Resistance Quantitative Trait Loci Originating from *Solanum sparsipilum* Act Independently on the Sex Ratio of *Globodera pallida* and Together for Developing a Necrotic Reaction

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Plant resistance to nematodes is related to the ability of the host to reduce the development of nematode juveniles into females. Resistance to the potato cyst nematode (PCN) *Globodera pallida*, originating from the wild species *Solanum sparsipilum*, was dissected by a quantitative trait loci (QTL) approach. Two QTL explained 89% of the phenotypic variation. The QTL *GpaV^{spl}* on chromosome V displayed the major effect on the cyst number (coefficient of determination [R^2] = 76.6%). It restricted *G. pallida* development to 16.2% of juveniles, 81.5% of males, and 2.3% of females. The QTL *GpaXI^{spl}* on chromosome XI displayed a lower effect on the cyst number (R^2 = 12.7%). It restricted *G. pallida* development to 13.8% of juveniles, 35.4% of males, and 50.8% of females. Clones carrying both QTL restricted the nematode development to 58.1% juveniles, 41.1% of males, and 0.8% of females. We demonstrated that potato clones carrying both QTL showed a strong necrotic reaction in roots infected by nematodes, while no such reaction was observed in clones carrying a single QTL. This result underlines the importance to introgress together *GpaV^{spl}* and *GpaXI^{spl}* into potato cultivars, in order to reduce the density of this quarantine pest in soil and to decrease the risk of selecting overcoming *G. pallida* subpopulations.

The potato cyst nematodes (PCN), *Globodera pallida* (Stone) and *G. rostochiensis* (Wollenweber) are one of the most serious potato pests in temperate climates. Crop rotation is difficult to apply for economical reasons. Nematicide treatments are undesirable from the environmental point of view. Breeding for resistant cultivars is the most desirable method of control.

Resistance to *G. pallida* was described in several accessions issued from cultivated and wild tuber-bearing *Solanum* species, such as *S. tuberosum* subsp. *andigena*, *S. vernei*, *S. spegazzinii*

and *S. sparsipilum* (Ross 1986; Rousselle-Bourgeois and Mugniéry 1995). Host resistance to PCN is defined as the ability of the host to restrict the number of developed females (Trudgill 1991) correlated with the reproductive potential of the parasite.

The PCN are biotrophic sedentary endoparasites with obligatory sexual reproduction. Their life cycle involves the J1 to J4 juvenile stages and the adult stage, with each stage separated by molts. The first molt occurs in the eggs contained in the female. Potato root exudates stimulate the hatching of the infective second stage juveniles (J2). The J2 penetrate the roots, migrate intracellularly, establish a permanent feeding site forming a syncytium close to the vascular tissues, and become sedentary. The J2 undergo three molts during development to the adult stage. Males leave the roots to fertilize the swollen females. Females produce hundreds of eggs, which remain enclosed in a cyst constituted of the hardened phenolized dead female body (Gheysen and Fenoll 2002). Nematodes remain viable in the cyst for many years. Sex is determined epigenetically, with the frequency of males increasing under crowding or poor nutrition conditions at the J2 stage (Mugniéry and Fayet 1981, 1984; Trudgill 1967).

Several authors described the effect of resistance sources on the life cycle of the nematode (Arntzen et al. 1993; Mugniéry et al. 2001; Rice et al. 1987), demonstrating that resistance influences the hatching rate, nematode development at juvenile stages, or the adult sex ratio. In parallel, the genetic dissection highlighted an oligo- or polygenic inheritance for most of the resistance sources to *G. pallida*. Large-effect QTL originating from *S. spegazzinii* (Caromel et al. 2003; Kreike et al. 1994) and *S. vernei* (Bryan et al. 2002; Rouppe van der Voort et al. 1998, 2000) have been mapped on the short arm (Dong et al. 2000) of chromosome V. QTL with lower effect originating from *S. tuberosum* subsp. *andigena*, *S. vernei*, and *S. spegazzinii* have been mapped on chromosomes IV, VI, IX, and XII (Bradshaw et al. 1998; Bryan et al. 2002; Caromel et al. 2003; Rouppe van der Voort et al. 2000). But until now, the effect of different QTL on the PCN life cycle was not determined.

