from Sylvania GroLux fluorescent tubes with a wavelength interval between 400 and 700 nm. The photoperiod was 14 light hours. Inoculum was prepared by grinding infected leaves of the susceptible tomato line NE-1 in cold 0.1 M phosphate buffer, pH 7.0, containing 0.2% Na₂S₂O₅ and 0.2% sodium diethyldithiocarbamate in a proportion of 1:5 (wt/vol) and 1% 600 mesh Carborundum (Soler et al. 1998). Seven days after the first inoculation plants were inoculated again to avoid escapes. Symptoms were evaluated at 15, 30, 45 and 60 days after the second inoculation. At the same dates, samples from inoculated and non-inoculated leaves were harvested and virus presence was detected using DAS-ELISA (Ding et al., 1995). Absorbance of serologic reaction was measured at a wavelength of 405 nm in a Titertek multiscan MCC/340 photometer. Samples with absorbance three times higher than the average absorbance of samples from non inoculated plants were considered positive or TSWV infected.

Inoculation and assessment for TYLCV

Clonal replicates of each hybrid, pseudo-F₂, pseudo-F₃-BC₁, pseudo-F₃-BC₁ x pseudo-F₃-BC₁ generations and controls were inoculated (Table 1). Both TYLCV and TYLCSV species were used in IT1, while TYLCV was the species used in IT2. Isolates used were TYLCV-Mld [ES:72:97] (accession L27708) and TYLCSV-ES[2] (accession L27708), kindly provided by Dr. E. Moriones (Estación Experimental “La Mayora”, Málaga), and Dr. E. R. Bejarano (Universidad de Málaga), respectively. Agroinoculation at four true-leaves state was used in IT1, following the methodology described by Picó et al. (2002). Plants in IT2 were whitefly-inoculated with *Bemisia*

tabaci Genn. biotype Q, (provided by Dr. F. Beitia, Instituto Valenciano de Investigaciones Agrarias, Valencia) inside muslin-covered cages for seven days. Symptom severity was scored at 15, 30, 45 and 60 days post inoculation (dpi). Moreover, virus DNA accumulation was measured on each date. Leaf tissue samples were harvested and total DNA was extracted following the protocol described by Crespi et al. (1991). Viral DNA was detected by dot-blot and molecular hybridization with specific digoxigenin-labelled probes for TYLCSV and TYLCV provided by Dr. E.R. Bejarano (Universidad de Málaga) and chemiluminescent detection, following the protocol described in “The DIG system user’s guide for filter hybridization” of Roche Molecular Biochemicals. Viral DNA was quantified according to a standard curve. Total plant DNA extracted was also quantified by agarose gel electrophoresis using the software Image Gauge V.4.0., to relate viral amounts detected to plant DNA present at each sample.

Genotyping

A total of 117 plants were genotyped, belonging to the following generations: one plant of each tomato parent (NE-1 and FC), F₁-A and F₁-B (F₁-E was not available), three BC₁, two BC₂, 13 BC₃, two pseudo-F₂, 18 pseudo-F₄, 17 pseudo-F₅, one pseudo-F₂-BC₁, four pseudo-F₃-BC₁, one pseudo-F₃-BC₂, two pseudo-F₃-BC₁ x BC₁ and 50 pseudo-F₃-BC₁ x pseudo-F₃-BC₁ generations (Fig. 1). Leaf tissue samples were harvested and total DNA was extracted following the protocol described by Doyle and Doyle (1990).

A total number of 263 markers were analyzed. Polymorphism was revealed by 105 out of the marker set (Online Resource 2; only the 105 polymorphic ones are shown). The polymorphic marker set consisted of 61 Simple Sequence Repeat (SSR) and 12 Conserved Ortholog Set (COSII) described and mapped in Sol Genomics Network (<http://solgenomics.net/>), 29 SSR designed from the sequences available in that database, using the free access programmes WebSat (<http://wsmartins.net/websat/>) and Primer 3 (<http://frodo.wi.mit.edu/primer3/>), and three Restriction Fragment Length Polymorphism (RFLP) converted in Cleaved Amplified Polymorphic Sequences (CAPS) (Bai et al. 2004).

For SSR markers Polymerase Chain Reaction (PCR) consisted of an initial incubation at 94°C 5 min, 30 cycles of denaturation at 94°C 30 s, annealing at temperatures between 40-60°C 30 s (see Online Resource 2), and elongation at 72°C 1 min, with a final elongation step at 72°C 10 min. PCR for COS II and CAPS markers consisted of an initial incubation at 94°C 5 min, 35 cycles of denaturation at 95°C 30 s, annealing at temperatures between 55-56°C 1 min (see Online Resource 2), and elongation at 72°C 2 min, with a final elongation step at 72°C 10 min.

