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Chapter 6

APPLICATION OF CAPS MARKERS TO THE MAPPING AND MARKER-ASSISTED BREEDING OF GENES FOR RESISTANCE TO FUSARIUM WILT IN THE TOMATO

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ABSTRACT

CAPS markers have been useful tools for the mapping and marker-assisted breeding of desirable genetic traits in tomato for many years. However, two recent advances have dramatically increased the availability and power of CAPS markers to the tomato breeder. The first is the advent of near-complete genome sequences for tomato, *Solanum lycopersicum*, and its close wild relative, *S. pimpinellifolium*, as well as incomplete genome sequences for other wild relatives used as sources of traits for tomato improvement. The second is the identification of a multitude of SNPs not only between tomato and its wild relatives, but also between tomato cultivars. Both of these resources can be mined for SNPs that can be converted readily into CAPS markers. In this chapter, we present our work on mapping and marker-assisted breeding of genes for Fusarium wilt resistance to provide examples of the pre- and post-genomic application of CAPS markers in tomato, ranging from genome-wide surveys and crude-mapping to the fine-mapping and marker-assisted breeding of specific disease resistance genes.

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INTRODUCTION

Fusarium wilt caused by the fungus *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is a devastating disease of tomato (*Solanum lycopersicum*) that affects tomato crops worldwide. The fungus penetrates the roots and colonises the xylem of tomato plants where it blocks water flow, resulting in the wilting and eventual death of infected plants (Figure 1). *Fol* overcomes host plant defences by secreting an array of host-specific disease effector proteins during infection (reviewed by Michielse and Rep 2009; Catanzariti and Jones 2010; Takken and Rep 2010).

These effector proteins, originally termed SIX (Secreted In Xylem) proteins, were recovered from the xylem of *Fol*-infected tomato plants (Rep et al., 2002, 2004; Houterman et al., 2007). Several of these effectors are required for full pathogenicity and some can be recognised by specific tomato disease resistance proteins, thereby also functioning as avirulence (Avr) proteins e.g. SIX4 = Avr1, SIX3 = Avr2 and SIX1 = Avr3 (Rep et al., 2004; Houterman et al., 2008, 2009; Table 1).

Remarkably, the Avr1 effector has been shown to function as a meta-effector able to suppress the detection of the Avr2 and Avr3 effectors by tomato plants carrying the corresponding resistance genes (Houterman et al., 2008). Also remarkably, analysis of the *Fol* genome sequence has revealed that the genes encoding these host-specific effectors are carried on a supernumerary pathogenicity chromosome that can be transferred horizontally within the genus *Fusarium oxysporum* (Ma et al., 2010).

Figure 1. Tomato plants growing in a field infested with *Fol* race 3 in Bowen, Australia, showing the devastating effects of *Fol* infection on a susceptible tomato cultivar (front left) and the protection provided by *I-3* in resistant tomato cultivars (front right).

Table 1. Effectors present in the three known races of *Fol* and their pattern of recognition by tomato genes for Fusarium wilt resistance

Race	Effectors	Recognised by
1	Avr1 Avr2 Avr3 Avr7?	I, I-7? (Avr1 suppresses detection by I-2 and I-3)
2	— Avr2 Avr3 Avr7?	I-2, I-3, I-7? (Avr1 deleted in races 2 and 3)
3	— Avr2* Avr3 Avr7?	I-3, I-7 (mutant Avr2* effector avoids recognition by I-2)

