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Identification of RAPD markers linked to a locus involved in quantitative resistance to TYLCV in tomato by bulked segregant analysis

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Abstract In tomato, Bulked Segregant Analysis was used to identify random amplified polymorphic DNA (RAPD) markers linked to a quantitative trait locus (QTL) involved in the resistance to the Tomato Yellow Leaf Curl Virus. F_4 lines were distributed into two pools, each consisting of the most resistant and of the most susceptible individuals, respectively. Both pools were screened using 600 random primers. Four RAPD markers were found to be linked to a QTL responsible for up to 27.7% of the resistance. These markers, localized in the same linkage group within a distance of 17.3 cM, were mapped to chromosome 6 on the tomato RFLP map.

Key words Bulked Segregant Analysis · Marker-assisted selection · QTL · RAPD · Tomato Yellow Leaf Curl Virus

Introduction

The Tomato Yellow Leaf Curl Virus (TYLCV), which is transmitted by the whitefly *Bemisia tabaci* Gennadius, is responsible for severe losses in tomato production in the Eastern Mediterranean, Spain, North-West and East Africa, Dominican Republic, Jamaica and Cuba (Czosnek et al. 1990; Czosnek and Laterrot 1997). Recently, the virus has been detected in Italy also (south Italy, Sicily and Sardinia). Since *B. tabaci* is resistant to insecticides (Dittrich et al., 1990) breeding for resistance to TYLCV appears to be a promising and ecological approach to control the disease. Resistances to TYLCV have been reported in wild relatives of *Lycopersicon esculentum*, namely in some accessions of *L. peruvianum*, *L. hirsutum*, *L. pimpinellifolium* (Hassan et al. 1984; Kasrawi et al. 1988) and *L. cheesmanii* (Hassan et al. 1984). The genetic bases of the resistances vary from a single dominant gene to a polygenic recessive pattern (for a review see Laterrot 1992). Recently, the *Ty-1 L. chilense* gene involved in an oligogenic tolerance to TYLCV was mapped to chromosome 6 (Zamir et al. 1994).

The identification of loci involved in quantitative traits are usually based on restriction fragment length polymorphism (RFLP) linkage maps. Quantitative trait loci (QTLs) associated with disease resistance have been identified in different species: (1) for resistance to the downy mildew in pearl millet (Jones et al. 1995), to *Exserohilum turticum* in maize (Freymark et al. 1993), to *Rhynchosporium secalis* and *Erysiphe graminis* in barley (Backes et al. 1995), to *Globodera rostochiensis* (Kreike et al. 1993) and *Phytophthora infestans* in potato (Leonard-Schippers et al. 1994), to *Globodera pallida* in wild relatives of potato (Kreike et al. 1994), to *Ascochyta pisi* in pea (Dirlewanger et al. 1994); and (2) for tolerance to TYLCV in tomato (Zamir et al. 1994).

In this paper, we show that the Bulked Segregant Analysis (BSA) method (Michelmore et al. 1991) that is, widely used in the analysis of simply inherited traits, and particularly of disease resistances (de Vries et al. 1992; Miklas et al. 1993; Haley et al. 1993; Van der Beek et al. 1994; Chagué et al. 1996), is a valid and reliable method for targetting QTLs. Furthermore, and in support of our results, a theoretical analysis by Wang and Paterson (1994) demonstrated the feasibility of the method for quantitative inherited traits by selecting

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individuals with extremely contrasted phenotypes to form the two pools used in the BSA.

The resistance of the breeding material described in this paper originated in *L. pimpinellifolium* hirsute INRA. This genotype was used as a starting point for a preliminary identification of loci having major effects on TYLCV resistance. Segregation analysis revealed a quantitative inherited pattern of the resistance (Chague 1996, Laterrot unpublished). Our objective was to rapidly identify random amplified polymorphic DNA (RAPD) markers linked to TYLCV resistance loci for further utilization in breeding programs. Towards this aim, we applied the BSA method using pooled DNA from F_4 lines. Data supporting the successful identification of four RAPD markers linked to a QTL associated to TYLCV quantitative resistance are presented.