SSR markers were analyzed on a LICOR 4300 DNA sequencer. Digestion products of CAPS markers were analyzed by 1.5% agarose gel electrophoresis and visualized by GelRed (Biotium) or ethidium bromide staining.

Results

Population development

In a previous work developed by our group, crosses between tomato and some plants of *S. peruvianum* PI 126944 were carried out. From these crosses, three interspecific and self-incompatible hybrids (F₁-A, F₁-B and F₁-E) were obtained by embryo rescue (Picó et al. 2002). At present work, a collection of generations derived from these hybrids were developed (Fig. 1). Due to the high incompatibility existent between *S. lycopersicum* and PI 126944, a lot of crosses and embryo rescue were required to obtain the backcross generations (Online Resource 3). In the first backcross, a total number of 129 embryos were obtained from 65 fruits and four of them developed until plants. Most of the embryos were rescued at globular stage, reaching a few of them heart or torpedo stages. From the second backcross (BC₂ and BC₃), some mature fruits produced few viable seeds, although embryo rescue was also employed to obtain progeny. In the BC₂, most embryos developed until torpedo stage.

As only a few plants were obtained by direct backcrosses, additional crosses were made in order to increase the number of descendants. Self-incompatibility did not allow obtaining progenies by selfing, so crosses between pseudo-F₂ plants were made (and this strategy was used until pseudo-F₆ generations). A high incompatibility was also found in crosses between pseudo-F₂ plants. From more than one hundred pseudo-F₂ plants, only crosses involving 20 of them allowed obtaining 19 pseudo-F₃ generations. These 20 pseudo-F₂ plants showed different levels of incompatibility: some of them produced compatible crosses with several plants while others were only able to give descendants when crossed with one or two of the plants. Plants of the 19 pseudo-F₃ generations obtained were intercrossed in many combinations to obtain pseudo-F₄ generations. Only three pseudo-F₃ plants were involved in crosses which produced the pseudo-F₄ progeny.

As an attempt to continue with the introgression in the tomato genetic background, a lot of crosses between tomato and 10 pseudo-F₃ plants were also made but only one of them produced pseudo-F₃-BC₁ descendants by embryo rescue. A total number of 136 embryos were obtained from 10 fruits derived from this cross, and 15 of them developed until plants (Online Resource 3; Fig. 1). In these generations several embryos developed until torpedo stage, although some of them had an abnormal development consisting in an irregular growth of cotyledons (Online Resource 1). These abnormal embryos did not give plants. In pseudo-F₂-BC₁ and pseudo-F₃-BC₂ generations several backcrosses to tomato followed by embryo rescue were carried out, but only one plant of each generation was obtained. Most of the embryos found in pseudo-F₂-BC₁ generations were globular. Several plants of pseudo-F₅ and pseudo-F₆ generations were also backcrossed once to tomato. Some embryos were found, being most of them at globular stage, but no plants were obtained from these embryos. Crosses between pseudo-F₃-BC₁ generations were more compatible and allowed to obtain a lot of progeny. Several plants from these crosses were backcrossed to tomato (Fc x [pseudo-F₃-BC₁ x pseudo-F₃-BC₁]), but it was not possible to obtain progeny from these crosses by embryo rescue. However, several fruits from this backcross were allowed to mature, being able to produce few viable seeds.

Inoculation response to TSWV

Inoculation trial 1

Plants of the susceptible control were infected at 15 dpi showing severe symptoms and high absorbance values. Plants of the resistant control PI 126944

remained symptomless during all the assay, and did not accumulate virus. Clonal replicates of the three hybrids behaved as the resistant control.

Inoculation trial 2

All susceptible controls (NE-1, FC and RDD) showed systemic infection, but symptom severity and absorbance values were higher in NE-1 than in FC and RDD. Systemic infection was detected only in 9% of the plants of the resistant control PI 126944 showing only slight symptoms. All clonal replicates of pseudo-F₃-BC₁ generations behaved as resistant PI 126944 plants, so symptoms were mild and systemic infection was not detected. The percentage of systemically infected plants in crosses between pseudo-F₃BC₁ generations ranged between 21% (71-4 x 71-2) and 28% (71-4 x 71-3). These results could suggest a monogenic control of the resistance. Symptoms in infected plants were more severe than the ones shown by the resistant control but markedly lower than the ones exhibited by the susceptible ones.

