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The development of an improved PCR-based marker system for *Sw-5*, an important TSWV resistance gene of tomato

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Abstract. *Sw-5* is an important disease resistance gene of tomato, providing broad resistance to *Tomato spotted wilt virus* (TSWV). A cleaved amplified polymorphic sequence (CAPS) marker, closely linked to the gene, has been reported. Although the *Sw-5* locus has been characterised, a gene-specific marker has not been developed. This paper presents a PCR-based marker-system that consists of the co-amplification of a dominant marker representing the *Sw-5* gene sequence, and the modified CAPS marker as a positive control and indicator of genotype.

Additional keyword: rapid DNA extraction.

Introduction

Tomato spotted wilt virus (TSWV) (genus *Tospovirus*, Family Bunyaviridae) causes major losses in tomato (*Lycopersicon esculentum*) production throughout the world (Roselló *et al*. 1996). The very wide host range of TSWV among crop and weed species and the efficient transmission by western flower thrips (*Frankliniella occidentalis*) and other thrip species, contribute to the worldwide importance of TSWV (Prins and Goldbach 1998). Host resistance is an effective means of managing TSWV and several sources of resistance have been found in *Lycopersicon* species (Stevens *et al*. 1994). The dominant *Sw-5* gene is the most widely used source of resistance (Spassova *et al*. 2001). This gene was derived from *L. peruvianum* and introgressed into the fresh market tomato cultivar, Stevens (Stevens 1964; Thompson and van Zijl 1996).

The gene *Sw-5* is closely linked to the restriction fragment length polymorphism (RFLP) markers CT220 and CT71 and is located near the telomere of chromosome 9 (Stevens *et al*. 1995). *Sw-5* is a member of a loosely clustered gene family, members of which have been genetically mapped close to *Sw-5*, to other regions of chromosome 9, and to chromosome 12 (Brommonschenkel *et al*. 2000).

Spassova *et al*. (2001) characterised 2 highly homologous *Sw-5* gene candidates within 40 kb of CT220. The genes, tentatively named *Sw-5a* and *Sw-5b*, are members of the coiled-coil, nucleotide-binding, leucine-rich repeat class of resistance genes. The *Sw-5b* gene was found to be vital for resistance to TSWV in transformed tobacco plants (Spassova *et al*. 2001).

The *Sw-5* locus was shown to be effective against many TSWV isolates (Cho *et al*. 1996; Roselló *et al*. 1996) and to provide resistance against 2 other tospoviruses infecting tomato, *Groundnut ringspot virus* and *Tomato chlorotic spot virus* (Boiteux and Giordano 1993; Brommonschenkel *et al*. 2000). Spassova *et al*. (2001) suggested that the broad resistance may be due to the presence of *Sw-5* homologues, and those authors characterised a further 3 related genes near CT220, designated as *Sw-5 c*, *d*, and *e*.

Folkertsma *et al*. (1999) developed a cleaved amplified polymorphic sequence (CAPS) marker for *Sw-5* based on CT220 sequence. However, no marker has been developed from the available gene sequence. We present a polymerase chain reaction (PCR)-based marker system that consists of a dominant marker representing the *Sw-5b* gene sequence and incorporates the modified CAPS marker as a positive control and potential indicator of genotype (homozygous resistant *v.* heterozygote).

Materials and methods

Sw-5 analogues (GenBank accessions *Sw-5a* and *b* AY007366; *Sw-5c*, *d*, and *e* AY007367) were aligned using ClustalW (WWW Service at the European Bioinformatics Institute http://www.ebi.ac.uk/clustalw; Higgins *et al*. 1994) in order to identify polymorphic sections, and sequences specific to *Sw-5b*. The leucine-rich-repeat (LRR) domain was targetted for the production of *Sw-5b* sequence-specific PCR primers. The LRR region was selected because it is a characteristic feature of the NBS (nucleotide binding site)-LRR class of disease resistance genes, and possesses high levels of polymorphism. *Sw-5a* has been identified as the most similar analogue to *Sw-5b* (Spassova *et al*. 2001), with only 2.2% of bases differing. In the LRR domain, 5.6% of bases were found to differ, whereas 1.1% of bases were different between *Sw-5a* and *Sw-5b* for the remainder of the gene sequence.