Fortunately, Fusarium wilt disease of tomato has so far been controlled by major genes for resistance introgressed from wild species of tomato (Figure 1; Table 1). The *I* (*Immunity*) gene, introgressed from *S. pimpinellifolium,* confers resistance to *Fol* race 1 through recognition of Avr1 (Houterman et al., 2008). *Fol* race 2, able to infect tomato plants carrying the *I* gene, arose through a deletion of the *Avr1* gene. The *I-2* gene, also introgressed from *S. pimpinellifolium*, confers resistance to *Fol* race 2 through recognition of the Avr2 effector protein (Houterman et al., 2009). *Fol* race 3, able to infect tomato plants carrying *I-2*, then arose through a mutation of the *Avr2* gene enabling the Avr2 effector protein to retain its pathogenic function but evade recognition (Houterman et al., 2009). Two different resistance genes, *I-3* and *I-7*, both conferring resistance to *Fol* race 3, were then introgressed from *S. pennellii* (McGrath et al., 1987; Scott and Jones, 1989; Lim et al., 2006). *I-3* enables recognition of the Avr3 effector protein (Rep et al., 2004), but an Avr7 effector protein corresponding to *I-7* has yet to be identified.

The *I-2* resistance gene has been isolated and shown to encode a coiled-coil nucleotide-binding leucine-rich-repeat (CC-NB-LRR) protein typical of a large class of resistance proteins from a broad range of plants and effective against a wide range of pathogens (Ori et al., 1997; Simons et al., 1998). Recently, we identified the *I-3* resistance gene (Catanzariti et al., in preparation) and a candidate for the *I-7* resistance gene (Gonzalez et al., unpublished). We made extensive use of CAPS markers to help locate the *I-3* and *I-7* genes and develop markers suitable for their marker-assisted breeding. More recently still, we have begun the process of identifying the *I* gene and are again making extensive use of CAPS markers to help locate the gene and develop markers suitable for marker-assisted breeding. However, our development and use of CAPS markers has changed over the last ten years with the advent of new tomato genomic and genetic resources. This chapter describes the various approaches we have used for the development of CAPS markers linked to the tomato *I*, *I-3* and *I-7* genes for Fusarium wilt resistance and describes CAPS markers suitable for the marker-assisted breeding of each of these genes.

CAPS Markers in the *I-3* Region of Tomato Chromosome *7*

When we began mapping *I-3* on the long arm of chromosome *7*, there were few genomic or molecular marker resources available for tomato apart from molecular genetic maps based on RFLP (Restriction Fragment Length Polymorphism), SSR (Simple Sequence Repeat) and isozyme markers. Initially, we developed CAPS and SCAR (Sequence Characterised Amplified Region) markers from the sequences of tomato or potato gDNA (genomic DNA) or cDNA (complementary DNA) clones used as RFLP probes, or the sequences of cloned genes mapping to the long arm of chromosome *7* (Hemming et al., 2004; Lim et al., 2008).

Sequences were either obtained from public databases or by sequencing the ends of gDNA or cDNA clones obtained from colleagues. These sequences were used to design oligonucleotide primers for PCR amplification of genomic DNA fragments from a line carrying *I-3* on an introgressed segment of *S. pennellii* chromosome *7* and a line lacking *I-3*. Where possible, primers were designed to flank introns in order to capitalise on the greater sequence variation present in introns relative to exons. In some cases, PCR products were obtained only from the line lacking *I-3*, thereby generating dominant SCAR markers. In other cases, PCR products of different sizes were obtained, thereby generating co-dominant SCAR markers. However, in the majority of cases, products of the same size were obtained. These PCR products were developed into CAPS markers, either by trial and error using a panel of restriction enzymes, or by sequencing the PCR products and using sequence alignment tools to detect restriction site polymorphisms *in silico*.

We also developed CAPS markers from isozyme markers. The *Got-2* isozyme marker had previously been shown to be closely linked to *I-3* (Bournival et al., 1989). *Got-2* is a member of a multigene family encoding glutamate oxaloacetate transaminases (also known as aspartate aminotransferases). To develop a CAPS marker for *Got-2*, oligonucleotide primers were designed using the sequences of all the available tomato unigenes annotated as glutamate oxaloacetate transaminases or aspartate aminotransferases. These primers were then used to amplify *Got* gene sequences from *S. lycopersicum* cultivar M82 and *S. pennellii* accession LA716 (the source of *I-3*). Using the same approach to marker development described above, the resulting PCR products enabled the development of two SCAR and nine CAPS markers corresponding to at least six *Got* genes (Wang et al., 2007). These markers were bin-mapped onto the tomato genome using a set of introgression lines each carrying a unique sub-chromosomal segment of *S. pennellii* DNA derived from LA716 in a M82 background (Eshed and Zamir 1994). This enabled identification of a *Got-2* CAPS marker (GOT-B; Table 2) on the long arm of chromosome *7* suitable for marker-assisted breeding of *I-3* and more accessible to tomato breeders than the *Got-2* isozyme marker (Wang et al., 2007).