Materials and methods

Plant material

Bulk RAPD analyses were performed on 11 F_4 lines obtained from a cross 'Rty Azur' (resistant) × 'S Harmony' (susceptible). 'Rty Azur' and 'S Harmony' are *L. esculentum* cultivars developed by Clause Semences (St Remy de Provence, France). 'Rty Azur' carries resistance to TYLCV derived from *L. pimpinellifolium* hirsute INRA (Montfavet, INRA, France). These 11 F_4 lines were selected from a set of 40 lines derived from this cross. Thirty individuals of each F_4 line were evaluated for resistance/susceptibility to TYLCV by agroinoculation. From this test, the 5 most resistant F_4 lines (numbered R5, R6, R7, R9 and R13) and the 6 most susceptible ones (referred as S3, S4, S8, S10, S11 and S12) were used for the BSA analysis, each line consisting of 30 individuals.

Two bulks were prepared using DNA from F_4 lines: a resistant bulk consisting of DNA from 100 individuals from the 5 resistant F_4 lines, scored 1 according to the pathological scale, and a susceptible bulk consisting of DNA from 29 individuals scored 5 plus 35 individuals scored 4 from the 6 susceptible F_4 lines.

ANOVA analyses were performed on 10 F_4 lines (each line consisting of 20–30 individuals: 245 plants in total) developed by Clause Semences (Saint Rémy de Provence, France) from different crosses involving the same resistant parent, 'Rty Azur' or a derived resistance from *L. pimpinellifolium* hirsute INRA:

5 lines from a cross 'Rty Azur' × S Sunny
3 lines from a cross 'Rty Azur' × Rty LA 121 pimhir.SBC2
1 line from a cross 'Rty Azur' × S Carolina
1 line from a cross Rty pimhir × S Fantasy

S Sunny, S Carolina and S Fantasy are susceptible *L. esculentum* lines; Rty pimhir is a resistant line derived from *L. pimpinellifolium* hirsute INRA and Rty LA121pimhir.SBC2 is a resistant line derived from crosses involving *L. pimpinellifolium* hirsute INRA and *L. pimpinellifolium* LA 121.

Linkage analysis was performed on two F_2 populations:

1) One F_2 population from a cross 'Rty Azur' × 'S Harmony', consisting of 120 individuals, was used for RAPD analyses.
(2) RFLP analysis was performed on a F_2 population consisting of 85 individuals obtained from a cross Venise (*L. esculentum*) × *L. hirsutum*. This population was already mapped for 130 RFLP markers (unpublished results, markers from Tanksley et al. 1992) covering the whole genome, with 1 marker localized every 9–10 cM on average.

Resistance tests

Pathogenicity phenotype scoring

Resistance tests were performed by the agroinoculation of young tomato plants (1 month old, 3–4 leaf stage) with an LBA 40.44.04/pBin19 *Agrobacterium* strain bearing a dimeric genome of the extremely virulent Jordanian TYLCV isolate (Kheyr-Pour and Gronenborn, unpublished results), as described by Kheyr-Pour et al. (1991).

Symptom severity was scored according to the following scale: 1 = no symptom; 2 = slight yellowing of leaflet margins of the apical leaves; 3 = leaf yellowing, decrease in leaf size, plant stunting and upward twisting of leaf; 4 = yellowing of leaf, substantial reduction in leaflet size, severe leaf curl and plant stunting; 5 = very severe plant stunting, erected branches, leaves with very small leaflets exhibiting severe interveinal chlorosis, and upward cupping to assume an erected position.

Molecular detection of TYLCV

Infected leaf samples were squashed on a nylon membrane (Hybond N, Amersham) through 5-mm-diameter holes of a superimposed Saranwrap film, allowing extraction from approximately 25 mg of fresh material. The teflon pestle was protected with Saranwrap that was changed between each squash to avoid cross contamination. Dotted DNAs were fixed to the membrane by exposing the blots to UV light (312 nm) for 5 min. Following pre-hybridization of the blots (Sambrook et al. 1989), squash dot membranes were hybridized using the whole TYLCV genome as a probe: the 2750-bp TYLCV DNA was released from the LBA 40.44.04/pBin19 vector following restriction with *Eco*RI and *Hin*dIII and purification in a 0.8% agarose gel (Zhu et al. 1985). Labelling was performed by random priming, and hybridizations were carried out at 42°C according to the manufacturer (Radprime DNA labeling system, Gibco BRL). Blots were washed twice at 42°C in 2 × SSC − 0.1% SDS for 15 min and twice in 1 × SSC − 0.1% SDS for 15 min. All membranes were autoradiographed using X-ray films (Hyberfilm MP, Amersham) and intensifying screens for 48 h to standardize exposure. Dot signals were quantified using an image-analyser (MasterScan I, Computer Signal Processing, Billerca, Mass.). Signals were standardized as follows: the above-used blots were dehybridized (2 × 15 min in boiling water) and further re-used for hybridization using a standard 18S-rDNA probe (Falconet 1987). Quantification of the 18S signal was as above. TYLCV/18S signal ratios were used in ANOVA analyses.