Inoculation response to TYLCV and TYLCSV

Inoculation trial 1

The susceptible control NE-1 showed severe symptoms from 15 dpi. Viral DNA of both TYLCV and TYLCSV species was detected in all plants from this date. Plants of resistant control TY-197 remained symptomless although virus was detected from 25 dpi. PI 126944 plants showed mild symptoms and only accumulated TYLCV. Hybrids did not show a consistent behaviour. F₁-A hybrid plants were susceptible from 15 dpi. The number of infected plants was higher for TYLCV than for TYLCSV. Both F₁-B and F₁-E hybrids were resistant: plants were symptomless and viral DNA was detected only in three plants.

Inoculation trial 2

Susceptible controls showed severe symptoms and high DNA accumulation as expected. Most of TY-197 and PI 126944 plants remained asymptomatic, showing the rest very mild symptoms. However, viral accumulation in PI 126944 was comparable to accumulation detected in NE-1 and FC. TY-197 showed a marked reduction in viral DNA accumulation. Some of the plants of pseudo-F₂ generation could not be evaluated given that they showed an abnormal growth habit and symptoms were not distinguishable. Most of the tested plants remained asymptomatic or showed mild symptoms. Only two plants displayed severe symptoms at 60 dpi. Viral accumulation was not detected in 14 plants. Average viral accumulation in plants in which virus was detected was similar to that of the resistant control TY-197 and lower than the amount of virus in NE-1 and FC at 35 dpi, when accumulation was maximum. Symptoms developed in pseudo-F₃BC₁ plants were lower than in susceptible controls. The most severe symptoms were shown by replicates from plants 71-2 and 71-4. Clonal replicates from plants 71-1 and 71-7 displayed milder symptoms. Clone 71-1 had the best response to TYLCV and it was similar to that of PI 126944. Clonal replicates from 71-2 and 71-3 accumulated viral amounts similar to those detected in the most resistant control, TY-197. Plants of the cross (71-1 x 71-3) remained asymptomatic or displayed mild symptoms. Percentage of asymptomatic plants in crosses (71-4 x 71-2) and (71-4 x 71-3) was lower. It was possible to select plants which remained asymptomatic and also accumulated low amounts of virus in all generations evaluated.

Genotyping

Among the 263 markers tested, 95 resulted monomorphic and 63 did not amplify. Polymorphism between tomato and *S. peruvianum* was revealed by 53% of the markers that amplified. Different plants of PI 126944 were used to produce the three interspecific hybrids. This caused the existence of several *S. peruvianum* alleles in the different generations for 39% of the polymorphic markers analyzed. Similarly, as two different tomato parents (NE-1 and FC) were employed for the populations development, two different tomato alleles were found for 10% of the polymorphic markers analyzed. Although several markers exhibited various *S. peruvianum* alleles, these alleles were not present in all generations. In some cases all plants of the most advanced generations exhibited only one of the possible *S. peruvianum* alleles. Anyway, the other allele was always present in less advanced generations such as pseudo-F₄ or pseudo-F₅. Markers with different *S. peruvianum* alleles were mostly at chromosomes 1, 2, 3, 7 and 12. However, only one *S. peruvianum* allele was found for most of the markers analyzed in chromosomes 4, 5, 8 and 10. In a similar way, the same tomato allele was found in both tomato parents for all markers analyzed at chromosomes 4, 5, 8, 10 and 11. For the rest of chromosomes there was at least one marker in which the tomato allele was different in both tomato parents.

First plant set genotyping

Thirteen plants of different generations (two F₁, two pseudo-F₂, three BC₁, one pseudo-F₂-BC₁, four pseudo-F₃-BC₁ and one pseudo-F₃-BC₂) were genotyped with the 105 polymorphic markers (Fig. 1). With them, almost 60% of genome was covered, according an average spacing between markers of 10 cM (Table 2).

As average, 79% of *S. peruvianum* genome was represented in pseudo-F₂ generations (20% in homozygous state and 59% in heterozygous state). Considering both pseudo-F₂ plants analyzed, most of *S. peruvianum* genome was present (Fig. 2). Only regions covered by markers TAHINA-3-123, in chromosome 3, and TAHINA-8-2, in chromosome 8, were fixed for the tomato alleles in both plants.