Subsequently, the advent of COS (Conserved Orthologue Set) and COSII markers (Fulton et al., 2002; Wu et al., 2006) enabled the development of additional CAPS markers in the *I-3* region. Marker location and often primer information could be obtained from the SOL Genomics Network (SGN) website (http://solgenomics.net/) but, until recently, restriction enzyme information was often unavailable. As outlined above, the choice of restriction enzyme was often determined by trial and error using a panel of restriction enzymes or by sequencing PCR products and aligning their sequences. However, the convention of naming COSII markers according to the names of their Arabidopsis orthologues also allowed us to detect microsynteny between Arabidopsis and tomato for markers mapping in the vicinity of *I-3*. This was confirmed by using additional Arabidopsis genes from these regions of microsynteny to identify orthologous tomato unigenes for development into new CAPS markers that also mapped to the *I-3* region of tomato chromosome *7* (Lim et al., 2008).

With all these markers (mainly CAPS), we were able to fine map *I-3* and move from genetic to physical mapping using BAC (bacterial artificial chromosome) clones covering the *I-3* region. We were then able to design new CAPS markers by aligning *S. lycopersicum* BAC sequences with a *S. pennellii* BAC sequence carrying *I-3* (Lim et al., 2008) to further refine the location of *I-3*. This high-resolution mapping enabled the identification of three members of a small multigene family as candidates for *I-3* and ultimately the identification of *I-3* itself

through the generation of transgenic tomato plants resistant to *Fol* race 3 (Catanzariti et al., in preparation). This analysis allowed the identification of a CAPS marker based on the tomato cDNA probe CT226 (Table 2; Hemming et al., 2004; Lim et al., 2006) as a better marker for marker-assisted breeding of *I-3* than the GOT-B (*Got*-2) marker because CT226 is about 150 kb closer to *I-3* than GOT-B (*Got*-2). However, CT226 is still located about 130 kb away from *I-3* and a better marker still would be one located within the *I-3* gene itself, such as the S5 CAPS marker we report here for the first time (Figure 2; Table 2).

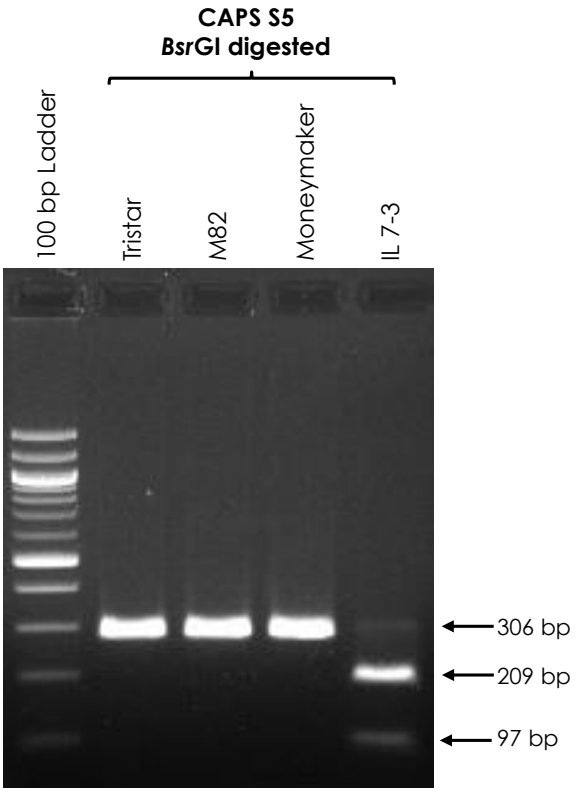

Figure 2. Agarose (2%) gel with CAPS marker S5 showing the presence of the *I-3* resistance gene in the tomato introgression line IL7-3 but its absence in the tomato cultivars Tristar, M82 and Moneymaker. Primer details are provided in Table 2. PCR reactions were performed using Phire Hot Start II DNA polymerase (Thermo Scientific) with an annealing temperature of 58°C.