RAPD analysis

Total DNA was extracted from plants (each individual of the different lines, F_2 population and parents) before inoculation, as described in Chagué et al. (1996). Bulked genomic DNA was used as template for polymerase chain reaction (PCR) amplification primary screening. RAPD amplifications were performed using single 10-mer oligonucleotides (Operon Technologies), and amplification products were separated as described in Williams et al. (1990).

Since RAPDs are multi-loci markers, a polymorphic band in one population does not necessarily reflect the same locus in different populations. To avoid misinterpretation, we used the polymorphic bands detected between parents and bulks of lines as probes for RFLP analyses in the populations used for statistical and linkage analyses.

RFLP analysis

For RFLP analysis, DNA was extracted from dessicated leaves of each individual as described in Saghai-Maroof et al. (1984). RFLP analysis was carried out as described in Chagué et al. (1996).

Statistical analysis

The link between markers and resistance to TYLCV was assessed with one-way ANOVA based on both pathological tests and squash dot results. Magnitude of the marker-associated phenotypic effect is described by the coefficient of determination, R^2, which represents the fraction of total variance explained by the polymorphism at the marker locus. Heritabilities of the two tests were estimated from assays performed in duplicate.

Linkage analysis

Marker order and map distances were estimated using MAPMAKER version 2.0 (Lander et al. 1987). Markers were first ordered with a LOD score >3. Recombination fractions were transformed by the Kosambi map function to estimate the map distance (Kosambi 1944).

Results

RAPD markers

Parents and bulks were screened using 600 random primers of which 90% allowed amplification of a RAPD pattern of approximately 10 DNA fragments ranging from 200 to 3000 bp in both parents and bulks. Further analysis allowed the identification of 254 primers revealing 330 polymorphic DNA fragments between the parents ('S Harmony' and 'Rty Azur'). The large amount of polymorphism found between 'S Harmony' and 'Rty Azur' can be explained by their different origins. Both cultivars contain several resistance genes as a result of different introgressions: *Ve*, *I*, *I-2*, *Tm-2²* and resistance to TYLCV for 'Rty Azur'; *Ve*, *I* and *Sm* for 'S Harmony'. For example, *Tm-2²* comes from *L. peruvianum* and represents a non-negligible source of polymorphism. Of these 254 primers, only 43 of them generated 49 polymorphic DNA fragments observed in both resistant or susceptible parents and bulks. In order to confirm the polymorphisms observed in the parents and bulks, we used the 43 primers in a secondary screening in which each F_4 line was analyzed as a bulk of their 30 individuals. Four of these primers (ra, rb, rc and rd, Table 1) generated markers potentially linked to a resistance locus, whereas the 45 other markers segregated randomly between resistant and susceptible lines. Primers generated markers Ra, Rb, Rc and Rd (450, 800, 400 and 1100 bp in size, respectively, Fig. 1) segregating in the same way in all F_4 lines. All 4 markers were present in the resistant lines R5, R6, R7 and R9.

Table 1 Sequence of 10-mer oligonucleotide primers for each RAPD marker linked to the TYLCV resistance QTL

Locus	Primer	Sequence
Ra	ra	CTGGCGTGTC
Rb	rb	GGGTGGGTAA
Rc	rc	GGAGTGCCTC
Rd	rd	GTGCTCCCTC

Statistical analysis

One-way ANOVAs were carried out in order to confirm an association between the 4 Ra, Rb, Rc and Rd RAPD markers and resistance to TYLCV in the F_4 lines. Results of the ANOVAs are shown in Table 2. ANOVAs between markers and scores established by pathological tests showed a correlation with variation in resistance only for Ra, Rb and Rc. On other hand, ANOVAs between markers and scores derived from molecular detection of the virus showed an association of resistance and all 4 markers, Ra, Rb, Rc and Rd. In addition, the latter analysis gave a similar R^2 value ranging between 11.2 to 22.8% for these markers (Table 2) in contrast to scores of symptom severity where R^2 ranged from 4 to 27.7% (Table 2). Heritability (h^2) of the two tests was: $h^2 \# 0.3$ using the pathological test scoring, and $h^2 \# 0.8$ using the squash dot scoring.