A reduction in *S. peruvianum* genome was observed in more advanced generations like BC₁ (56%), pseudo-F₂-BC₁ (60%) and pseudo-F₃-BC₁ (70%). A higher reduction was observed in the pseudo-F₃-BC₂ generation (33%). *S. peruvianum* alleles were represented in all regions analyzed for the group of plants belonging to BC₁, pseudo-F₂-BC₁ and pseudo-F₃-BC₁ generations. Pseudo-F₃-BC₂ generation was composed only of one plant. Several regions in all chromosomes were fixed for *S. lycopersicum* alleles in this generation.

In general, recombination was variable depending on the chromosome considered. For example, as average in the pseudo-F₂ and BC₁ generations, recombination was higher at chromosomes 1, 6, 7, 9, 11 and 12, and lower at chromosome 2.

Second plant set genotyping

A set of 83 plants (18 pseudo-F₄ plants, 50 pseudo-F₃-BC₁ x pseudo-F₃-BC₁, two BC₁ x pseudo-F₃-BC₁ and 13 BC₃) (Fig. 1), was analyzed with 64 of the polymorphic markers, selected to be equally distributed in the genome. With this marker set, 40% of genome is covered (Table 2).

As average, *S. peruvianum* genome was represented in 78% of markers analyzed in these generations (39% in homozygous state and 39% in heterozygous state) (Table 2). Considering all pseudo-F₄ plants analyzed, most of *S. peruvianum* genome was

present. The wild species alleles predominated in some chromosomes in all plants while other presented a higher tomato genome proportion (Fig. 3a). Most of chromosome 8 was covered by tomato alleles. Anyway, *S. peruvianum* alleles were found for all markers analyzed for this chromosome in at least one plant of this generation, with the exception of the distal end of the short arm, covered by the marker TAHINA-8-2.

In pseudo-F₃-BC₁ x pseudo-F₃-BC₁ generations, *S. peruvianum* allele was present for 47% of markers analyzed (11% in homozygous state and 36% in heterozygous state) (Table 2). Only for some regions of chromosomes 1, 6, 7, 8, and 9 *S. peruvianum* alleles were not represented (Fig. 3b). As occurred with pseudo-F₄ generations, tomato and *S. peruvianum* alleles were almost fixed for some regions.

In plants of BC₁ x pseudo-F₃-BC₁ generations, the average percentage of *S. peruvianum* genome was 63% (19% in homozygous state and 44% in heterozygous state) for the markers analyzed (Table 2). *S. peruvianum* alleles were represented in most of the regions analyzed in one of the two plants. Only for a few regions of chromosomes 2, 4, 5, 6, 7, 8, 9 and 12, *S. peruvianum* alleles were not represented. On the other hand, some regions of chromosome 3, 6 and 8 were fixed for *S. peruvianum* alleles in those generations (data not shown in Fig. 3).

S. peruvianum alleles were not found in BC₃ generations for the 64 markers analyzed. However, these plants presented small and orange fruits, probably due to the presence of *S. peruvianum* alleles.

Third plant set genotyping

A total number of 17 plants of pseudo-F₅ generation were analyzed with 43 polymorphic markers, covering 28% of genome. As average, *S. peruvianum* genome was represented in 84% of markers analyzed (50% in homozygous state and 34% in heterozygous state). Considering all plants of this generation, *S. peruvianum* alleles were present in all regions analyzed, with the exception of the region covered by the markers SSRB105694, on chromosome 8, and C2_At1g07310, on chromosome 9 (Fig. 3c).

Discussion

The aim of this work was to develop a set of ILs derived from PI 126944 due to the resistance to different pathogens reported in this accession. Several generations derived from crosses between this accession and cultivated tomato have been obtained despite the high incompatibility existing between *S. peruvianum* and the cultivated species. Concretely, PI 126944 was strongly incompatible in the initial crosses made to obtain the interspecific hybrids (Picó et al. 2002). These authors tested different methods and found the most efficient by combining the stigma and pistil treatments with immature seed culture. This procedure allowed obtaining some hybrid plants. In the present work, a similar efficiency was observed when immature seed culture was carried out without the stigma and pistil treatments employed previously. For that reason, this step was skipped in order to make the protocol more time- and cost-effective. As a whole we have obtained a 'genotype-dependent' efficiency in the specific embryo rescue technique used. As an example, only one of the ten pseudo-F₃ plants crossed to tomato allowed obtaining descendants, being this cross completely compatible. Additionally, efficiency of distinct genotypes of the recurrent parent to overcome the crossability barriers has been reported to differ in previous works (Sacks et al. 1997). The study of these factors was not the main objective of this work; however, we do not discard the use of additional treatments of the stigma and style and

the exploration of other recurrent parents in order to facilitate the obtaining of more descendants. Although these improvements could be incorporated to our procedure, increasing the efficiency, we have obtained an abundant progeny from some crosses. Moreover, the incompatibility is starting to be lost in the most advanced generations allowing the obtaining of normal progeny and the construction of the set of ILs from this particular accession.