Map location of genetic factors conferring the resistance originating from *S. sparsipilum* was not yet determined. However, this resistance source is attractive, since it is effective against the most aggressive populations of *G. pallida* pathotype Pa2/3, which is the most frequently encountered pathotype in Europe. *S. sparsipilum* was shown to develop a necrotic

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reaction in roots infected with *G. pallida*, to restrict the nematode development at a juvenile stage, and to modify the sex ratio toward males (Mugniéry et al. 2001).

In this paper, we report i) the genetic map location of the loci involved in the resistance to *G. pallida* originating from *S. sparsipilum*, and ii) the phenotypic effects of the individual QTL or the combination of QTL on the development of the necrotic reaction and on the developmental stages of *G. pallida*.

RESULTS

Mapping resistance QTL to *G. pallida*.

The resistance to *G. pallida* was evaluated by the number of newly formed cysts on a progeny of 239 hybrid clones issued from the cross between the susceptible *S. tuberosum* Caspar H3 and the resistant *S. sparsipilum* spl329.18. The whole progeny was split out in two sets that were separately assessed for resistance. The mean adjusted value per clone (described below) ranged from 0 to 1,005. The log-transformed data showed a continuous distribution with a bimodal tendency, skewed toward susceptibility (Fig. 1). Heritability of the genotypic means, computed on the merged data, was estimated to be 0.96.

Molecular genetic linkage maps were constructed for each parental clone, Caspar H3 and spl329.18, according to the double pseudo-test cross design. A total of 366 marker loci segregating from Caspar H3 mapped into 13 linkage groups, ranging from 18 to 131 cM. Three markers remained unlinked. A total of 293 marker loci segregating from spl329.18 mapped into 12 linkage groups, ranging from 31 to 108 cM. A set of 108 well-distributed markers with an average intermarker distance of 9.6 cM (standard deviation [SD] = 6.3 cM) constituted the framework map of Caspar H3, spanning a total length of 958 cM. A set of 106 well-distributed markers with an average intermarker distance of 9.5 cM (SD = 6.4 cM) constituted the framework map of spl329.18, spanning a total length of 894 cM.

Because the progeny was split into two sets of 97 and 142 clones tested independently for resistance to *G. pallida*, QTL analyses were first performed separately on each set of clones. Since the QTL log of the likelihood ratio (LOD) score plots detected in both sets overlapped (data not shown), data of both sets were merged to perform the QTL analysis presented in this paper. QTL analyses were performed on the 239 hybrid clones, using the marker data of the two framework parental maps together. Composite interval mapping (CIM) analyses detected two QTL on chromosomes V and XI of the *S. sparsipilum* map (Fig. 2) that we named *GpaV^{spl}* and *GpaXI^{spl}*. The QTL nomenclature indicates the nematode genus and species, the potato chromosome in Roman numerals, with the chromosome arm in superscript ('s' for short and 'l' for long, according to Dong and associates [2000]), and the species of origin in subscript. *GpaV^{spl}* displayed a major effect (coefficient of determination [R^2] = 76.6%), whereas *GpaXI^{spl}* displayed a weak effect (R^2 = 12.7%). No QTL was detected on the Caspar H3 map. No epistatic interaction was detected at a threshold of 10^{-4} . According to a two-factor analysis of variance (ANOVA), *GpaV^{spl}* and *GpaXI^{spl}* explained 89% of the total variation altogether.

Clones of the progeny were assumed to carry the resistance allele at a QTL when both flanking markers of this QTL possessed the alleles in coupling with the resistance phenotype. Double recombination events between the flanking markers were ignored. The clones carrying different combinations of alleles at both QTL displayed significantly different resistance phenotypes at a 95% confidence level. Their distribution is illustrated in Figure 3. The clones without resistance alleles at both QTL have the highest mean number of cysts (mean = 475.0). The clones with the resistance allele at the *GpaV^{spl}*

QTL formed a smaller mean number of cysts (mean = 14.5) than the clones with the resistance allele at the *GpaXI^{spl}* QTL (mean = 122.6). The clones carrying resistance alleles at both QTL have the smallest number of cysts (mean = 1.7). This result illustrates the additivity of QTL effects.