CAPS Markers in a Genome-Wide Search for *I-7*

During the *I-3* mapping work described above, the *I-7* gene was found not to be located on chromosome *7* and therefore to be distinct from *I-3* (Lim et al., 2006). Since the chromosomal location of *I-7* was unknown, no markers were available for the marker-assisted breeding of *I-7*. Markers for *I-7* would be especially useful because they would allow *I-7* to be combined with *I-3*, an otherwise difficult and time consuming task because both genes confer the same phenotype of resistance to *Fol* race 3.

Table 2. Primer sequences, restriction enzymes and band sizes for CAPS markers suitable for marker-assisted breeding of the tomato *I*, *I-3* and *I-7* genes for resistance to Fusarium wilt (as shown in parenthesis)

CAPS marker	Forward primer (5'-3')	Reverse primer (5'-3')	Restriction enzyme	Digestion products (bp)			
				Tristar	M82	Moneymaker	IL7-3
GOT-B (*I-3*)	AGTGGCAGTGAAAAGTCAGTTG	CCAAGTAACCAACATTTCCAGTAG	*Hpa*II	ND*	440+210	ND	650
CT226 (*I-3*)	GTGAAGGAGTGTCAAAGGCAAC	GGAATGAACAATTTATATGCAGCAG	*Mae*III	ND	287+114	ND	401
CAPS_S5 (*I-3*)	CAATGGTCTGTAGTTGATTGGAATG	CTGCCCAAGCCACAATTTAG	*Bsr*GI	306	306	306	209+97
Solyc08g077780 (*I-7*)	GCAATGACTTTGCTGTTTATGTC	AATTATAATCGAGACCACTTTACCC	*Tsp*509I	192+56+33	136+56+56+33	ND	ND
Solyc08g077730 (*I-7*)	GCTTCTGCTTTAACCCCACTT	TCTGAACTTCAATATTCTTCTGATGC	*Hin*fI	177+69	246	ND	ND
Solyc08g077560 (*I-7*)	TTTTCTCATATGGGTTTCTCTGG	TCCCCTCTCCTACGAGACCT	*Dra*I	463	292+100	ND	ND
Solyc08g077540 (*I-7*)	CCCAATTCCTAAAACCTCAGAA	CCAAACAAAACCCATTTTTCA	*Ase*I	~109+83+80	292	ND	ND
CAPS_21099 (*I*)	GTTGAAGTGCAACACGGATG	TTTCAATGGCTCATGCAACT	*Nco*I	584	584	325+259	ND
CAPS_100072 (*I*)	TTTGGAAAACTCCGAAAAGG	GGCACTATTTCACCTGACACG	*Eco*RV	836	836	517+319	ND
CAPS_21040 (*I*)	CCCAAGAAGTTATCCGATGC	TTCCAAGCAGCATTTGTGAG	*Bgl*II	ND	343+342	685	ND

* ND = band size not determined

Table 3. Primer sequences, annealing temperatures, restriction enzymes and digestion product sizes for CAPS markers developed for use in the genome-wide survey for markers linked to the *I-7* gene in the tomato cultivar Tristar, and for which no information is available on the SGN website (http://solgenomics.net/) about restriction enzymes used, and no or differing information is available about digestion product sizes