PCR primers were designed for a 305-bp section of the LRR domain of *Sw-5b*, using the computer program Primer3 (Rozen and Skaletsky 2000). This DNA fragment had 7.9% of bases different between

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Sw-5b and *Sw-5a*. The sequence differences included a 3-bp deletion in *Sw-5a*. Primer details are provided in Table 1. Both primers were polymorphic for the 3′ end when compared with the *Sw-5a* sequence. The eighth base, from the 5′ end of the forward primer (Sw-5b-LRR-F), and the seventh base of the reverse primer (Sw-5b-LRR-R), were also polymorphic for the same comparison. Initial testing with this primer pair indicated that specificity was obtained at a relatively low annealing temperature of 50°C and therefore it was not necessary to incorporate artificial mismatches near the 3′ end of the primers. Specificity was tested by achieving positive amplification in resistant lines and no apparent amplification from a susceptible cultivar.

Folkertsma *et al*. (1999) provided primer details for the CT220 fragment, a locus within 25 kb of *Sw-5b*, and indicated that a *Mse*I digestion was suitable for the production of a CAPS-based marker for the identification of *Sw-5* (recognition sequence T▼TAA). *Mse*I digestion of the product from *L. esculentum* produces a fragment equivalent to 94 bp and 4 small fragments less than 40 bp. *Mse*I digestion of a fragment from a resistant line (assuming linkage has been maintained) produces a fragment equivalent to 119 bp and 4 small fragments. The *Sw-5b* sequence contains a *Mse*I restriction site but not the recognition sequence for *Ase*I (AT▼TAAT). To facilitate co-amplification of the CT220 fragment and the *Sw-5b*-LRR fragment, followed by a restriction digest for genotyping of the CT220 locus, the *Ase*I enzyme was used.

The CT220 fragment from *L. esculentum* (sequence ID cTOF-14-J16.TH.B, Solanaceae Genomics Network http://www.sgn.cornell.edu/cgi-bin/SGN/) possessed 1 restriction site for *Ase*I and, after digestion, would be expected to produce 1 fragment equivalent to 94 bp and 1 equivalent to 119 bp. The 213-bp fragment from a resistant line (assuming linkage has been maintained) would not be cut by *Ase*I. Primer details for the CT220 fragment are provided in Table 1.

Mapping and virus resistance assays

Fifty F_2 individuals from a mapping-population segregating for resistance to TSWV were genotyped by screening F_3 plants for reaction to TSWV inoculation. The same 50 F_2 individuals were genotyped using the marker system to test for linkage between the marker and TSWV resistance.

The 50 F_2 plants were genotyped by inoculating more than 30 (in most cases) F_3 plants per line, with TSWV isolate 1423, originally obtained from *Capsicum annuum* in Queensland, Australia. The virus was propagated in tomato cv. Grosse Lisse. Inoculum was prepared by grinding symptomatic leaves with a chilled mortar and pestle with cold 0.1 M phosphate buffer to which 0.1% sodium sulfite had been added immediately prior to use. Diatomaceous earth (Celite) and carborundum abrasives were added to the inoculum, which was then applied to plants. Plants were inoculated when the first true leaves had emerged, approximately 20 days after planting. Plants were rinsed with water after inoculation to remove excess inoculum and abrasives. Plants were inoculated twice, several days apart, to reduce the false identification of resistant individuals. The cultivar Grosse Lisse was included as a susceptible control in all experiments and any apparently resistant lines were usually retested, bringing the total number of individual plants tested for these resistant lines to greater than 50.