Effects of *GpaV^{spl}* and *GpaXI^{spl}* on development of a necrotic reaction.

Three to four clones for each allelic combination at the resistance QTL *GpaV^{spl}* and *GpaXI^{spl}* were chosen in the progeny between Caspar H3 and spl329.18 to study the individual and combined effects of both QTL on the development of a necrotic reaction. In the infected roots of the three clones with a resistance allele at the major-effect QTL *GpaV^{spl}* and in the infected roots of the four clones with a resistance allele at the minor-effect QTL *GpaXI^{spl}*, a thin layer of necrotic cells was observed in the immediate vicinity of nematode body (Fig. 4B and C). This necrotic reaction was similar to the one observed in the infected roots of the three clones carrying susceptibility alleles at both QTL (Fig. 4A). By contrast, in the infected roots of the four clones carrying resistance alleles at both QTL in addition to the thin layer of necrotic cells around the nematode body, a strong necrotic reaction always developed ahead of the head of juveniles (Fig. 4D). This strong necrotic reaction encompassed cortical cells from the juvenile head to the vascular vessels (Fig. 5), suggesting that it was initiated after syncytial induction.

Effects of *GpaV^{spl}* and *GpaXI^{spl}* on nematode development.

Four clones of the progeny between Caspar H3 and spl329.18, carrying different allelic combinations at the resistance QTL *GpaV^{spl}* and *GpaXI^{spl}*, were chosen to study their effects on nematode development. For each infected clone, the

Fig. 1. Frequency distribution of *Globodera pallida* Pa2/3 resistance scores expressed by $\log_{10}$ (adjusted mean cyst number + 1), measured in the 239 clones derived from crosses between *Solanum tuberosum* Caspar H3 and *S. sparsipilum* spl329.18. The cyst numbers given on the x axis of the figure follow a logarithmic scale. Positions of the parental clones are indicated on the histogram.

developmental stage of the retrieved nematodes was noted as J2/J3 undeveloped nematodes, males, or females. ANOVA detected a highly significant effect of the clone on the developmental stage ($P < 2.2 \cdot 10^{-16}$, $R^2 = 53\%$). The respective proportion of nematodes for each developmental stage showed obvious differences between the four clones (Fig. 6). Two criteria are particularly favorable for the resistance expression, the percentage of undeveloped J2 and J3 nematodes and the percentage of adult nematodes developed into males. In the clone with resistance alleles at both QTL, 58.1% of the nematodes were restricted to a J2/J3 juvenile stage, whereas the clones with a resistance allele at a single QTL harbored three- to fivefold fewer juveniles, and the clone without any resistance allele harbored 37-fold fewer juveniles. This result indicates that a single QTL confers a small effect for blocking the development of the juvenile nematodes and that the joined presence of both QTL confers a large effect on this trait.

In the clone without any resistance allele at the QTL, 89.4% of the nematodes developed in females and 9.2% developed in males. In the clone with the resistance allele at the minor-effect *GpaXI^s_{spl}* QTL, 50.8% of the nematodes developed into females and 35.4% into males. In the clone with the resistance allele at the major-effect *GpaV^s_{spl}* QTL, 2.3% of the nematodes developed into females and 81.5% into males. In the clone with resistance alleles at both QTL, 0.8% of the nematodes developed into female and 41.1% into males. This result underlines the large effect of the *GpaV^s_{spl}* QTL on the sex ratio imbalance toward males.

DISCUSSION

To our knowledge, this study is the first report of i) QTL associated with resistance to *G. pallida* originating from *S. sparsipilum*, and ii) the effect of individual and combined QTL on the development of the nematode. Two additive QTL were detected on chromosomes V and XI. The *GpaV^s_{spl}* QTL displayed the largest effect ($R^2 = 76.6\%$). The *GpaXI^s_{spl}* QTL displayed a

smaller effect ($R^2 = 12.7\%$) that is perfectly additive to *GpaV^s_{spl}*, since both QTL explained 89% of the total observed variation. This value is close to the broad-sense heritability value (0.96), indicating that most of the genetic variation is explained by both those QTL.