CAPS Marker	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temp (°C)	Restriction enzyme	Digestion products (bp)		
					Tristar	M82	LA716
C2_At5g41760	TTGACGACTCTATCGCAAAG TAATGG	TAGATGGAATTGGGTATA AACGAACAC	52	*Eco*RI	866+171	866+171	1061
U237757	ATCGGCTGCTGATGTTTATG ATCG	ACAACATCCCTCCATAGA GTTTCAAG	50	*Hin*dIII	969+640+472	969+640+472	~1112+969
C2_At1g61150	ATGGAAAGATTGAGGAGGC TTTGGAG	ATCTTCAATAGGCTTGGA AGCTTTGG	55	*Bst*NI	401+292+291	401+292+291	984
C2_At5g37290	TCGTCATCCAGTGTTTGTCA AGTCCTGT	TTTAAGATCTCTTCCTTGT TTGATGC	53	*Taq*I	370+242+138	370+242+138	~500+350
C2_At3g55800	TTTGAAATCAAGCTCATTAT TTGG	AGCTGTTCCTCCACAAGA AGCTG	50	*Hin*cII	197+137	197+137	334
C2_At1g18640	AATTCCGTTGTTGCTTCAGTT CAGCC	TCGTCTATGCACACAGTG CTATCCAC	55	*Rsa*I	310+161	310+161	471
C2_At1g16870	ACCCTGAAGAAATGCTCCGG TC	TCACCGAATTCCTTAAGC ATCCC	55	*Hin*dIII	501+167	501+167	668
C2_At1g56050	TCTCCTAGGGTTGCGTACAT ACTTCAC	TGCCCTGATGAAGCCCTT TTCAAAGTC	53	*Dra*I	670+463	670+463	~463+400+300
C2_At1g32220	ACCCCTAAGTTCATATTGAT TTCTGTGC	TCTTCCCATATATAAAGG CAGGTCTAAG	55	*Alu*I	199+115+56	199+115+56	~270+100
C2_At1g04190	TCATTTCTTCGACAGTATGC TGAAGATT	ATTCCATCATTTTGTCCAT GCTTCC	58	*Apo*I	1120+86	1120+86	1206
C2_At3g23400	TGGGCTAAACAGAGGTCTTG CTGC	TATAAGTTCAAATTTGTG GGCTAAAG	55	*Dra*I	494+415	494+415	909
C2_At4g37510	ATGGAATTATGTTCATCTTT CAAACC	ATTAGTCCTCTCACACTTA GGATGTTG	55	*Dra*I	306+213	306+213	519
C2_At1g17410	ATTGGCTATAATAAAGCCAG ATGG	AATTAATGCACGCCAATC AGC	55	*Taq*I	971+449+335	971+449+335	~971+350+335
C2_At4g31150	TACTGAAGATGACTTCAAAT GGAGATT	TCATTTTATCCAAAAGTTC CAGAA	60	*Eco*RV	344+63	344+63	407

