Transgenic Tomato Hybrids Resistant to Tomato Spotted Wilt Virus Infection

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Tomato spotted wilt virus (TSWV) infections cause significant economic losses in the commercial culture of tomato (Lycopersicon esculentum). Culture practices have only been marginally effective in controlling TSWV. The ultimate way to minimize losses caused by TSWV is resistant varieties. These can be obtained by introgression of natural sources of resistance from wild relatives or by expressing viral sequences in transgenic tomato plants. We report high levels of resistance to TSWV obtained in an inbred tomato line, transformed with a DNA construct comprising the TSWV nucleoprotein (NP) gene. The high levels of resistance were maintained in hybrids derived from the parental transgenic tomato lines. Moreover, the transgenic hybrids remained completely free of TSWV symptoms in field trials under high virus pressure.

Culture practices such as rotation, thrips control, and removal of alternate weed hosts have only been marginally effective in disease management of TSWV (Cho et al. 1989). Host-plant resistance to the virus is the most promising means of controlling the disease in the long term. Several accessions of *Lycopersicon* germplasm and tomato cultivars descended from such accessions are resistant to TSWV (Smith 1944; Finlay 1952, 1953; Paterson et al. 1989; Kumar et al. 1993). The resistance to TSWV derived from *L. peruvianum* was found to behave as a single dominant gene (Stevens et al. 1992; Boiteux and Giordano 1993). Since then, this resistance gene has been referred to as the *Sw-5* gene. So far however, it is not known whether the *Sw-5* gene confers durable resistance to TSWV in tomato. This uncertainty, together with the threat of new tomato-infecting tospoviruses, urges the need for identification or development of new sources of TSWV-resistance, especially considering the desirable reductions in the application of insecticides.

Over the past decade, numerous publications have shown the successful generation of virus resistance through transgenic expression of viral sequences in plants (Hull and Davies 1992; Scholthof et al. 1993; Wilson 1993; de Haan 1996). Transformation of tobacco with the TSWV NP gene confers resistance against TSWV infections (Gielen et al. 1991; MacKenzie and Ellis 1992; Pang et al. 1992). Expression of a translationally defective NP gene cassette also generates high levels of resistance, which indicates that the observed resistance is partly RNA-mediated (de Haan et al. 1992). To study the application of this technology in crops of agronomic importance, we transferred the TSWV NP gene cassette to an inbred tomato line used in the production of fresh-market hybrids.

Materials and Methods

Virus and plant material

TSWV isolate BR-01 was maintained in tomato by grafting to prevent the generation of defective mutants by repeated mechanical passages (Resende et al. 1991a). Parental tomato line ATV847 was used in the transformation. This inbred line is used as the male parent for the production of a number of hybrids for the southern European market (represents fresh-market tomatoes of the determinant type). Transgenic tomato plants were grown under certified greenhouse conditions according to the legislation imposed by the Dutch authorities (Voorlopige Commissie Genetische Modificatie: VCOGEM).

Transformation of parental tomato line ATV847

The construction of transformation vector pTSWVN-B and transformation of line ATV847 with this vector have been described by Ultzen et al. (1995). After transfer to the greenhouse, the transgenic plants were analyzed for their ploidy level using flow cytometry (de Laat et al. 1987). Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was subsequently employed to detect the accumulation of TSWV NP in transgenic plants and the integrity of the TSWV NP in these plants was verified by Western blots (Resende et al. 1991b). The transgene copy numbers of all diploid NP expressing transformants was determined using Southern blots (Ultzen et al. 1995).

Analysis of protection to TSWV infection

After emergence of the first leaf, about 3–4 weeks after sowing, tomato seedlings were analyzed for resistance to TSWV using mechanical inoculation (Gielen et al. 1991; de Haan et al. 1992; Ultzen et al. 1995). After mechanical inoculation, resistant transformant lines were identified, which were subsequently cross-pollinated to produce experimental hybrids.

