Y15_999*Fw*, a dominant SCAR marker linked to the Fusarium wilt race 1 (*Fw*) resistance gene in pea

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Introduction

Fusarium wilt, caused by *Fusarium oxysporum* Schlecht. emend. f. sp. *pisi* (van Hall) Snyd. & Hans., poses significant yield losses to *Pisum sativum* L. (green pea) in the Pacific Northwest and other pea-growing regions of the world (4, 10). Fusarium wilt race 1 is one of four major pathogenicity groups on pea (6). A locus designated *Fw* that confers resistance to Fusarium wilt race 1 has been mapped to linkage group III in the pea cultivar Green Arrow (9).

The objective of this study was to develop a sequence characterized amplified region (SCAR) marker for *Fw* to be used in marker assisted selection (MAS). If closely linked to a trait of interest, such a molecular marker provides a high probability that the trait is present. Use of molecular markers can conserve resources if the desired trait is exhibited late in development (requires time), or scoring of the phenotype is complicated or requires a high degree of expertise. McClendon *et al.* (9) identified three dominant markers for *Fw*, two amplified fragment length polymorphism (AFLP) markers ACC:CTG_159 and ACG:CAT_222 that flank *Fw* over a 4.0 cM interval, and a random amplified polymorphic DNA (RAPD) marker Y15_1050 that lies approximately 4.6 cM upstream of *Fw*. Here, we report the nucleotide sequence of RAPD-derived Y15_1050 PCR product, the development of a 999 bp SCAR marker, and the identification of a Y15 allele linked in coupling to susceptiblity.

Materials and Methods

Plant materials and DNA were generated as previously described (9). Eighty F_8-derived recombinant inbred lines (RIL) from a cross of PI 179449 (susceptible) and cv. Green Arrow (PI 614141, resistant) were tested for resistance to Fusarium wilt race 1 in two greenhouse trials and one field test. The RIL mapping population contained six individuals that showed single recombination events between Y15_999*Fw* and the *Fw* locus (Table 2); four recombinants displayed the Green Arrow phenotype at Y15_999*Fw* (PRIL 7-10, PRIL 7-11, PRIL 7-44, PRIL 7-71) and two displayed the susceptible (null) phenotype (PRIL 7-36 and PRIL 7-69). Genomic DNA was prepared from young leaf tissue of the parents and RILs using a modification (16) of the DNA extraction method of Murray and Thompson (11). DNA concentration was estimated by comparing ethidium bromide staining intensity to that of DNA ladder standards of known concentrations. Leaf DNA concentration was adjusted to 25 ng/□l, then screened using RAPD oligonucleotides (OPERON Technologies, Alameda, CA) as described by Paran and Michelmore (13).

Primer Y15 (Table 1) amplified a ~1050 bp product that co-segregated with *Fw* (7) in the resistant parent Green Arrow, but not in the susceptible parent, PI 179449. To clone the Y15_1050 PCR amplicon, the RAPD

Table 1. RAPD and SCAR primers used in this study. All primers are shown in the 5' to 3' orientation.

Amplicon	Assay	Forward primer	Reverse primer	Position[a]	Tm (°C)	Product (bp)
Y15_1050	RAPD	5'-AGTCGCCCTT-3'	same	1	36	1082
Y15_999*Fw*	SCAR	5'-ATGAGGGTAGC GCTTCATTG-3'	5'-GCCCTTTGTTG TCTCACCTG-3'	81	60	999

[a] Starting nucleotide position of the forward primer on the Y15_1050 sequence (GenBank DQ189096).

Table 2. RILs showing recombination between Fusarium wilt race 1 resistance (*Fw*) and two markers, RAPD Y15_1050 and SCAR Y15_999*Fw*, in the PI 179449 (susceptible) x Green Arrow (resistant) F_8 RIL mapping population. A, Green Arrow parental type; B, PI 179449 parental type.

Line	Type	*Fw* phenotype[a]	Y15_1050	Y15_999*Fw*
Green Arrow	R parent	A	A	A
PI 179449	S parent	B	B	B
PRIL7-10	RIL	B	A	A
PRIL7-11	RIL	B	A	A
PRIL7-36	RIL	A	B	B
PRIL7-44	RIL	B	A	A
PRIL7-69	RIL	A	B	B
PRIL7-71	RIL	B	A	A

[a]Scoring based on data from two greenhouse trials conducted in 1999 and 2000, and one replicated field trial (three plots) conducted in 2000.

PCR reaction (9) was run at an annealing temperature of 36 C. The PCR product was separated on a 1% agarose gel (low OEO, Fisher Scientific, Pittsburg, PA), excised from the gel, and purified using the Concert kit (Invitrogen, Carlsbad, CA). Purified single-band amplicons were ligated to vector DNA, and 2 □l of the ligation mixture was used to transform chemically competent *E. coli* cells (TOPO pCR 4.0 cloning kit, Invitrogen, Carlsbad, CA). Plasmid DNA containing Y15_1050 was isolated using the Perfectprep Plasmid Mini kit (Brinkman,Westbury, NY) from 2-ml overnight cultures containing 25 □g/ml kanamycin. Y15 inserts from four independent clones were excised from the vector with the restriction enzyme *Eco*RI, and sized on an agarose gel. The four Y15 inserts were also sequenced using M13F and M13R primers (Center for Gene Research, Oregon State University, Corvallis, OR).