The low heritability of the symptom severity test cannot lead to an interpretation that R^2 is a good indication of the importance of other genes that may contribute to the trait but which were not detected. In contrast, the high heritability of the virus detection test relates to the reliable results of the R^2. Therefore, in spite of this difference and in the view of the heritability values, ANOVA results appear to be consistent since the 4 markers had significant R^2 and α values with the more reproducible test, and thus allowed the detection of one QTL for TYLCV resistance.

Mapping

Linkage between the 4 RAPD markers was analyzed on the F_2 population of the 'Rty Azur' × 'S Harmony' cross. All markers were mapped to the same linkage group, within a 17.3-cM fragment (Fig. 2), supporting the idea of a single QTL being localized within this genomic region. In order to localize these RAPD markers on the genetic map of tomato (Tanksley et al. 1992), we analyzed the segregation of RAPD and RFLP markers in a Venise × *L. hirsutum* F_2 population. Only the Rc marker could be detected in the population, the 3 other markers being likely more specific of a *L. pimpinellifolium* hirsute INRA background. The segregation data suggested that this marker is localized on chromosome 6 between TG153 (LOD

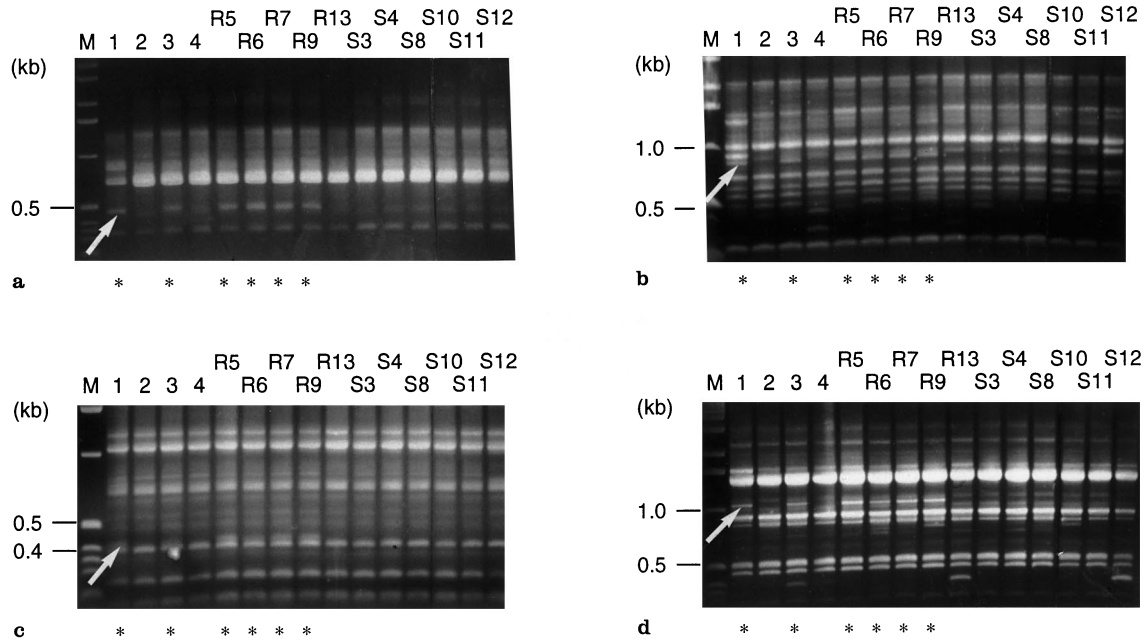

Fig. 1a–d Identification of Ra (**a**), Rb (**b**), Rc (**c**) and Rd (**d**) markers (arrows) by RAPD. Fragments were separated in 1.4% Bet-stained agarose gels. *Stars* denote lanes in which Ra, Rb, Rc and Rd are visible. *Lane M* 1-kb molecular weight ladder, *lane 1* resistant parent 'Rty Azur', *lane 2* susceptible parent 'S Harmony', *lane 3* resistant F_4 bulk, *lane 4* susceptible F_4 bulk. *R5*, *R6*, *R7*, *R9* and *R13* are resistant F_4 lines used to form the resistant bulk; *S3*, *S4*, *S8*, *S10*, *S11* and *S12* are susceptible F_4 lines used to form the susceptible bulk

value was 3.14) and CT83 (LOD value was 3.82) at a distance of 14.6 and 14.3 cM (Fig. 2), respectively, from these loci. Our map distances differ from the map established by Tanksley et al. (1992), but mapping based on different species from the original cross mapped by Tanksley (i.e. *L. esculentum* cv 'VF-36 *Tm-2²*' × *L. pennellii*) could alter crossover frequencies. The use of *L. hirsutum* in our cross could explain some variation in crossover frequencies, as reported in Chagué et al. (1996).