Two kinds of necrotic reactions were observed, in the infected roots of the clones studied, according to their allelic combination at both QTL *GpaV^s_{spl}* and *GpaXI^s_{spl}*. In the infected roots of the clones carrying a resistance allele at a single QTL as well as in the infected roots of the clones carrying susceptibility alleles at both QTL, the necrotic reaction was restricted to a thin layer of cells around the nematode body. This necrotic reaction is similar to those described in compatible interactions in other plant-nematode pathosystems (Golinowski et al. 1996; Rice et al. 1987). It resulted from host wound response to mechanical damage induced by juveniles during their migration through the cortex and would not be attributed to the individual QTL effects. Conversely, in the infected roots of the clones combining the resistance alleles at both QTL *GpaV^s_{spl}* and *GpaXI^s_{spl}*, a strong necrotic reaction developed around the head of the nematode. Since this strong necrotic reaction was not observed on the path of the nematode but only near the vascular vessels, it was supposed to be induced after syncytial initiation, as observed in the case of the *H1* gene conferring resistance to *G. rostochiensis* (Rice et al. 1985). Thus, PCN establish a syncytium by injecting secretions from esophageal gland cells through their stylet (Davis et al. 2000; Williamson and Gleason 2003). The necrotic reaction observed in roots of the clones carrying resistance alleles at both QTL was probably elicited, directly or indirectly, by nematode avirulence product secreted for syncytium initiation or development. Due to its location near the head of the nematode and in the vicinity of vascular vessels, this strong necrotic reaction is assumed to encompass most of the syncytium, resulting in a drastic starvation of juveniles. Consequently, most of them (58.1%) did not reach the adult stage, and those who succeeded to develop shifted to males (41.1%).

Fig. 2. Quantitative trait loci (QTL) for the *Globodera pallida* Pa2/3 resistance detected by composite interval mapping on the *Solanum sparsipilum* spl329.18 map. The log of the likelihood ratio (LOD) score is shown on the x axis. Markers and scaled distances are given on the y axis. Dotted lines indicate flanking markers of the most likely position of the QTL.

Development of a necrotic reaction, termed hypersensitive response, was frequently described in cases of monogenic resistances. In our study, we demonstrated that a complementation between both *GpaV^{spl}* and *GpaXI^{spl}* QTL is required to provoke a strong necrotic reaction in infected roots, near the head of the nematode. How two QTL, without individual necrotic effect, could produce a strong necrotic reaction when they are joined in the same potato clone remains unclear. One explanation to the phenomenon observed in our study could be that at least one of the two *GpaV^{spl}* and *GpaXI^{spl}* QTL has potential to individually activate a necrotic reaction but that this reaction is too slow to be observed in our experimental conditions. Conversely, once the QTL are both present, the reaction is boosted due to a synergic effect conferred by the interaction of both QTL, so that the necrotic reaction is visible 5 days postinoculation. Another explanation could be that both *GpaV^{spl}* and *GpaXI^{spl}* QTL are members of a resistance complex, as described in other pathosystems (Belkhadir et al. 2004). Molecular interactions between resistance protein domains may function as activation switches to trigger a necrotic reaction (Moffett et al. 2002). When a single QTL is present, the necrotic reaction is not activated and defense reactions affect more weakly the syncytium functionality.

Both QTL *GpaV^{spl}* and *GpaXI^{spl}* mapped in resistance-gene clusters common to the family Solanaceae (Gebhardt and Valkonen 2001). On the cluster of chromosome V containing *GpaV^{spl}*, the cloned resistance genes potato *R1* to *Phytophthora infestans* (Ballvora et al. 2002), potato *Rx2* to *Potato virus X* (Bendahmane et al. 2000), and tomato *Bs4* to *Xanthomonas campestris* pv. *vesicatoria* (Schornack et al. 2004) belong to the nucleotide binding site-leucine rich repeat (NBS-LRR) superfamily. On the cluster of the short arm of chromosome XI containing *GpaXI^{spl}*, the potato *R3a* to *P. infestans* (Huang et al. 2005) and tomato *I2* to *Fusarium oxysporum* (Simons et al. 1998) also belong to the NBS-LRR superfamily. As hypothesized by Rouppe van der Voort and associates (2000) for both nematode resistance QTL originating from *S. vernei*, *Gpa5* (on potato chromosome V) and *Gpa6* (on potato chromosome IX), the map location of both QTL *GpaV^{spl}* and *GpaXI^{spl}* suggests that they could encode NBS-LRR proteins, despite the fact that they have a quantitative effect.