Resistance to TSWV under field conditions

In the summer of 1994, a field trial was performed on the premises of S&G Seeds in El Ejido, Spain, according to the legislation imposed by the Spanish Ministry of Public Works and Environment. This location was known to have high TSWV incidence during the previous 5 years. The tomatoes were planted in a randomized split-plot design. Twenty to forty plants per line were plotted in two replications. Six transgenic entries were tested for TSWV resistance, together with nontransformed parental line ATV847, and the hybrids Radja and Yaiza, which were included as susceptible controls. Hybrids containing the *Sw-5* gene from *L. peruvianum* were tested in the same field to enable a comparison between these two sources of TSWV resistance.

Four-week-old seedlings (about 10 cm in height) were planted in the open field and exposed to thrips throughout their crop cycle. The trial was cultured under netting conditions, routine measurements, including the application of pesticides were taken. Chemical control against thrips was only applied after 85% of the susceptible controls showed systemic disease symptoms.

The field was visually monitored for appearance of systemic TSWV symptoms and for agronomic performance every week. At 16 weeks after transplanting, when the plants were bearing several red fruits, final observations were made. One week later, samples were taken from plants showing systemic symptoms and checked for the presence of TSWV using ELISA.

Results

Transformation of parental tomato line ATV847

The TSWV NP gene cassette pTSWVN-B, which is already proven to confer TSWV resistance in tobacco, has been transferred to the genome of tomato line ATV847 by *Agrobacterium*-mediated leaf disc transformation. About 45% of the transformants accumulated the TSWV NP at detectable levels in an ELISA assay. Western blots showed that this transgenically expressed protein comigrated with protein extracted from tomato plants systemically infected with TSWV, demonstrating the integrity of the NP produced in transgenic plants. Using flow cytometry, it was shown that 60% of the transgenic plants retained the diploid ploidy level. Diploid transgenic tomato plants were subsequently maintained to produce S1 offspring by self-pollination. None of them, or their progeny, exhibited phenotypic aberrations that could be assigned to the accumulation of NP or to the insertion of the NP gene cassette into the genome.

Protection of transgenic tomato against TSWV infections

The S1 progeny plants were analyzed for resistance to TSWV using mechanical inoculation. The susceptible control plants reached infection of 90%or higher within 2–4 weeks after inoculation. The final observation was made 6 weeks after the first inoculation (Figure 1). Out of 17 progeny populations challenged with the virus, 9 transformant lines could be identified that showed reduced susceptibility to TSWV infection, ranging from complete resistance in transformant lines 780 and 815 to moderate levels of resistance in lines 531 and 698. Plants without visual symptoms were free of virus when tested by ELISA using an antiserum raised against a nonstructural viral protein (NSs) that accumulates to high levels in TSWV-infected plant cells (Kormelink et al. 1991). From each S1 transformant line that showed