Discussion

QTL detection

We observed a significant positive association between squash dot scoring and pathological test scoring with $r = 0.59$ (correlation coefficient) and $\alpha < 0.001$ (significance). This observation is in agreement with data from Rom et al. (1993), who have established a positive correlation between virus quantity and symptoms severity. In addition, as observed by Kasrawi et al. (1988), all symptomless individuals (scored 1) carried the virus at a low level (100- to 300-fold less than a susceptible scored as 5), suggesting that the resistance mechanism may involve a reduction in the capability of the virus to replicate.

Genetic mechanisms determining the level of virus multiplication and degree severity of the symptoms may involve common but also distinct loci. In the case of a typical QTL analysis, one would expect to identify several unlinked markers. Our data suggests that we have found 4 linked markers dealing with one QTL due to the way the bulks were constituted. Whatever its role, the detected locus is located within a region spanning 17.3 cM of chromosome 6. However, since only a maximum of 27.7% of the resistance could be attributed to this locus it should be possible to identify other chromosomal regions conferring additional resistance in further studies. Indeed, the discrepancy in R^2 values of ANOVAs for all 4 markers between the two tests used for scoring TYLCV infection (pathogenicity and molecular detection of the virus) may also indicate that other loci may play a role in virus resistance. On the other hand, several environmental factors may influence the pathological test, such as room temperature, water uptake and attacks by other pathogens that result in TYLCV-like symptoms. This may account for the low heritability of this test, rendering the squash dot test more reliable. Indeed, this latter test (1) allowed a direct estimate of virus multiplication in plants and (2) did not appear to depend on morphological variations of plants due to external factors, thus resulting in a better heritability than the pathogenicity test.

Only part of the variation in resistance can be associated with the reported QTL. Several explanations can be proposed for this observation. Firstly, as suggested by Kreike et al. (1993), the distance between the

Table 2 Significant association between resistance and marker loci detected using ANOVAS. Marker Rd showed no significant values with the symptom severity test. The same plants were evaluated for virus resistance with both methods

Disease scoring	Marker	r[a]	α[b]	R^{2}[c]	F[d]	Prob > F[e]	h^{2}[f]
Symptom severity							0.3
	Ra	−19.1	<0.05	4	3.70	0.06	
	Rb	−52.6	<0.001	27.7	38.28	<0.001	
	Rc	−38.5	<0.001	14.8	17.40	<0.001	
Virus detection							0.8
	Ra	−42.9	<0.001	18.4	14.44	<0.001	
	Rb	−41.3	<0.001	17	13.14	0.001	
	Rc	−47.7	<0.001	22.8	18.86	<0.001	
	Rd	−33.5	<0.001	11.2	8.10	<0.001	

[a] Correlation coefficient
[b] Significance of r
[c] Percentage of resistance variation explained by the marker locus
[d] value from Fisher test
[e] Significance of F
[f] Estimated heritability

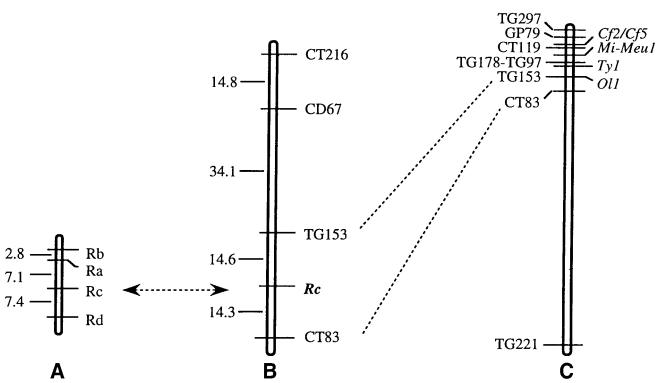

Fig. 2A–C Marker linkage group and chromsome 6 integrated map. Map distances (*left*) expressed in centiMorgans have been estimated using the Kosambi map function. **A** Linkage group obtained by RAPD analyses on the 'S Harmony' × 'Rty Azur' F_2 progeny. **B** Chromosome 6 integrated map obtained by RFLP and RAPD analyses of 'Venise' × *L. hirsutum* F_2 progeny. **C** Comparative localization of Rc with mapped resistance genes on chromosome 6 (Messeguer et al. 1991; Tanksley et al. 1992; Dickinson et al. 1993; Van der Beek et al. 1994; Zamir et al. 1994; Kaloshian et al. 1995)