Two sets of SCAR primers (Table 1) were designed using Primer3 software at the default parameters (14). The SCAR assays (25 □l) consisted of 1 unit of *Taq* polymerase in 1X buffer (Roche Applied Science, Indianapolis, IN), 0.2 mM dNTPs, 250 nM each of the forward and reverse primers, and 75 to 125 ng of genomic DNA from the RILs. PCR was conducted using the ABI 9700 thermocycler (Applied Biosystems, Foster City, CA) and the following amplification profile: 1 min 94 C, 1 min 60 C and 2 min 72 C for 30 cycles. Alternatively, the PTC 200 thermocycler (MJ Research/Bio-Rad Laboratories, South San Francisco, CA) and an amplification profile of 30 s 94 C, 30 s 60 C and 1 min 72 C for 30 cycles was used. Amplicons were separated on 1% agarose gels and visualized using ethidium bromide and UV light. DNA from the resistant and susceptible parents served as positive and negative controls, respectively.

The Y15_1050 nucleotide sequence was analyzed for open reading frames using MapDraw and the Blastx algorithm (1) of GenBank. The *Medicago truncatula* gene index of TIGR (www.tigr.org/tdb/tgi) and GenBank were searched for Y15_1050 homologs using Blastn (1).

Results and Discussion

To increase its utility in MAS, the Y15_1050 RAPD marker from the *Fw* pea cultivar Green Arrow was cloned, sequenced and used to develop SCAR primers. Three of four Y15 inserts were of the expected size of ~1050 bp and had an identical 1082 bp nucleotide sequence (GenBank accession no. DQ189096), whereas the fourth insert was approximately 1000 bp.

Initially, two SCAR primer pairs (Table 1) were designed from the 1082 bp Y15_1050 sequence and tested on the parents and RIL mapping population. One of the primer pairs amplified a 999 bp fragment, designated Y15_999*Fw* (Fig. 1, Table 1), from Green Arrow and 38 RILs that displayed the Green Arrow genotype at Y15_1050 (data not shown). All 37 RILs that showed the PI 179449 (susceptible) genotype at Y15_1050 were negative for the 999 bp SCAR amplicon. In addition, these SCAR primers resolved ambiguous RAPD scores in three other RILs (data not shown). In initial screens, two RILs displayed discrepancies between the RAPD and SCAR results. The RILs shown in Fig. 1, exhibiting single crossover events between Y15_1050 and *Fw*, showed

the expected Y15_999*Fw* SCAR genotype. In short, the Y15_999*Fw* SCAR marker remained polymorphic (dominant) and reliably reflected the presence of the original RAPD marker in the parental lines and the RIL mapping population. The second SCAR primer pair generated a 201 bp amplicon that was monomorphic in both resistant and susceptible parents, and from the six RILs in the Y15_1050 - *Fw* interval (data not shown).

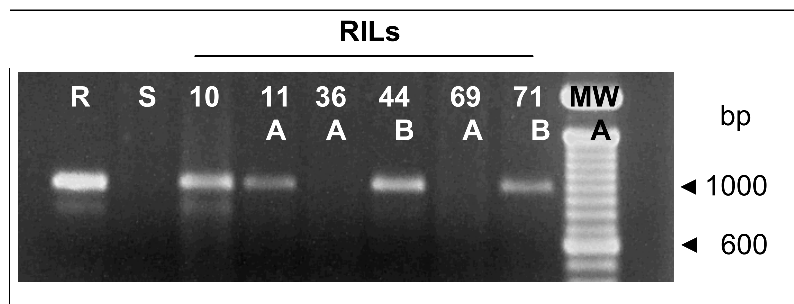

Fig. 1. Co-segregation of the Y15_999Fw SCAR with the Fw locus in six RILs of PI 179449 (S) x cv. Green Arrow (PI 614141; R. The selected RILs, designated 10, 11, 36, 44, 69 and 71, showed single crossover events between Y15_1050 and Fw. A indicates the Green Arrow phenotype at Y15_1050; B indicates the PI 179449 phenotype.

The absence of long open reading frames within the 1082 bp Y15 RAPD amplicon, the lack of significant matches to proteins in GenBank, and the dominant nature of the Y15_999*Fw* SCAR indicated that this molecular marker was not derived from an open reading frame. However, two segments of the Y15_1050 sequence totaling 150 bp showed an average of 86% nucleotide sequence identity (E value 4e-09) to the non-coding region of a *mariner*-like transposable element from *P. sativum* (GenBank AY833550). *Mariner*-like elements have been observed in a wide range of eukaryotes (2), including a member of the family Fabaceae (5), and the detection of transposons by RAPD primers is not surprising. More genomic sequence data will be needed to determine whether the Y15_1050 and Y15_201*Fw* amplicons are located in *mariner*-like elements in Green Arrow and PI 179449.

The Y15_999*Fw* SCAR will provide breeders with a robust, dominant molecular marker with which to follow the *Fw* locus in approximately 95% of the progeny. This marker is one of several that can be used in MAS and in further characterization of the *Fw* locus. The AFLP markers ACC:CTG_159 and ACG:CAT_222 that flank *Fw* are being sequenced, and segregation analyses using these markers is in progress. Additional markers in pea, including resistance gene analogs (17) and microsatellites (8), can also be adapted for MAS.

Inverse PCR and primer walking strategies will be undertaken to obtain additional sequence information for developing a co-dominant Y15 marker. Such a marker could be primer-based (e.g., SCAR), a RFLP, or a single nucleotide or conformational polymorphism. For example, insertions and deletions are observed at the Y15_999*Fw* locus in two of 96 individuals sampled from the *Pisum* core collection (15) (Coyne and Timmerman-Vaughn, unpublished data). A dramatic migration shift between the one of these variants and the parental amplicon was observed in a SSCP gel (Coyne and Timmerman-Vaughan, unpublished data), making this type of marker highly scorable. These observations indicate several approaches for the development of a co-dominant Y15 marker that could be used to distinguish *Fw* homozygotes from heterozygotes in breeding programs.

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