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Molecular mapping of the potato virus Y resistance gene *Ry$_{sto}$* in potato

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Abstract *Ry$_{sto}$* is a dominant gene which confers resistance to potato virus Y (PVY) in potato. We have used bulked segregant analysis of an F_1 tetraploid potato population to identify three AFLP markers linked to and on either side of *Ry$_{sto}$*. The tomato homologue of one of these AFLP markers was assigned to linkage group XI by analysis of an F_2 mapping population of tomato, suggesting that *Ry$_{sto}$* is also on chromosome XI of the potato genome. This map position was confirmed by the demonstration that *Ry$_{sto}$* was linked to markers which had been previously mapped to chromosome XI of the potato genome. Four additional AFLP markers were identified that were closely linked to *Ry$_{sto}$* in a population of 360 segregating progeny of a potato cross between a resistant (*Ry$_{sto}$*) and a susceptible parent. Two of these markers were on either side of *Ry$_{sto}$*, separated by only a single recombination event. The other two markers co-segregated with *Ry$_{sto}$*.

Key words Potato virus Y · *Ry$_{sto}$* extreme resistance · *Solanum tuberosum* · High-resolution map · AFLP

Introduction

Immunity or extreme resistance to potato virus Y (PVY) was first reported by Cockerham (1943) in *Solanum stoloniferum*. This trait is controlled by a single dominant gene (*Ry$_{sto}$*) which has been introgressed into *Solanum tuberosum* (Ross 1952, 1958). Extreme resistance to PVY has also been found in *S. tuberosum* spp. *andigena* (Muñoz et al. 1975; Gálvez and Brown 1980; Gálvez et al. 1992) and *S. hougasii* (Cockerham 1970).The *Ry*-mediated resistance has practical importance because it is effective against all strains of PVY (Ross 1952; Fernández-Northcote 1983).

Ry$_{sto}$-mediated resistance is similar in several ways to the extreme resistance to potato virus X conferred by the potato *Rx* gene. With both types of resistance, the resistant plants challenged with the respective virus do not develop visible symptoms and virus accumulation cannot be detected by either ELISA (Adams et al. 1986; Jones 1990) or RNA hybridisation (Goulden et al. 1993; Brigneti, unpublished). A further similarity is that both *Rx*- and *Ry*-mediated resistances are active at the protoplast level (Barker and Harrison 1984; Adams et al. 1986) suggesting a mechanism that involves either suppression ofvirus replication or virus destabilisation. We have therefore initiated an analysis of *Ry*-mediated resistance to complement our previous and current studies of *Rx*-mediated resistance in potato (Köhm et al. 1993; Bendahmane et al. 1995; Bendahmane and Kanyuka, unpublished).

In the present paper we describe a chromosomal assignment for *Ry$_{sto}$* and the first steps towards the molecular cloning of *Ry$_{sto}$* from potato by a map based approach. We have exploited the availability of high-density molecular-linkage maps of the potato and tomato genomes (Bonierbale et al. 1988; Gebhardt et al. 1989, 1991; Tanksley et al. 1992) and the development of amplified fragment length polymorphism (AFLP) technology (Vos et al. 1995) as a source of molecular markers linked to *Ry$_{sto}$*.

Materials and methods

Plant material and virus

True seed from cross 90V-241 (83W28-50×I-1039; the I-1039 parent carries *Ry$_{sto}$*) was obtained from Robert Boulton of Plant Breeding International, Cambridge, UK.

PVY^N was propagated on *Nicotiana occidentalis* and *N. clevelandii* by mechanical inoculation and infected plants were kept as a source of virus. PVY^N-infected potato plants were also kept as sources of scions for the graft inoculation experiments.