The shift of the nematode sex ratio toward males was described in cases of monogenic and polygenic resistances (Bakker 2003; Luedders 1987; Rice et al. 1987). We demonstrated that both resistance QTL originating from *S. sparsipilum* independently modified the sex ratio toward males, with the largest effect attributed to *GpaV^{spl}*, which increased the number of males two to threefold more than did *GpaXI^{spl}*. Moreover, the association of both QTL reduced the percentage of females to almost zero. These conclusions were drawn from the observation of a single genotype per allelic combination, because of the time-consuming and laborious experiments. These results give an indication of the QTL effect on the sex ratio, whereas it is not excluded that the measured effect was also influenced by the genetic background. However, Mugniery and associates (2001) demonstrated that the number of females (counted in petri dishes) is highly correlated to the cyst number (counted in pots). Since both QTL explained 92.7% (total $R^2 = 0.89$, $h^2 = 0.96$) of the genetic variation associated with the cyst number, we assumed that the number of females is also due to the genetic effect of both QTL and that the genetic background has a negligible effect on the shift of the sex-ratio.

Our results provide an explanation why, in practical breeding programs based on introgression of resistance from wild species, the recovery of fully resistant offspring is very difficult. After several backcrosses, the best breeding lines are, at best, highly resistant, but these clones still allow the develop-

ment of a few cysts. Loss of a minor QTL as described in this paper is the most likely explanation for the enigmatic inability of practical breeding programs to retain the level of resistance of the donor wild species. In another pathosystem, Thabuis and associates (2004) demonstrated that phenotypic selection did not retain low-effect QTL and hindered the creation of fully resistant lines.

Our results also support arguments to banish mono-QTL cultivars for disease resistance. The introgression of the *S. sparsipilum* resistance to *G. pallida* into potato cultivars must take into account both QTL together. Despite its low effect, *GpaXI^{spl}* amplified and stabilized the action of the major effect of *GpaV^{spl}*. Potato clones with resistance alleles at both QTL displayed less than five cysts, whereas clones with resistance alleles at the single *GpaV^{spl}* QTL allowed development of up to 45 cysts. Reducing the reproductive potential to a minimal number of cysts is of primary importance in the case of a quarantine pest such as *G. pallida*. Moreover, current experiments conducted in our laboratories showed that the combined QTL reduced considerably the total cyst number indifferently towards

Fig. 3. Frequency distribution of *Globodera pallida* Pa2/3 adjusted mean cyst number for clones possessing different allele combinations at the *GpaV^{spl}* and *GpaXI^{spl}*. The clones that recombined between markers flanking the quantitative trait loci were considered as missing data. The cyst number given on the x axis follows a logarithmic scale. "Mean" indicates the mean value of the clones with each allele combination.

eight nematode populations, whereas the single *GpaV^{spl}* highly interacts with the nematode population (*unpublished data*), which could promote the selection of virulent populations.

Reducing the reproductive potential to a minimal number of cysts is crucial in a durability point of view. The fewer the number of cysts, the lower the risk of selecting for subpopulations of *G. pallida* that overcome the resistance. It has been demonstrated that *G. pallida* rapidly overcame partial resistance originating from several wild potato species (Beniers et al. 1995; Schouten and Beniers 1997; Turner and Fleming 2000, 2002; Turner et al. 1983; Whitehead 1991), but overcoming was not reported in *S. sparsipilum* species, even after a five-year experiment (Turner and Fleming 2000). Thus, the *GpaV^{spl}* and *GpaXF^{spl}* QTL together are, a priori, good candidates for assuring durable resistance to *G. pallida*.

MATERIALS AND METHODS

Plant material.

Analyses were performed on 239 diploid hybrid clones originating from crosses between the same parental accessions. spl329.18, a diploid clone of the *S. sparsipilum* PI 310984 accession from the Sturgeon Bay collection used as male, is resistant to *G. pallida*. It did not develop any cysts in previous tests

(Rousselle-Bourgeois and Mugniéry 1995). Caspar H3, a dihaploid of *S. tuberosum* cv. Caspar used as female, is susceptible.