The inoculated plants were maintained in a glasshouse and assessed for symptoms over 4 weeks. Plants from apparently resistant F_2 lines and a selection of plants with symptoms typical of TSWV were tested by ELISA using TSWV antiserum (Bio-Rad Cat. No.51267). The tests essentially followed the supplier's protocols. Both the antibodies and conjugated antibodies were used at 1 : 500 in carbonate coating buffer and PBS-T, respectively. Samples of tip leaves were extracted (1/10 dilution) in PBS-T (containing 2% PVP) and assays were carried out in polystyrene microplates (Nunc-ImmunoMaxisorp, Nalge Nunc International, Roskilde, Denmark) with reaction volumes of 100 μL. Overnight incubations were at 5°C and all other incubations were at room temperature for 2–3 h. Absorbance values at 410 nm ($A_{410\,nm}$) were measured using a Dynatech MR 7000 plate reader. Samples were considered positive when mean absorbance values exceeded twice that of appropriate healthy controls.

Table 1. Primers specific to the LRR domain of the *Sw-5b* gene and the CT220 marker

Primer name	Oligonucleotide sequence
Sw-5b-LRR-F	TCTTATATTGTGGAGTTTTTGTCG
Sw-5b-LRR-R	TCCACCCTATCAAATCCAAC
ZUP641[A]	AAGCCGAATTATCTGTCAAC
ZUP642[A]	GTTCCTGACCATTACAAAAGTAC

[A]Primer details for the CT220 fragment (Folkertsma *et al*. 1999).

DNA extraction

DNA was extracted from approximately 0.03 g of fresh leaf tissue and ground with 1 mL of extraction buffer (Edwards *et al*. 1991) at 60°C. The extract was added to 700 μL of chloroform/isoamyl alcohol (24 : 1), mixed, left on ice for at least 30 min, and centrifuged at 11 200 rcf for 3 min. DNA was precipitated from the supernatant after adding 1 volume of isopropanol followed by 5 min centrifugation at full speed. The pellet was washed twice with 70% ethanol and resuspended in 100 μL of Tris-EDTA (TE) buffer at pH 8.

The marker system was also tested using a rapid DNA-extraction protocol. The DNA extraction protocol was modified from that described in the Matrix Mill Manual, Harvester Technology Inc., USA. Small discs of fresh or frozen leaf tissue were cut and contained within 0.6-mL microcentrifuge tubes by placing a leaf between the lid and the top of the tube, and closing the lid. Then, 200 μL of 0.25 N NaOH was added to the tube. The leaf was ground in the NaOH solution, with a pipette tip, until the solution was a pale green colour. Fifty μL of the extract was then neutralised in 200 μL of 0.17 M Tris-HCl with 1 mM EDTA (pH 7.0) (see Fig. 3 for optimisation of the relative proportion of NaOH extract solution to neutralising solution).

PCR

Primers were synthesised by Proligo Pty Ltd, SCU, Lismore, NSW, Australia. PCR reactions were carried out on a Perkin Elmer, Gene Amp PCR System 9700. The reaction volume was 20 μL containing 1 × Roche PCR Buffer, approximately 50 ng of genomic DNA, and 188 μM dNTPs. The PCR reaction mix also included 100 nM of each of the 4 primers, 5.75 mM total $MgCl_2$, and 0.1 units of *Taq* DNA Polymerase (Roche) (see Fig. 1 for magnesium titration). The temperature cycling conditions were 3 min at 94°C; followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; with a final hold at 72°C for 1 min. *Ase*I (NEB) digestion followed the manufacturer's instructions.

PCR conditions for the rapidly extracted DNA template were as described above except for the following differences: 2 μL of the neutralised DNA extract was used as PCR template, 200 μM dNTPs, 150 nM of each of the 4 primers, 8.25 mM total $MgCl_2$, and 0.2 units of *Taq* DNA Polymerase (Roche).