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PVY resistance assay

Plants were screened for resistance to PVY^N by mechanical and graft inoculation. Three cuttings from each individual plant were used in the mechanical inoculation experiments and infection was assayed 3 weeks post-inoculation by RNA-blot hybridisation of systemic tissue. The probe was the PVY negative-strand RNA produced by transcription of a PVY cDNA clone (pY19A-B99) with SP6 RNA polymerase in the presence of ^{32}P-αUTP (800 Ci/mmol). If the plants failed to accumulate PVY RNA they were graft inoculated and tested 3 weeks post-inoculation by RNA-blot hybridisation. It was considered that plants carried Ry_{sto} if they failed to become infected with PVY^N after both graft and mechanical inoculation.

Preparation of genomic DNA for AFLP analysis

The protocol used was provided by Bendahmane et al. (unpublished). Ten grams of young leaves were harvested and ground in the presence of liquid nitrogen. Leaf powder was transferred to a tube containing 23 ml of DNA extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 1.25% SDS, 8.3 mM NaOH, 0.38% sodium bisulphite and 0.38% sodium diethyldithiocarbamate). The homogenate was incubated at 65°C for 30 min. Potassium acetate was added to a final concentration of 1.25 M and the mixture was left on ice for 60 min. After a low-speed centrifugation the supernatant was filtered through Miracloth (Calbiochem) and the DNA was precipitated with isopropanol. DNA was re-suspended in a small volume of water and further purified by centrifugation to equilibrium on caesium chloride gradients containing ethidium bromide (Sambrook et al. 1989). DNA was re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4°C. DNA samples from 20 individual resistant (R) and 20 individual susceptible (S) plants were pooled for the bulked segregant analysis (Giovannoni et al. 1991; Michelmore et al. 1991).

Five micrograms of genomic DNA from each pool or from individual plants were digested with 25 U of the restriction enzymes *Mse*I (New England Biolabs) and *Pst*I (Boehringer) in a total volume of 100 µl. The ligation of adapters and the selection of biotinylated DNA fragments by streptavidine-coated beads (Dynal) were performed as described elsewhere (Thomas et al. 1995; Vos et al. 1995).

AFLP analysis

*Pst*I primers were labelled with either ^{32}P-γATP, for the preliminary screening, or ^{33}P-γATP (2000 Ci/mmol), using T4 polynucleotide kinase (Pharmacia, 9.3 U/µl). PCR reactions were performed in either a 9600 Thermal Cycler (Perkin Elmer Cetus) or a PTC-200 DNA Engine (MJ Research). The AFLP PCR step was performed essentially as described by Thomas et al. (1995) and Vos et al. (1995). AFLP reaction products were denatured and fractionated by electrophoresis on a 4.5% acrylamide, 7.5 M urea, 0.5×TBE buffer (45 mM Tris-Borate, 1 mM EDTA) gel at 100 W for 2.5 h. After electrophoresis, the gels were transferred to Whatman 3MM paper, dried without prior fixing and exposed to X-ray film (X-OMAT AR, Kodak) overnight.

Cloning of AFLP fragments

Polymorphic AFLP fragments were cut from the sequencing gel and rehydrated in 1 ml of TE buffer for 3 h. The gel was transferred to 100 µl of TE buffer and squashed with a sealed micropipette tip. One microliter of the total volume was used in a 20-µl PCR reaction using the same conditions and primers as for the original AFLP reactions. The amplified DNA was run on an agarose gel and the AFLP fragment was excised, purified with a Qiaquick gel extraction kit (Qiagen) and cloned into a pGEM-T vector (Promega). To verify that the cloned DNA was the same size as the original AFLP marker, an AFLP reaction was carried out on the plasmid DNA and the products were fractionated by electrophoresis alongside the original PCR reactions on the pools of genomic DNA. Two independent clones from each AFLP marker were sequenced (ABI Prism 377 DNA sequencer) and primers were designed and synthesised accordingly (Expedite Nucleic Acid Synthesis System).