Resistance assessment for QTL analysis.

Resistance assessment was performed by artificial tests in controlled conditions on both parental clones and the 239 clones of the progeny. Four tubers per clone were planted separately in plastic pots filled with 400 cm³ of a mixture of soil and sand. Ten cysts of *G. pallida* pathotype Pa2/3 (Chavornay population) were added to each pot, in order to achieve an infestation of five to ten juveniles per gram of soil. Plants were grown in a greenhouse for four months. Newly-formed cysts were recovered from the soil by elutriation. Cysts were counted for each plant individually.

To assess the resistance, the whole progeny was split out in two sets of 97 and 142 clones tested independently. Caspar H3, the susceptible parent, 96D31.137, a moderately resistant clone, and 96D31.145, a fully resistant clone, were used as controls in both resistance tests.

Phenotypic effects of the QTL on development of a necrotic reaction.

The QTL effect on the development of a necrotic reaction was assessed in infected roots of three to four clones per allelic

Fig. 4. Potato roots infected by *Globodera pallida* J2 juveniles, stained with acid fuchsin 5 days postinoculation and observed under 100× magnification. **A**, Potato clone carrying susceptibility alleles at both quantitative trait loci (QTL) *GpaV^{spl}* and *GpaXF^{spl}*, acting on the resistance to *G. pallida*. **B**, Potato clone carrying resistance allele at the major effect *GpaV^{spl}* QTL. **C**, Potato clone carrying resistance allele at the minor effect *GpaXF^{spl}* QTL. **D**, Potato clone carrying resistance alleles at both QTL. The juvenile (J) has achieved his migration and is located in cortical cells near the vascular vessels (xylem = X). Intact cortical cells (CC) appear in light red. In the four clones, a thin layer of necrotic cells (N), envisioned as dark-red cells, is visible around the juvenile body. In the clone carrying resistance alleles at both QTL, the area of necrotic cells ahead of the nematode is much more extended than the one observed along the nematode body.

combination at QTL $GpaV_{spl}^s$ and $GpaXI_{spl}^s$, chosen in the mapping progeny. Two tubers per clone were grown at 20°C in petri dishes on 2% agar solidified water (Mugniéry and Person 1976). Three roots per tuber were inoculated with 10 J2 of *G. pallida* (Chavornay population) per root. The root system was cut off five days after inoculation, was washed out and stained with a mixture of acid fuchsin in acetic acid with ethanol (1:1), and then, was rehydrated in a mixture of water with chloral hydrate (1:4), according to Daykin and Hussey (1985). Roots were gently squashed between two glass slides and were examined with a light microscope under 100× and 400× magnifications.

Phenotypic effects of the QTL on nematode development.

Four clones of the progeny were chosen according to their alleles at both QTL. Ten tubers per clone were grown at 20°C in petri dishes on water solidified with 2% agar (Mugniéry and Person 1976). One to three roots per tuber were inoculated with 10 J2 per root. A total of 20 inoculated roots per clone were dissected under 40× magnification 15 days after inoculation (Mugniéry et al. 2001). For each retrieved nematode among the ten inoculated per root, the developmental stage was noted according to the following classification: J2/J3 undeveloped nematodes, male, or female. The distinction male vs. female was determined on J4 or adult nematodes. Presence versus absence of a necrosis around the nematode was noted. Nonretrieved nematodes were considered as nonpenetrated or missing data.

Molecular analysis.

Molecular markers well distributed on published potato genetic linkage maps (Gebhardt et al. 1991; Isidore et al. 2003; Milbourne et al. 1998; Tanksley et al. 1992) were analyzed on the 239 diploid clones. Total nuclear DNA was extracted from 100 mg of fresh leaves of 3-week-old plants, following the protocol described by Fulton and associates (1995).

Twenty amplification fragment length polymorphism (AFLP) primer combinations, delivering markers covering the whole genome of the ultra high density (UHD) potato map (Isidore et al. 2003) were chosen. They were generated with *EcoRI* or *SacI* as rare cutter and *MseI* as frequent cutter restriction enzymes and were analyzed according to the method described by Vos and associates (1995), except that the anodal

electrophoresis buffer was 1× tris-borate-EDTA, 0.5 M NaAc. Segregating fragments were scored visually and independently by two people. Only markers heterozygous for a single parent were considered. They were identified by the name of the primer combination and the molecular weight of the fragment, as indicated on the UHD potato database.