Results

The CT220 and *Sw-5b* fragments were found to co-amplify when using a template prepared with the Edwards *et al*. (1991) extraction buffer (Figs 1 and 2). Digestion of

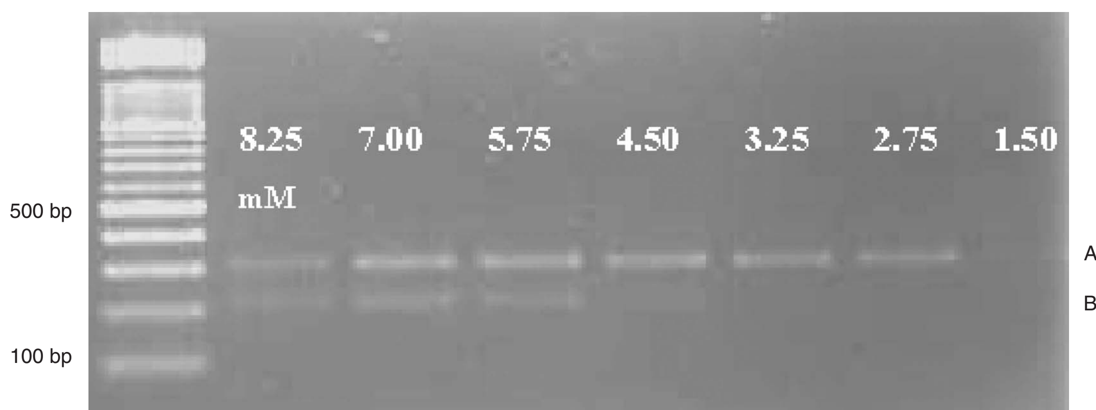

Fig. 1. Results of co-amplification of the *Sw-5b*-LRR (A) and CT220 (B) fragments at various magnesium concentrations. Concentrations (mM) are indicated. A 100-bp ladder is at the left of the gel image. PCR products are undigested. Good amplification of both products was obtained between 5.75 and 7.00 mM. The CT220 fragment did not amplify below 4.50 mM.

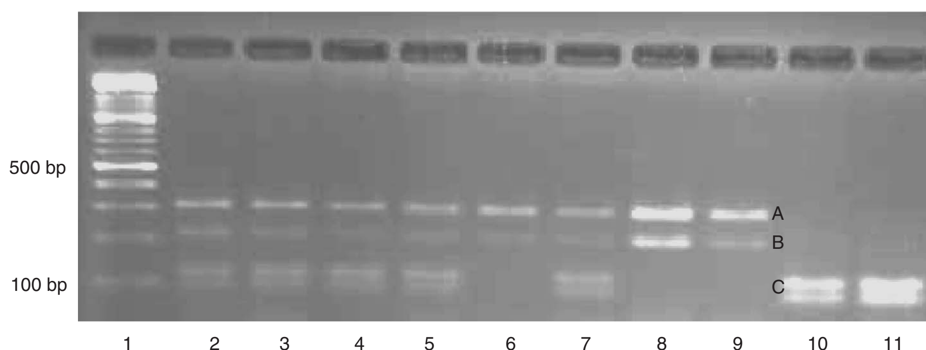

Fig. 2. Results of co-amplification of the *Sw-5b*-LRR and CT220 fragments after digestion with the *Ase*I restriction enzyme. (A) The *Sw-5b*-LRR fragment at approximately 300 bp. (B) The uncut CT220 fragment at approximately 200 bp. (C) The 2 fragments at approximately 100 bp, produced by the *Ase*I restriction of the CT220 fragment. (1) 100-bp ladder, (2–5, 7) heterozygous individuals from an F_2 population, (6) homozygous resistant individual from an F_2 population, (8–9) separate PCR amplifications for *Lycopersicon esculentum* accession LA3667 (known to possess *Sw-5*), (10) *Lycopersicon pennellii* LA716, (11) *Lycopersicon esculentum* susceptible line.

the fragments with *Ase*I produced the expected results (Fig. 2). When the rapid extraction protocol was used, positive amplification of the marker system was also obtained (Fig. 3) and product was successfully digested using the *Ase*I restriction enzyme (Fig. 4). However, amplification of the *Sw-5b*-LRR fragment was unreliable, alone or in combination with the CT220 fragment, when using the rapidly extracted template. Amplification of the CT220 fragment was routinely produced, alone or in combination with the *Sw-5b*-LRR fragment, when using the rapid extraction protocol.