Inverse PCR

Primers were designed based on the ends of the AFLP clone M39b (described in the Results section) and 1.25 µg of genomic DNA from both the R pool and the S pool (as a control) were digested with *Alu*I or *Rsa*I. After phenol extraction, the DNA was ethanol-precipitated and re-suspended in 20 µl of water. The DNA was then self-ligated with 5 U of T4 DNA ligase (Boehringer) at 12°C overnight, phenol-extracted, ethanol-precipitated and re-suspended in a final volume of 20 µl in water. This DNA was used as a template in a PCR reaction in a total volume of 100 µl (100 pmole each primer, 200 µM dNTPs, 0.005% Tween 20, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl_2, 0.05% Nonidet P-40). The PCR products obtained were cloned directly into a pGEM-T vector and sequenced.

PCR markers

Potato markers GP163 and GP259 and the tomato marker CT182 have been previously described by Gebhardt et al. (1991) and Tanksley et al. (1992), respectively. These markers and various cloned AFLP markers were assayed in the present study as PCR fragments produced by amplification of DNA from R and S pools or individual progeny plants. In those cases in which the amplified DNA was not polymorphic (the primers used were not allele-specific), the PCR products were digested with 4-base-cutter restriction enzymes and the digestion products were fractionated on 2–3% agarose gels. Table 1 shows the primer sequences used for each marker and the restriction enzyme used to detect the sequence polymorphism.

Genomic DNA extraction for PCR

Potato genomic DNA from individual plants was extracted from a leaf disk in an Eppendorf tube with 100 µl of extraction buffer (140 mM d-sorbitol, 220 mM Tris-HCl pH 8.0, 22 mM EDTA pH 8.0, 800 mM NaCl, 0.8% hexadecyltrimethylammonium bromide, 1% N-lauroylsarcosine) and sterile sand (BDH). Samples were ground with a heat-sealed micropipette tip. Then 100 µl of chloroform were added and the mixture was incubated at 65°C for 10 min. After a 5-min centrifugation, the supernatant was recovered and the DNA was precipitated with 100 µl of isopropanol. The pellet was washed with 70% ethanol and re-suspended in 50 µl of water; 2 µl of DNA was used for each PCR reaction.

Results and discussion

AFLP markers linked to Ry_{sto}

To identify AFLP markers linked to Ry_{sto} we first analyzed pooled DNA samples from 20 individual resistant (R) and 20 individual susceptible (S) plants. These plants were the progeny of cross 90V-241 in which the PVY-resistant parent carried Ry_{sto} in the simplex condition, giving a 1:1 segregation for extreme resistance and susceptibility to PVY^N (Brigneti, unpublished). Of 216 primer combinations tested for AFLP markers linked to Ry_{sto}, there were 33 that produced DNA fragments present only in the DNA pool from the resistant plants.

To facilitate the analysis of these markers in large populations of individual plants, three AFLP fragments

Table 1 Primer sequences derived from Ry_{sto}-linked markers. Restriction enzymes used to detect polymorphism are shown in brackets beside each marker. A.S.: allele-specific primers (no enzyme used). For markers in italics (not cloned) the sequence of the original AFLP primer combinations used is given