Resistance to TSWV and to TYLCV derived from PI 126944 has been successfully introgressed into several of the generations obtained in the present work.

There was no systemic infection to TSWV in the three hybrids, as previously reported by Picó et al. (2002). On the other hand, response was variable in plants derived from crosses between pseudo-F₃-BC₁ generations, giving approximately a third of susceptible plants. This segregation points to a simple dominant gene controlling the resistance, although this assumption will be confirmed in the future. Resistance to TSWV derived from *S. peruvianum* has been previously reported as controlled by the single dominant gene *Sw-5* (Stevens et al. 1992). The marker SSR599, located on chromosome 9, is the closest marker to *Sw-5* of all the polymorphic markers analyzed in the present work. Several TSWV resistant plants of generations pseudo-F₃-BC₁ and pseudo-F₃-BC₁ x pseudo-F₃-BC₁ displayed tomato alleles for this marker. Therefore, these results suggest that the gene controlling TSWV resistance in PI 126944 may not be *Sw-5*.

Regarding the response to TYLCV, two hybrids were resistant while the third was susceptible. Variability was also observed among plants of the resistant parent PI 126944. Although Pilowsky and Cohen (2000) tested this accession for resistance to TYLCV and obtained a consistent resistant response in the 21 tested plants, our results suggest the existence of genetic variability for the genes of resistance in the set of plants used in this study. The different levels of resistance in all generations tested points to a quantitative nature of the resistance, which agrees with the reported genetic control for the resistance in some *S. peruvianum* accessions (Pilowsky and Cohen, 1990).

Polymorphism between tomato and *S. peruvianum* was revealed by 53% of the markers analyzed. This result is slightly lower than the one obtained by Fulton et al. (1997), in which 65% of markers analyzed were polymorphic. This difference can be due in part to the different types of molecular markers used in both studies: RFLPs (assayed with different restriction enzymes) were the markers used by Fulton et al. (1997) and mostly SSR in our study. Interestingly, 39% of the polymorphic markers exhibited different *S. peruvianum* alleles in our set of genotyped plants. This will allow the development of different sets of ILs containing each allele in order to have represented the higher amount of variability existing in the plants of the original accession crossed. In those cases that all the possible *S. peruvianum* alleles are not present in the most advanced generations, it will be necessary to make use of less advanced generations such as pseudo-F₄ or pseudo-F₅ to have them all represented. The loss of *S. peruvianum* alleles in more advanced generations was probably caused by random, as the number of plants involved in the construction of these generations was small, so the genotype is limited to their parent genotypes. On the other hand, the proportion of markers with different *S. peruvianum* alleles was variable depending of the chromosome considered. The same occurred with markers that exhibited different tomato alleles. In general, chromosomes 4, 5, 8, and 10 had the lowest proportion of markers that exhibit different *S. peruvianum* or tomato alleles. It may be due to a higher conservation of the genomic regions on these chromosomes.

A progressive reduction in the proportion of *S. peruvianum* genome and also in the size of the introgressed fragments was observed in generations obtained with one or

more backcrosses to tomato. As a whole, the percentage of *S. peruvianum* genome in these generations was 55% compared to the 80% for pseudo-F₂, pseudo-F₄ and pseudo-F₅ generations (Table 2). Different authors observed that recombination rate, which determines the fragment sizes, depends not only on the species but also on the region of the genome considered. Bonnema et al. (1997) compared recombination rates in an F₂ population obtained from a cross between tomato and *S. peruvianum*, with recombination rates of the F₂ population derived from *S. pennellii* LA716 (Tanksley et al. 1992). They observed that recombination was reduced at chromosome 2 and 5, while at chromosomes 1, 7, 9, 10 and 11 recombination rates were higher. We obtained similar results with our pseudo-F₂ and BC₁ generations. Recombination was lower at chromosome 2 and higher at chromosomes 1, 6, 7, 9, 11 and 12. Fragmentation of the introgressed segments after some selfing or backcross generations has been also demonstrated by Eshed et al. (1992) and Tanksley et al. (1996) respectively. Fulton et al. (1997) also found the progressive decrease of the size of the introgressed fragments of *S. peruvianum* in *S. lycopersicum*. Consequently, an efficient introgression of *S. peruvianum* genes into cultivated tomato was shown possible, in spite of the high distance between both species.