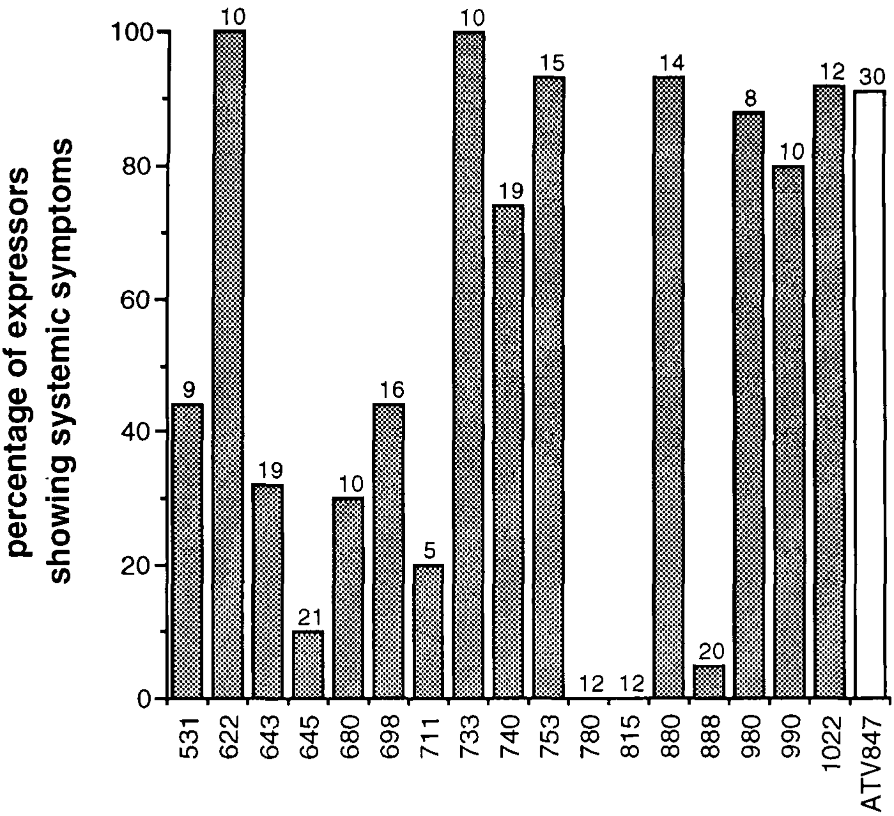

Figure 1. Resistance to TSWV infections of S1 progeny using mechanical inoculation. Plants were challenged twice with an inoculum prepared from systemically infected tomato plants. Progeny that did not inherit the NP gene cassette through segregation were left out of the analyses. Figures on top of the bars refer to the number of tomato plants of that particular line that were analyzed for resistance in the final observation about 8 weeks after the first inoculation.

reduced susceptibility against TSWV infections, a number of individual plants was maintained and self-pollinated to produce S2 offspring. The copy number of the NP gene in selected S1 plants was determined by Southern blots. The majority appeared to carry multiple copies of the transgene (2–7 copies), except for transformant line 698, which carried a single copy (results not shown).

The complex inheritance of multiple independent transgene copies makes it difficult to identify lines homozygous for all transgenes. Therefore, only lines 645, 698, 815, and 888 were used to produce experimental hybrids. The S2 lines (females) were pollinated with another parental line (males). This cross represents the reciprocal cross of a successful fresh-market hybrid. The reciprocal hybrids and their corresponding S3 lines were challenged with TSWV by mechanical inoculation