Marker	Primer name	Sequence
M33 (*Alu*I)	M33U	5′ GGCGAAGAATTGTCATCGCCGTTG 3′
	M33L	5′ CCAGTGTAATCCCATAGGTCCGGG 3′
M39b (*Dde*I)	M39bU	5′ AATCTTGAGGAGGTTTTCAAACTC 3′
	M39bL	5′ AATTAGGTGACAACCAATATGAG 3′
M35 (A.S.)	M35U	5′ GACTGCGTACATGCAGAGTTAG 3′
	M35L	5′ CGTATTTGGAGACTTAGACCCATGCC 3′
GP163 (A.S)	GP163U	5′ CTGCAGTTTTGAAATTACCATCT 3′
	GP163L	5′ CTGCAGCCAACTGATAACTCTCA 3′
GP259 (*Alu*I)	GP259U	5′ CTCTAGCTTGTAAGAACTGAG 3′
	GP259L	5′ GTTTCTCATGTAAATTATCCA 3′
CT182 (A.S.)	CT182U	5′ GGGAGGGAACAAGTTACTCTA 3′
	CT182L	5′ GCCAACTTCTTAGGCCGTTTC 3′
M38	91D04	5′ GACTGCGTACATGCAGAC 3′
	92G26	5′ GATGAGTCCTGAGTAACGT 3′
M17	92E11	5′ GACTGCGTACATGCAGTG 3′
	92G24	5′ GATGAGTCCTGAGTAACAT 3′
M45	92E08	5′ GACTGCGTACATGCAGCT 3′
	92F44	5′ GATGAGTCCTGAGTAAGGA 3′
M5	92E11	5′ GACTGCGTACATGCAGTG 3′
	92F41	5′ GATGAGTCCTGAGTAACAA 3′
M6	92E08	5′ GACTGCGTACATGCAGCT 3′
	92F42	5′ GATGAGTCCTGAGTAAGAA 3′
M41	91R31	5′ GACTGCGTACATGCAGCA 3′
	92G14	5′ GATGAGTCCTGAGTAAGAC 3′
M53	92E12	5′ GACTGCGTACATGCAGTT 3′
	92G40	5′ GATGAGTCCTGAGTAATCT 3′
M43	91R31	5′ GACTGCGTACATGCAGCA 3′
	92G17	5′ GATGAGTCCTGAGTAAGCA 3′

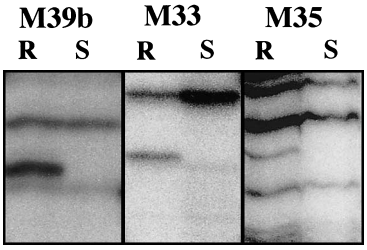

Fig. 1 Detail of an AFLP-gel autoradiograph showing the fragments corresponding to markers M39b, M33 and M35. AFLP reactions were performed using DNA pools from resistant (*R*) and susceptible (*S*) F_1 individuals

(Fig. 1) were cloned and sequenced and primers were designed for PCR. The primers based on the sequence of marker M35 selectively amplified DNA from the R pool (Fig. 2, lanes R and S). In contrast, the M33 primers amplified DNA in both the resistant and susceptible pools and it was necessary to digest the PCR fragments with *Alu*I to detect an Ry_{sto}-associated sequence polymorphism (Fig. 2, lanes R and S). The AFLP marker M39b was only 78 bp, and was unlikely to contain polymorphic restriction enzyme sites. For this marker, an additional inverse PCR step was performed in order to obtain the sequences flanking the original AFLP fragment. This extended sequence was used to generate primers for the PCR amplification of a 850-bp fragment with a *Dde*I-site polymorphism linked to Ry_{sto} (Fig. 2, lanes R and S).

PCR analysis of DNA from individual progeny (50 plants) of the F_1 population revealed that the M39b and M33 markers co-segregated with each other and, except in one plant, with Ry_{sto}. The M35 marker co-segregated with Ry_{sto} in all but four of the plants tested. It is likely that M35 and M39b/M33 are on opposite sides of Ry_{sto} because the plants in which there was recombination between Ry_{sto} and M35 did not include the plant in which there was recombination between Ry_{sto} and M39b/M33.