Deviation in the percentage of *S. peruvianum* genome in homozygous or heterozygous condition from expected values was patent for many generations. Thus, the percentage of *S. peruvianum* genome in the pseudo-F₄ generations (39% homozygous and 39% heterozygous) differed from the expected values for a F₄ generation (25% homozygous and 50% heterozygous). This percentage was higher than expected in the pseudo-F₅ generations (84% for the markers analyzed, 50% homozygous and 34% heterozygous). This is due to the specific genotype of the pseudo-F₄ plants involved in crosses to produce the pseudo-F₅ generation. Although hundreds of crosses between most pseudo-F₄ plants available were made in order to obtain the pseudo-F₅ generations, only two plants allowed obtaining progeny. These two pseudo-F₄ plants were the ones with the highest proportion of *S. peruvianum* genome. Maybe the success of these crosses was due to the higher wild species genome content and consequently the higher genetic similarity leading to compatibility between them. There is no information available about the genotype of the pseudo-F₃ plants from which these pseudo-F₄ generations were derived, but the reason of the percentage deviation could be the same than for the pseudo-F₅ generations.

Tomato and *S. peruvianum* alleles were not evenly distributed in the genome for the different generations. These deviations may be caused by chance, because of the small number of plants involved in the construction of the different generations due to the strong incompatibility. Additionally, it is also possible that for some loci a distortion of segregation exists. This distortion of segregation has been observed by other authors working with materials derived from interspecific crosses between tomato and some wild species (Fulton et al. 1997, Canady et al. 2005).

BC₃ generations are potentially very useful. No *S. peruvianum* genome has been found with the markers used. However, these plants presented some characters that could be related with the presence of *S. peruvianum* alleles, like small and orange fruits. Therefore, introgression fragments in these plants must be already very short. At present work, three BC₁ generations were genotyped and *S. peruvianum* fragments were already very short on them. So it is possible that most of *S. peruvianum* fragments have been lost on BC₃ generations and that the ones conserved are very short. Future work will include the analysis of these plants with the rest of the markers available in order to identify these fragments.

Conclusion

Introgression of the genes of interest into the cultivated species is necessary to make an efficient use of wild species. We have demonstrated that resistance to TSWV and to TYLCD from *S. peruvianum* PI 126944 can be successfully introgressed and expressed into tomato background. This accession is also resistant to other diseases, being thus interesting to test the final set of ILs developed for resistance to other pathogens.

S. peruvianum genome is almost completely represented considering the different plants of the most advanced generations. Development of the ILs will continue with backcrossing to tomato the most advanced generations available, like BC₁ x pseudo-F₃-BC₁ and pseudo-F₃-BC₁ x pseudo-F₃-BC₁. In any case, it will be necessary to make use of less advanced generations, such as pseudo-F₆ or BC₁ to introgress some fragments not present in these advanced generations. For this purpose it will be necessary to continue using embryo rescue technique due to the high incompatibility existent. Some of the improvements previously commented could be incorporated in our protocol in order to increase its efficiency. In any case, a reduction of this incompatibility, as a consequence of *S. peruvianum* genome reduction, has already been achieved.

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Fig. 1 Populations development. Generations written inside inverted commas are not true F₂, F₃, etc. as they were obtained by crossing different plants instead of self-pollinating. Numbers in brackets indicate the number of plants genotyped of each generation. See text for detailed information

Fig. 2 Genotype of generations analyzed for the 105 polymorphic markers. The top rows indicate chromosomes with markers (not to scale). White squares represent markers homozygous for *S. lycopersicum* alleles; grey ones, heterozygous; and black ones, homozygous for *S. peruvianum* alleles. White squares with a dash inside represent markers not determined

Fig. 3 Percentage of *S. peruvianum* genome considering all plants of pseudo-F₄ (a), pseudo-F₃-BC₁ x pseudo-F₃-BC₁ (b) and pseudo-F₅ (c) generations for each marker analyzed for each chromosome (Ch)