Five simple sequence repeat (SSR) markers were mapped. Stm0003, Stm0013, and Stm1020 were amplified according to the procedure described by Milbourne and associates (1997, 1998). Primer sequences for SSR4 and SSR19 were retrieved from the Solanaceae Genomic Network (SGN) database. Polymerase chain reaction (PCR) reactions were carried out as described by Milbourne and associates (1997) with an annealing temperature of 50°C.

Four cleaved amplified polymorphic sequence (CAPS) or sequence-characterized amplified region (SCAR) markers were mapped. PCR for CAPS marker GP179 were carried out as described by Meksem and associates (1995). PCR reaction (10 µl) was then digested with 3 U of *EcoRV*, following instructions provided by the manufacturer (Invitrogen, Cergy Pontoise, France). Primers and PCR conditions for markers TG432P-CAPS and GP21-SCAR were adapted. We designed new primers (Table 1) from sequences obtained in our laboratory. PCR were carried out using the Hot-Start version of TaKaRa Ex Taq enzyme, following instructions provided by the manufacturer (TaKaRa Bio Europe, Gennevilliers, France). Cycling condi-

Fig. 6. Percentage of *Globodera pallida* reaching the J2/J3, male, or female developmental stage in 20 roots of four chosen clones carrying different allele combinations at the $GpaV_{spl}^s$ and $GpaXI_{spl}^s$ quantitative trait loci (QTL) originating from *Solanum sparsipilum* ($GpaV_{spl}^s$ = resistance allele at $GpaV_{spl}^s$; $GpaXI_{spl}^s$ = resistance allele at $GpaXI_{spl}^s$; $GpaV_{spl}^s + GpaXI_{spl}^s$ = resistance alleles at $GpaV_{spl}^s$ and $GpaXI_{spl}^s$; No QTL = No resistance allele at both QTL). The total number of retrieved nematodes per clone ranged from 129 to 141. For the three developmental stages observed, different letters indicate significant differences between clones, assessed by the 21 multiple comparison test of Kullback (1959). The number of cysts counted in the four chosen clones during the resistance assay for QTL analysis is indicated on the top of the graph

Fig. 5. Root of potato clone carrying resistance alleles at both quantitative trait loci $GpaV_{spl}^s$ and $GpaXI_{spl}^s$, acting on the resistance to *Globodera pallida* under 400× magnification. The necrotic area (N) envisioned as dark red extends ahead of the juvenile head (JH) to the xylem vessels (X). Intact cortical cells (CC) are visible on the left, opposite to the xylem vessel.

Table 1. Polymerase chain reaction primers designed for markers of potato chromosome V

Marker ^a	DNA sequences used for designing primers	Primer sequences 5' → 3'	Enzyme ^b
TG432P-CAPS	DNA from Caspar H3 and spl329.18, amplified with TG432 primers (De Jong et al. 1997)	GGACAGTCATCAGATTGTGG GTACTCCTGCTTGAGCCATT	<i>DraI</i>
GP179-CAPS	Primers published by Meksem et al. 1995	GGTTTATGATTGTGCTGC AATTTCAGACGAGTAGGCACT	<i>EcoRV</i>
GP21-SCAR	DNA from Caspar H3 and spl329.18, amplified with GP21 primers (Meksem et al. 1995)	CTTTCATGTCATGAGGTAATGGC GTGTTAAATTTCTTATTAGTCTTTGTATTCA	–

^a CAPS, cleaved amplified polymorphic sequence; SCAR, sequence-characterized amplified region

^b Restriction enzyme for CAPS.

tions were as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 2 min 30 s (TG432P-CAPS) or 1 min 30 s (GP21-SCAR), followed by 72°C for 10 min. PCR using the GP21-SCAR primers amplified a single *S. sparsipilum* allele of 400 bp. GP179 primers (0.25 µM each) (Meksem et al. 1995) were added to the PCR reaction to get a fragment of 550 bp as amplification control.