Chromosomal location of Ry_{sto} in potato: use of a tomato mapping population

To assign a chromosomal position to Ry_{sto} the marker M39b was mapped in an F_2 population derived from the interspecific cross *Lycopersicon esculentum*×*Lycopersicon pen-*

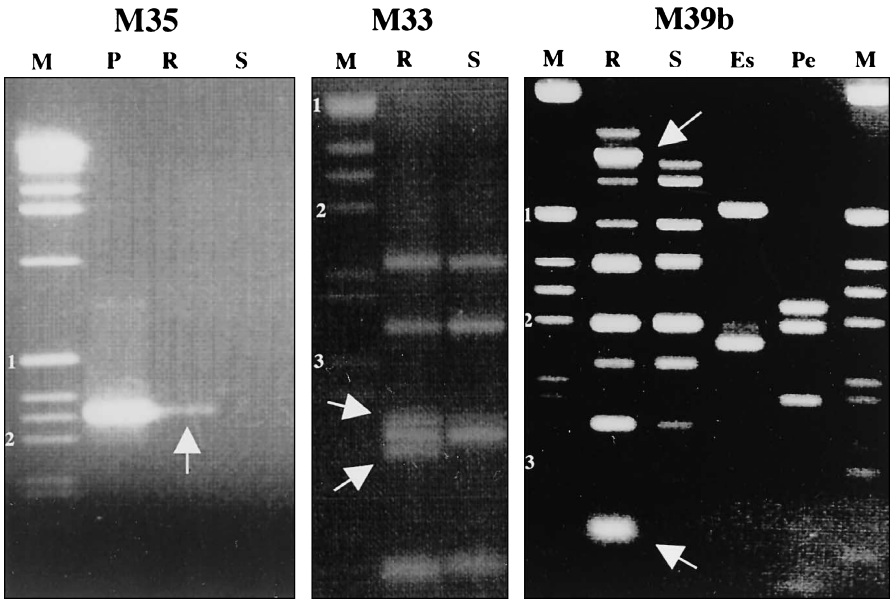

Fig. 2 PCR products obtained using M35, M33 and M39b primers. M33 and M39b products were digested with *Alu*I and *Dde*I, respectively, before being fractionated electrophoretically on an agarose gel and stained with ethidium bromide. DNA fragments polymorphic between the resistant (*R*) and susceptible (*S*) pools are marked with *arrows*. The polymorphism between *L. esculentum* (*Es*) and *L. pennellii* (*Pe*) with the M39b marker is also shown. *M* size marker, 1-kb DNA ladder (Gibco BRL). *1* 516–506 bp. *2* 298 bp. *3* 154 bp. *P* control PCR using M35 primers on plasmid DNA (pGEM-T) containing M35 insert

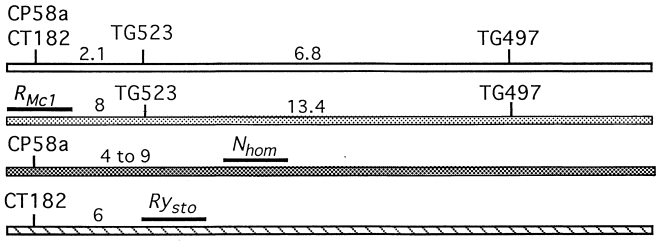

Fig. 3 Schematic representation of potato resistance genes mapped in the short arm of chromosome XI (N_{hom}: potato *N* homologue; R_{Mc1}: *Solanum bulbocastanum* root-knot nematode resistance; Ry_{sto}). Each resistance gene was mapped using different mapping populations but with some RFLP markers in common. The upper line shows markers as they are distributed in the potato genetic map (adapted from Tanksley et al. 1992) in order to permit a comparison between individual maps. Genetic distances are not to scale

nellii (Bernatzky and Tanksley 1986; Paul et al. 1994). It was anticipated that, because of synteny (Bonierbale et al. 1988; Gebhardt et al. 1991; Tanksley et al. 1992), the Ry_{sto}-linked markers would occupy homologous positions in the potato and tomato genomes.

The M39b primers amplified 750-bp fragments from the DNA of both *L. esculentum* and *L. pennellii* . Digestion of these PCR products with *Dde*I revealed a polymorphism between the two species (Fig. 2, lanes Es and Pe). The segregation of this M39b polymorphism in 41 F_2 individuals from the tomato mapping population was assessed, using the MapMaker software (Lander et al. 1987), relative to 1275 markers that have been mapped in this population. This analysis indicated that M39b is located in tomato linkage group XI close to the RFLP markers GP163 and GP259, that had been mapped previously in the potato genome (Gebhardt et al. 1991).