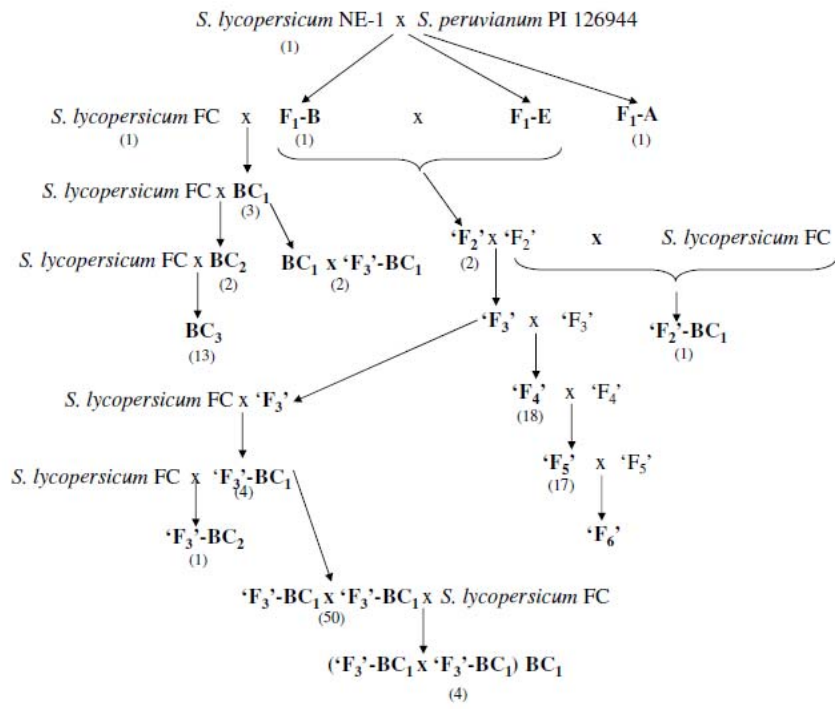


Figure 1

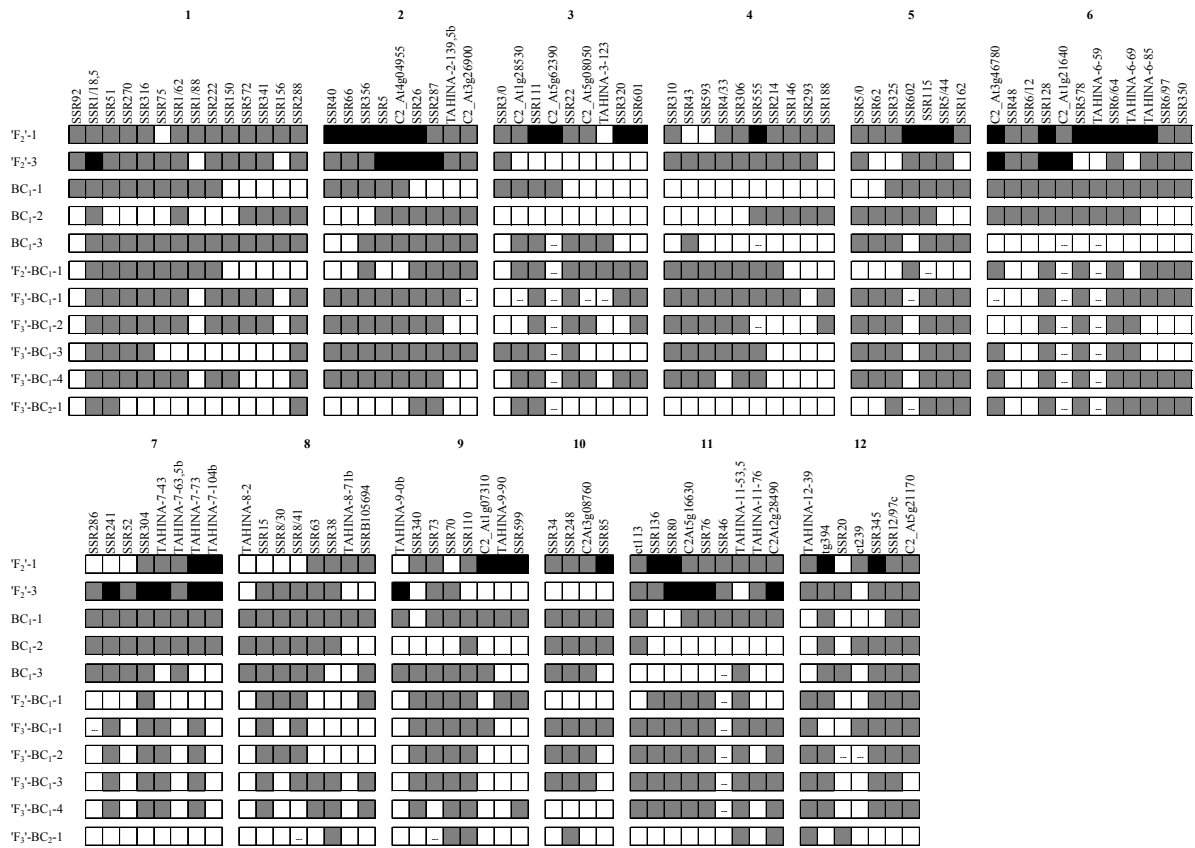