Assignment of linkage groups was realized thanks to comigrating AFLP markers (Roupe van der Voort et al. 1997) also mapped in the UHD potato map (Isidore et al. 2003) or the Rosa H1 or SPG 334.19 maps (Caromel et al. 2003) or thanks to SSR or CAPS markers of known locations. For one linkage group without previously mapped AFLP, SSR, or CAPS markers, AFLP fragments mapped on this linkage group were isolated as described by Roupe van der Voort and associates (1997) to produce restriction fragment length polymorphism probes. These probes were mapped on the Rosa H1 or SPG 334.19 maps on which all the linkage groups were assigned (Caromel et al. 2003).

Data analysis.

Statistical analyses were performed using the R software version 1.7.7 (Ihaka and Gentleman 1996).

The average cyst number of the clones, which were assessed in two independent tests, was adjusted using parameters of the linear regression, computed with the data of the control clones Caspar H3, 96D31.137, and 96D31.145 ($a = 1.2$, $b = 0$), in order to merge the two data sets. As currently observed with data issued from multiplication processes, the distribution of the untransformed numbers of cysts was skewed towards the low values. To improve the normalization of the data set, adjusted data were further transformed as $\log_{10}(x + 1)$, with x being the adjusted mean number of cysts. Further analyses were performed with these values. Broad-sense heritability was estimated with the following formula: $h^2_b = \sigma^2_g / (\sigma^2_g + \sigma^2_{en})$, where σ^2_g = genetic variance, σ^2_e = error variance, and n = number of replicates.

Molecular analyses and map constructions were performed in two steps. A set of 97 clones were genotyped with 20 AFLP primer combinations, four CAPS or SCAR markers, and five SSR markers. The 142 other clones were genotyped with a subset of evenly distributed markers covering most of the spl329.18 map. Two independent genetic linkage maps were derived from each parent, following the double pseudo-test cross design (Grattapaglia and Sederoff 1994), using the software MapMaker/Exp 3.0 (Lander et al. 1987). Linkage groups were determined with a minimum LOD score of 6 and a maximum recombination rate of 0.3. Recombination frequencies were converted into mapping distances (cM) using Kosambi's function (1944). Framework maps were constructed with the most informative markers evenly distributed on the maps. A total of 115 and 106 markers from Caspar H3 and spl329.18 framework maps were integrated in a single dataset.

QTL detection was performed using the CIM method with QTL Cartographer software (Basten et al. 1997). Two markers, selected by a forward-backward stepwise regression analysis,

were used as cofactors in the CIM procedure, with a window size of 10 cM and a walking speed of 2 cM. A 1,000-permutation test defined a significant threshold at LOD 3.0 (type I error = 0.05). R^2 describes the percentage of the total variation explained by the QTL. It was computed at the maximum of the LOD score plot.

Digenic interactions between all pairs of markers of the single dataset integrating the two framework maps were tested using a two-way ANOVA with an interaction component. A significance level of $P < 10^{-4}$ was employed.

A two-factor ANOVA was performed to estimate the total variation explained by both QTL. The most-tightly linked marker to each QTL maximum LOD score plot was used as factor.

Among the 239 clones of the whole progeny, 190 clones did not show any recombination event between the markers flanking each QTL. These 190 clones were classified according to their allelic combination at both QTL. For each QTL combination, the frequency distribution of the clones was compared for the $\log_{10}$ (adjusted mean cyst number + 1). QTL effect was assessed by ANOVA. The mean values of the clones possessing the different alleles at both QTL were compared using the multiple comparison test of Tukey (Tukey's honest significant differences) with a 95% family-wise confidence level.

For the four clones chosen for their different combination of alleles at both QTL, developmental stage of the retrieved nematodes was noted as J2/J3 undeveloped nematodes, male, or female. The effect of the QTL allele combination on the developmental stage was assessed by ANOVA. For each developmental stage, the number of retrieved nematodes was compared between the four clones, using the $2\hat{I}$ multiple comparison test of Kullback (1959) mentioned in Arbonnier (1966), with a 95% family-wise confidence level.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- The Solanaceae Genomic Network (SGN) database:
www.sgn.cornell.edu
- The ultra high density (UHD) potato map:
potatodbase.dpw.wau.nl/UHDdata.html