To confirm the map position of chromosome XI, the Ry_{sto} locus was mapped relative to GP163 and GP259 in the F_1 progeny of cross 90V-241. These markers were assayed by PCR in the 50 individual plants previously tested for the segregation of M35, M33 and M39b. The PCR primers based on the sequence of GP259 amplified DNA in both the R and S pools in which a polymorphism linked to Ry_{sto} was revealed by digestion with *Alu*I. The PCR primers obtained from the sequence of GP163 amplified DNA linked to Ry_{sto} in the R pool but not the S pool (allele-specific primers). There were two plants with recombination between GP163 and Ry_{sto}. Both of these plants also had recombination between Ry_{sto} and M35 and, in one of them, there was recombination between GP259 and Ry_{sto}. The likely order of markers on chromosome XI is therefore M39b, Ry_{sto}, GP259, GP163, M35.

Recently, a potato homologue of the tobacco *N* viral resistance gene (Leister 1995) and a root-knot nematode resistance gene derived from *Solanum bulbocastanum* (Brown et al. 1996) have been mapped to chromosome XI. It is not possible to assess precisely the relationship of these disease resistance genes and the *N* homologue because different mapping populations and markers were used (Fig. 3). However, even with this preliminary data, it is likely that *Ry*, like other disease resistance genes (Farrara et al. 1987; Ellis et al. 1988), is part of a resistance gene cluster .

High-resolution mapping of molecular markers relative to Ry_{sto}

To obtain a higher resolution map of molecular markers close to Ry_{sto} we screened an additional 310 F_1 progeny of

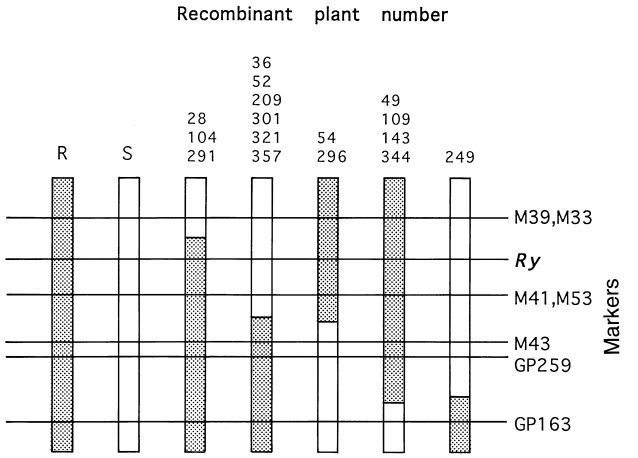

Fig. 4 Schematic representation of the 16 individual recombinant chromosomes and some linked markers. Plants with recombination events in different positions [identified as either *R* (resistant), *S* (susceptible) or by *number*] were used in AFLP analysis in order to locate new AFLP markers