Figure 2

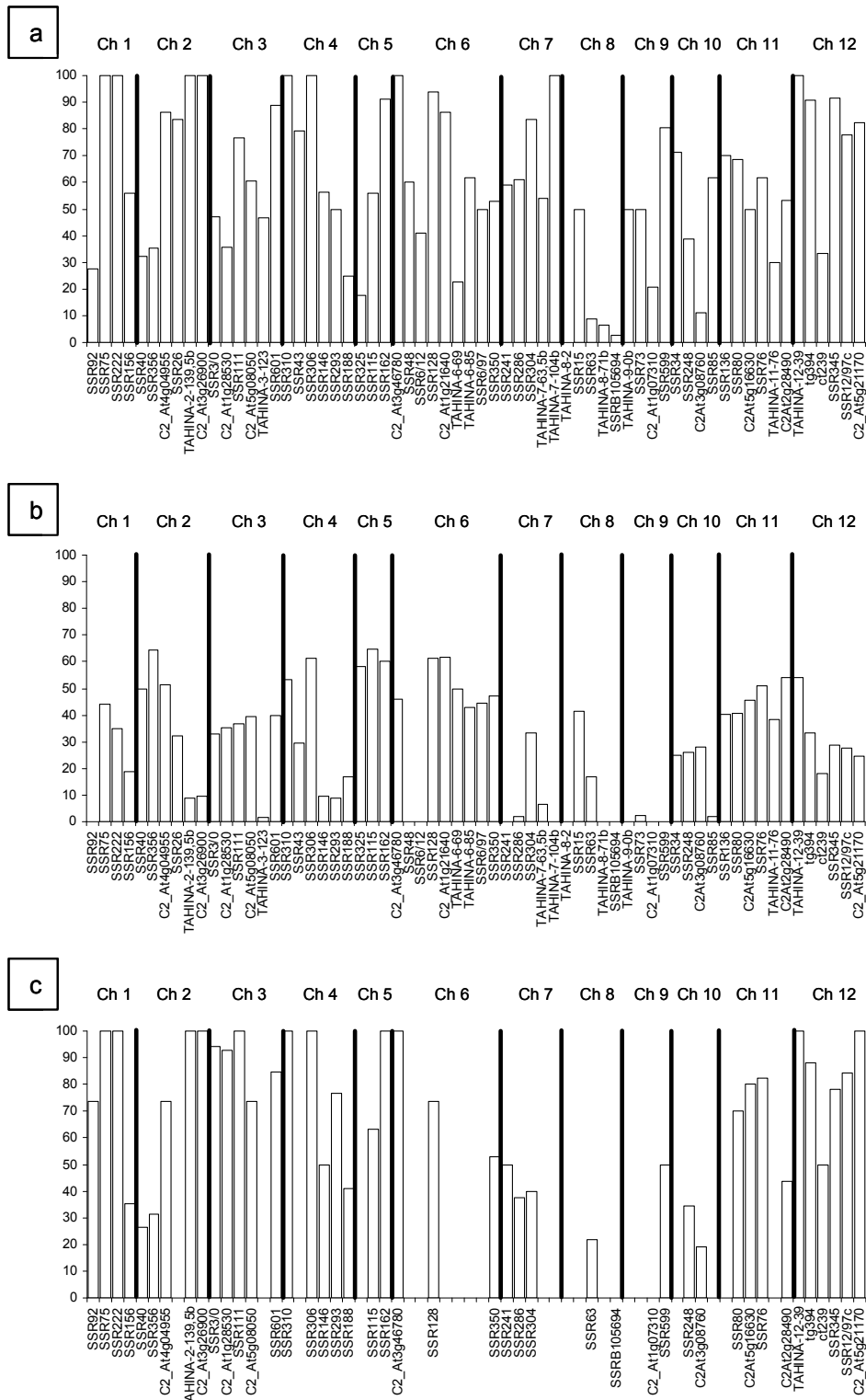


Figure 3