The results of the resistance screening and genotyping of F_2 individuals of the mapping population are summarised in Table 2. Ten F_3 families were uniformly resistant as determined by the absence of symptoms of TSWV up to 4 weeks after inoculation, and no virus detected by ELISA. Twenty-four families segregated for resistance with an average of 24.6% of plants developing typical systemic symptoms. Sixteen families were uniformly susceptible with an average of 85.9% of plants developing severe symptoms typical of TSWV. Infection levels in the susceptible cultivar Grosse Lisse were 68–100% in all experiments.

Marker and *Sw-5* genotypes agreed for 49 of the 50 F_2 plants from the mapping population. For one F_2 plant the marker system indicated a heterozygous individual, and resistance screening indicated it to be homozygous susceptible. CT220 alleles were found to segregate as a single locus in an F_2 mapping population as tested by χ^2 (0.50, d.f. = 2) analysis. The observed segregation ratio, homozygous for the *L. peruvianum* allele : heterozygous : homozygous for the *L. esculentum* allele, was 10 : 25 : 15. *Sw-5* alleles were found to segregate as a single locus in an F_2 mapping population as tested

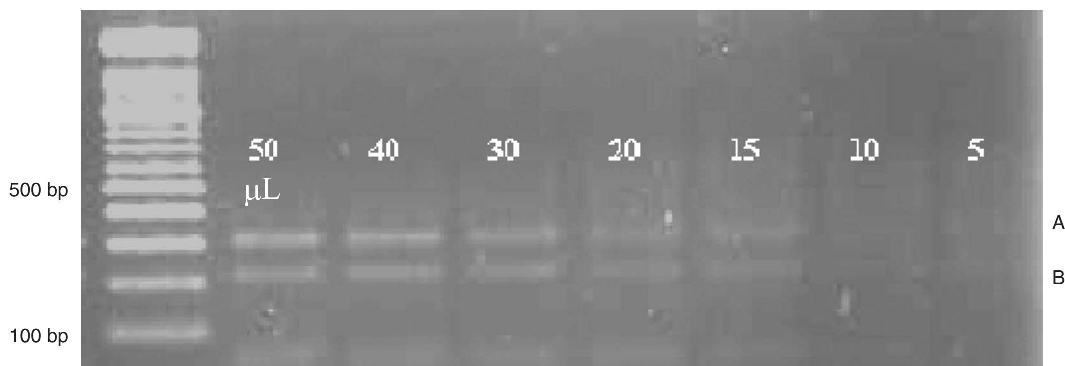

Fig. 3. Results of co-amplification of the *Sw-5b*-LRR (A) and CT220 (B) fragments, using rapidly extracted DNA templates prepared with different volumes of NaOH extract solution added to 200 µL of neutralising solution. A 100-bp ladder is at the left of the gel image. PCR products are undigested. Best amplification of both products was obtained when 50 µL of NaOH solution was added. Unrelated experimentation indicated that adding 80–50 µL of NaOH solution produced similar results. The pH of the extract solution ranged from 8.2 to 8.7 when 50–80 µL was added. Fifty µL of NaOH was the maximum volume tested in order to have the pH of the neutralised extract solution no higher than the pH of the PCR buffer.

by χ^2 (0.30, d.f. = 2) analysis. The observed segregation ratio, homozygous resistant : heterozygous : homozygous susceptible, was 10 : 24 : 16.

Discussion

The new marker system detected *Sw-5* without the need to perform a restriction digest, through the amplification of the *Sw-5b*-LRR fragment. The system incorporates a positive control for the PCR, in the form of the linked CT220 fragment, and therefore greatly reduces the chance of a false negative. The marker system also has the ability to predict the genotype of individuals possessing *Sw-5* (heterozygous or homozygous resistant) by performing a restriction digestion following PCR.