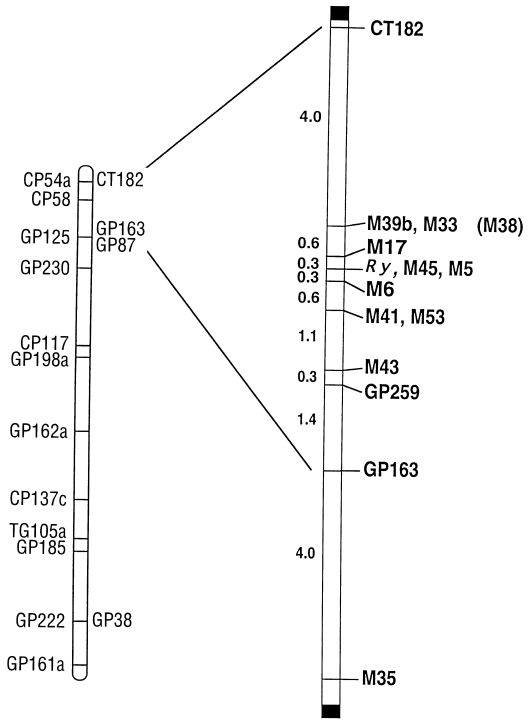

Fig. 5 High-resolution map of the Ry_{sto} region (on the right) relative to the genetic map of potato chromosome XI (on the left). The M38 marker (in *brackets*) was mapped using recombinant plants in the M39b/GP259 interval. The position of this marker in the interval CT182/M39b has not been tested. The sequence of the M38 marker showed homology with a potato UDP glucose pyrophosphorylase, which had previously been mapped in the same region under the name CP58 (potato RFLP marker; Gebhardt, personal communication). AFLP markers are identified with an *M* followed by a *number*. Map distances were calculated using two-point analysis and are represented in cM

the cross 90V-241 for recombination between M39b, GP259 and GP163. The progeny with recombination in this interval were tested for Ry_{sto} by inoculation with PVY^N. There were three recombination events between Ry_{sto} and M39b and 13 between Ry_{sto} and GP163. The identification of eight recombinants between Ry_{sto} and GP259 and five recombinants between GP259 and GP163 confirmed the order of markers deduced from the analysis of the smaller population.

AFLP analysis of DNA in these individual recombinant plants (Fig. 4) was carried out with 30 primer combinations shown previously to display markers linked to Ry_{sto} in pooled DNA samples of resistant and susceptible plants. Three of these primer combinations detected polymorphic DNA fragments (M41, M43 and M53) that mapped on the GP259 side of Ry_{sto} in the interval M39b/GP259. The markers M41 and M53 were separated from Ry_{sto} by three recombination events. M43 was separated from Ry_{sto} by seven recombination events and was therefore between M41/M53 and GP259.

To obtain closer linked markers to Ry_{sto}, the DNA of the R and S pools was analyzed with an additional 192 AFLP primer combinations (4 *Pst*I and 48 *Mse*I primers). Of these, there were 18 combinations that displayed markers linked to Ry_{sto}. These primer combinations were subsequently used to analyze DNA of the six plants with recombination events in the M39b/M41 interval. Four of the primer combinations identified markers that were closer to Ry_{sto} than M39b or M41. Two of these, M17 and M6, were separated by a single recombination event on each side of Ry_{sto}. Two other AFLP markers (M45 and M5) co-segregated with Ry_{sto}. The relative positions of these newly assigned AFLP markers to Ry_{sto}, and the previously determined map of potato chromosome XI (Gebhardt et al. 1991), are shown in Fig. 5.

Future prospects

It has been reported that, on average, a genetic distance of 1 cM corresponds to a physical distance of 1 Mb in the potato genome (Ballvora et al. 1995), the total length of the genome being 1000 cM (Gebhardt et al. 1991). Chromosome walking may not be a suitable way to clone genes in this large complex genome which has a high frequency of repetitive DNA (Tanksley et al. 1995). To clone *Ry*, we are therefore attempting to identify tightly linked flanking markers so that a single BAC or YAC clone can be generated which spans the interval between these markers. This approach has already proved successful in tomato (Martin et al. 1993).

We have estimated the interval between closest flanking markers to be 0.6 cM, which would correspond to a physical distance of 0.6 Mb. As a next step towards the positional cloning of Ry_{sto}, it will be necessary to attain a higher resolution in the molecular map around the gene. We estimate that an F_1 population of 1500–2000 individuals should be screened, with additional AFLP markers, to achieve a mapping resolution of at least 0.05 cM. This high

resolution is needed to clone the gene by chromosome landing using a bacterial artificial chromosome (BAC) genomic library. In the meantime, the linked PCR primers could be used for marker-assisted breeding of *Ry*.

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