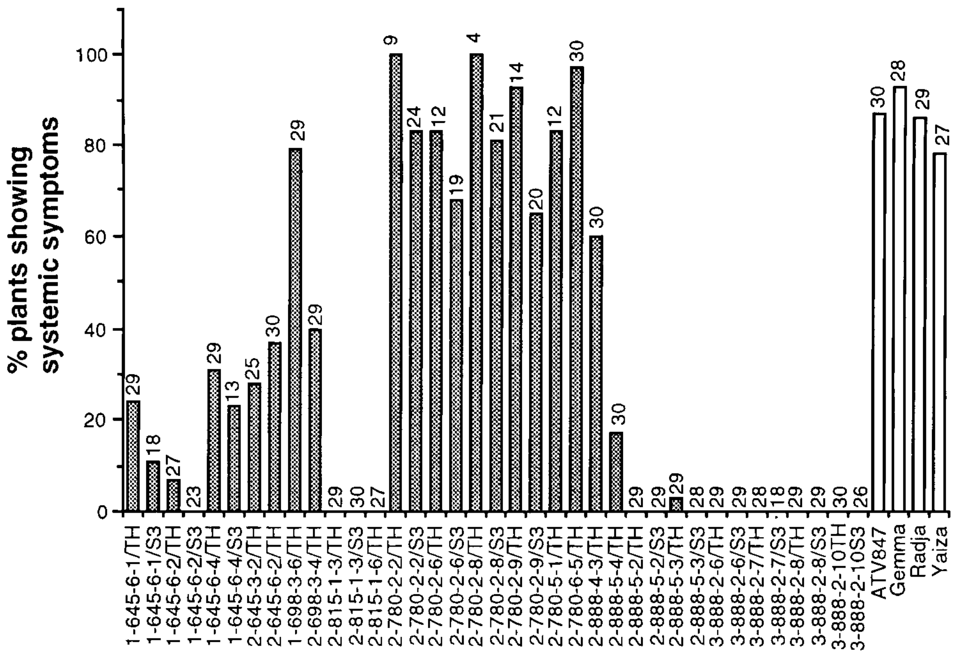

Figure 2. Resistance to TSWV infections of a selection of S3 populations and their corresponding hybrids. Control plants (white bars) consisted of nontransformed ATV847 plants, Gemma, Yaiza, and Radja hybrids. Plants were challenged twice by mechanical inoculation with an inoculum prepared from systemically infected tomato plants. Figures on top of the bars refer to the number of tomato plants of that particular line that were analyzed for resistance.

(Figure 2). The homozygous S3 line and the experimental hybrid descending from transformant lines 815 and 888 were both completely resistant to TSWV infection. Both lines carried two transgene copies that were closely linked and were inherited as single dominant genetic traits. The hybrids derived from primary transformant 645 (1-645-6-2 and 1-645-6-4) still segregated for the transgenes.

Most of the transgenic tomato lines were not completely protected from TSWV infection. For example, the homozygous S3 population and the experimental hybrid derived from transformant line 698 showed intermediate levels of resistance, as had been observed in the S1 population. Transformant 780 contained six copies of the NP gene. The S1 progeny of this plant were completely protected from TSWV. However, all S3 descendants and their corresponding hybrids were susceptible to TSWV. Southern blots revealed that all selected S2 lines lost two transgene copies. Obviously these two copies were responsible for the observed TSWV-resistance in the S1 progeny of this line.

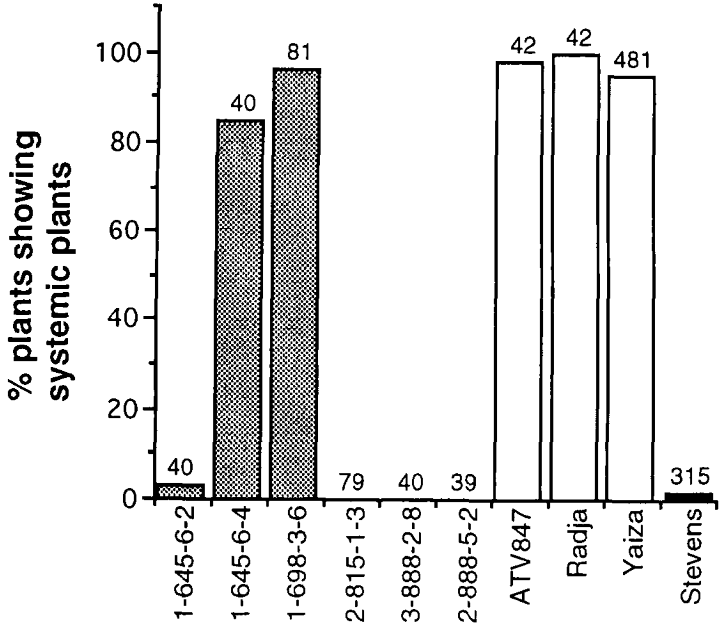

Figure 3. Resistance to TSWV infections of a selection of transgenic Radja hybrids under natural conditions, scored 16 weeks after planting. Control plants (white bars) consisted of nontransformed ATV847 plants, Radja, and Yaiza hybrids. Hybrids containing the *Sw-5* gene (black bar) were included in the same field trial. Figures on top of the bars refer to the number of tomato plants that were analyzed for resistance.

Field trials

Six different transgenic tomato hybrids were analyzed in open fields for resistance to TSWV under natural conditions. Eight weeks after planting, when plants were in their fruit-bearing stage, almost all nontransformed, susceptible control plants showed severe systemic symptoms (e.g., mosaic, bronzing, and wrinkling of leaves). The final observation was made 16 weeks after planting (Figure 3). The transgenic hybrids 2-815-1-3, 3-888-2-8, and 2-888-5-2, which were completely protected from TSWV after mechanical inoculation, were also resistant to TSWV under field conditions. In these immune transgenic hybrids, TSWV could not be detected in leaves or fruits using ELISA. The hybrids yielded healthy fruits of high quality. Transgenic hybrid 1-698-3-6, derived from a line with an intermediate level of resistance after mechanical inoculation, became almost completely infected in the field. As expected from the mechanical inoculation experiments, hybrid 1-645-6-4 also became infected in the field. Both hybrids did not yield marketable fruits.