markers and the resistance locus may still be great, and thus the QTL effect can be under-estimated. Secondly, detection of only one of several potential QTLs involved in this resistance may also be due to our use of starting material consisting of the most contrasting individuals in the population for pool construction: as emphasized by Wang and Paterson (1994), when a phenotype is influenced by multiple genetic loci and environment, individuals can exhibit extreme phenotypes due to different sets of QTLs or due to non-genetic factors. By using F_4 lines rather than a F_2 population, we have tried to minimize such non-genetic factor effects to target major QTLs involved in the resistance. In addition, the pooling of a large number of individuals increases the probability that the two pools will not differ for alleles other than those adjacent to the trait. Giovanni et al. (1991) suggested that increasing the pool size could also reduce the effect of false positives due to non-specific annealing. In this paper, we chose to use large bulks since our previous experience (Chagué et al. 1996) showed it minimized "one-off" individual effects. However, thirdly, several QTLs of lesser effect but accounting together for a non-negligible part of the genetic variation of the resistance trait probably escaped detection. Other reasons why we did not detect other loci may be (1) the lack of polymorphism in the region of these loci, (2) the possibility of *L. esculentum* genes segregating in our material and contributing together with *L. pimpinellifolium* hirsute INRA to the resistance.

In conclusion, while mapping of the whole genome using a segregating population has proven to be successful for the identification of loci involved in complex disease resistances (Landry et al. 1992; Kreike et al. 1993; Nodari et al. 1993; Leonard-Schippers et al. 1994), our results support the idea that BSA can provide fast detection of molecular markers linked to one QTL. Even though BSA is not as efficient as the classical QTL mapping analyses, this approach proved to be robust and efficient and provided a rapid starting point for complementary QTL analyses and identification of markers for use in selection programs.

TYLCV resistance loci

We mapped a new QTL to chromosome 6, between TG153 and CT83. Interestingly, Zamir et al. (1994) also mapped a major TYLCV tolerance locus (*Ty-1*) introgressed from the tomato wild relative *L. chilense* into *L. esculentum* to this chromosome by classical mapping. In addition, these authors mentioned another TYLCV tolerance locus from *L. cheesmanii* that also mapped to chromosome 6 (Zamir et al. unpublished). Taken together, these results support the idea that a region carrying major genes conferring resistance to TYLCV may be present on this chromosome as

a cluster of QTLs. Another possibility is that resistance/tolerance may be governed by the same QTL detected in different plant materials as allelic forms. Interestingly, several resistance loci have been mapped in this region of chromosome 6 (Fig. 2C): *Mi* (resistance to root knot nematode, Messeguer et al. 1991), *Cf-2* (resistance to *Cladosporium fulvum*, Jones et al. 1995; Dickinson et al. 1993), *Cf-5* (resistance to *C. fulvum*, Dickinson et al. 1993), *Ol-1* (resistance to *Oidium lycopersicum*, Van der Beek et al. 1994) and *Meu-1* (resistance to *Macrosiphum euphorbiae*, Kaloshian et al. 1995).

Implication for breeding programs

We have identified one major QTL for TYLCV resistance. The marker with the highest correlation, Rc, is the most closely linked to this QTL, and is the one now being used in a breeding program by Clause Semences. Further targeting of minor QTLs involved in the resistance will be performed in order to allow selection of the whole resistance capability carried by the genitor used in this work. Marker-assisted selection (1) enables the selection of loci involved in resistance without the drawbacks of pathological tests, and (2) allows the introgression of many resistances from different wild species into a single cultivar, enabling plants to resist a wider spectrum of isolates. Marker-tagging thus appears to be an essential step towards identifying better breeding strategies for TYLCV resistance and will facilitate the improvement of horizontal resistances which are polygenetically inherited and for which selection is often difficult.

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