It should also be noted that the modification of the previously published CT220 CAPS marker, through the substitution of *Mse*I for *Ase*I, produces a larger size difference between diagnostic bands. The *Mse*I digestion produces a 25-bp difference and the *Ase*I digestion creates a 94-bp difference between diagnostic bands.

The use of a rapid DNA extraction protocol would greatly increase the efficiency of the system. Unfortunately,

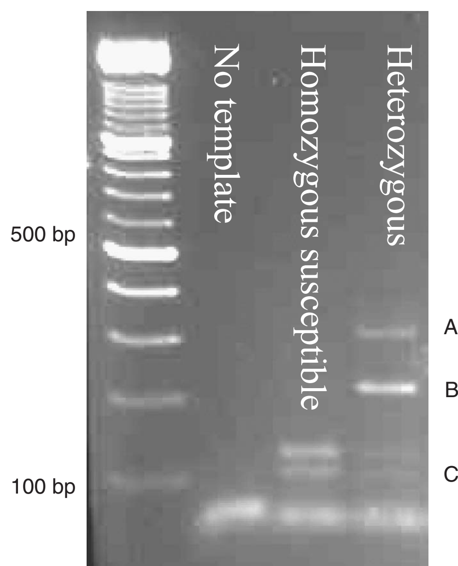

Fig. 4. Results of co-amplification of CT220 and *Sw-5b*-LRR fragments, using a rapid-extract DNA template, after digestion with the *Ase*I restriction enzyme. A 100-bp ladder is at the left of the gel image. (A) The *Sw-5b*-LRR fragment at approximately 300 bp. (B) The uncut CT220 fragment at approximately 200 bp. (C) Two fragments at approximately 100 bp produced by the *Ase*I restriction of the CT220 fragment.

Table 2. Summary of results for the screening of F_3 families for resistance to TSWV

The results for F_3 families are summarised for each F_2 genotype (heterozygous, resistant homozygous, susceptible homozygous)

Genotype of F_2 individuals or control	Infection rate (%)[A]			No. analysed[A]		
	Mean	s.d.	Range	Mean	s.d.	Range
Heterozygotes ($n = 24$)	24.6	7.26	10.3–41.9	38.4	8.94	24–68
Resistant homozygotes ($n = 10$)	0	0	0–0	51.7	16.38	30–84
Susceptible homozygotes ($n = 16$)	85.9	9.89	65.2–100	40.4	13.45	27–70
Susceptible control cv. Grosse Lisse	79.4	14.18	67.9–100	24.0	14.18	12–30

[A] F_3 plants per F_2 individual or control plants per experiment.

amplification of the *Sw-5b*-LRR fragment was found to be unreliable when using the rapid extraction procedure. However, the amplification of the CT220 fragment was routinely obtained using rapidly extracted DNA template. Without further optimisation, the CT220 fragment should be amplified alone, if the rapid DNA-extraction protocol is used.

Genetic mapping indicated one recombination event between the marker system and *Sw-5* (1 cM). For the 1 F_2 individual that indicated a recombination event, the marker system identified a heterozygous individual, and resistance screening indicated it to be homozygous susceptible. The recombination event probably did not occur, and the F_2 individual actually possessed the resistance gene as indicated by the positive amplification of the putative *Sw-5b* fragment and the heterozygous genotype of the CT220 marker, which is known to be within 25 kb and linked within 0.15 cM of *Sw-5* (Brommonschenkel and Tanksley 1997).

The apparent recombination is more likely to be a function of incorrect genotyping of the *Sw-5* locus through resistance screening. *Sw-5* does not produce 100% resistance (98.7% penetrance, reviewed by Stevens *et al*. 1996) and is semi-dominant (heterozygotes are less resistant than homozygotes, reviewed by Brommonschenkel *et al*. 2000). It will be difficult or impossible to detect the resistance gene by screening for resistance in some cases.

Acknowledgments

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