Six plants out of 315 hybrid plants containing the *Sw-5* gene developed characteristic TSWV symptoms on the leaves. When leaf extracts prepared from these symptomatic plants were inoculated to plants containing *Sw-5*, about 2% of the plants developed systemic symptoms. This indicates that, at least in this case, the field-infected plants containing *Sw-5* did not harbor resistance-breaking TSWV isolates. However, fruits of almost all plants containing *Sw-5* developed ringspot symptoms caused by TSWV.

Discussion

Resistance to virus infections can be obtained by introgression of resistance genes from wild relatives, by expressing antiviral agents in plants, or by expressing viral sequences in plants (Hull and Davies 1992; Scholthof et al. 1993; Wilson 1993; de Haan 1996). Sources of TSWV resistance have been identified in *L. chilense* and *L. pimpinellifolium* (Smith 1944; Finlay 1952, 1953; Paterson et al. 1989). However, tomato cultivars harboring these sources are resistant to only a few TSWV isolates and, as a consequence, their practical value is limited. A source of resistance derived from *L. peruvianum* seems to be more promising. The resistance is conferred by a single dominant gene, denoted *Sw-5* (Van Zijl et al. 1986; Kumar et al. 1993; Stevens et al. 1993).

The traditional (*Sw-5*) and the transgenic (TSWV NP gene) sources of resistance were field-tested under a high TSWV pressure. On average, 2% of hybrid plants containing *Sw-5* developed systemic symptoms after both mechanical inoculation and natural thrips-mediated inoculation in the field. In addition, in the field trial, almost all fruits containing *Sw-5* showed ringspot symptoms. These observations indicate that either the *Sw-5* gene confers tolerance to TSWV, or, as suggested by Cho et al. (1992), the *Sw-5* gene is a resistance gene triggering a hypersensitive defense response. If this is the case, the necrotic ringspot symptoms are caused by feeding of thrips on the fruit during the early stages of development.

Transformation of a parental tomato line ATV847 with a transformation vector comprising the TSWV NP gene, yielded a number of TSWV-resistant transgenic lines. After mechanical inoculation, 9 out of 17 tested transgenic tomato lines showed protection against TSWV infection, which ranged from high to intermediate levels of resistance. After both mechanical and thrips-mediated virus inoculation, the resistant transgenic tomatoes did not accumulate detectable amounts of TSWV (Wijkamp 1995). This indicates that the resistant plants are probably immune rather than tolerant to TSWV infection. The resistance is maintained in tomato hybrids in which the transgenes are in the hemozygous state. Moreover, under field conditions with high TSWV pressure, the TSWV-resistant transgenic hybrids were unaffected and yielded fruits of high quality.

Transgenic tobacco plants expressing the TSWV NP gene are only protected against TSWV infection, but remain susceptible to other tospoviruses (de Haan et al. 1992).

The emergence of new tospovirus species, some of which infect tomato (de Ávila et al. 1993), emphasizes the need for identification or development of additional sources of genetic resistance against such viruses. Expression of the NP genes from three tospoviruses in transgenic plants confers resistance to the homologous viruses (Prins et al. 1995). Therefore, new resistance genes based on NP gene sequences will become available to breeders as soon as novel tospoviruses emerge.

Because the transgenic TSWV resistance is primarily RNA-mediated (de Haan et al. 1992) and based on RNA–RNA interactions, it is likely that the resistance will not easily be overcome by mutant TSWV strains that carry single or few-point mutations in their NP gene. Therefore, the synthetic TSWV resistance gene is expected to be a durable and reliable source of genetic resistance to TSWV. It is also possible that the technology developed to obtain TSWV resistance can be applied to generate resistance to any tospovirus in any crop susceptible to the corresponding tospovirus.

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