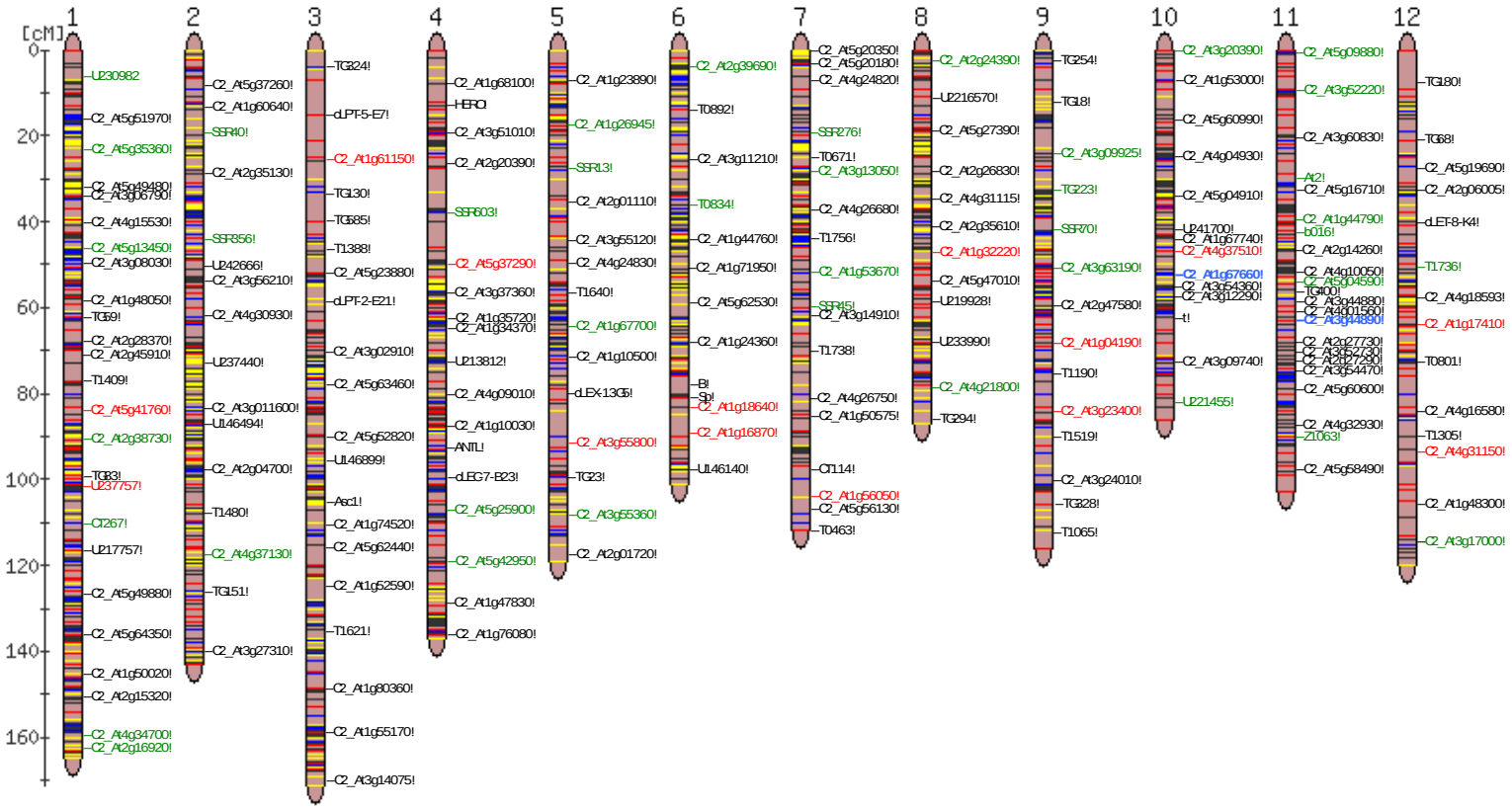

Figure 3. Approximate positions on the twelve tomato chromosomes of 160 CAPS and 40 SCAR markers used in the genome-wide survey for polymorphic markers linked to the *I-7* gene in the cultivar Tristar. Markers shown in black indicate CAPS markers for which there is complete information available on the SGN website (http://solgenomics.net/). Markers shown in red indicate CAPS markers that we developed (see Table 3) and for which marker information is incomplete or differs on the SGN website. Markers shown in green indicate SCAR markers for which information is available on the SGN website. Markers shown in blue indicate CAPS markers showing a polymorphism between *S. lycopersicum* cultivars Tristar and M82. The higher density of markers used around these two polymorphic markers reflects a more intensive but unproductive search for evidence of an introgressed region derived from a wild relative of tomato. This representation of the tomato chromosomes is derived from the Tomato EXPEN-2000 map available on the SGN website.

We therefore undertook a genome-wide marker survey of the tomato cultivar Tristar, which carries the *I-7* gene introgressed from *S. pennellii* accession PI414773, to try to identify the chromosomal location of *I-7*. To do this, we used CAPS and SCAR markers that had been used to construct the Tomato EXPEN-2000 map from an F_2 mapping population derived from the cross between *S. lycopersicum* accession LA925 and *S. pennellii* accession LA716.

Primer sequences and restriction enzyme information available from the SGN website allowed CAPS marker polymorphisms to be detected between *S. lycopersicum* cultivar M82 and *S. pennellii* accession LA716. When information about restriction enzymes was not available, restriction sites likely to be present in M82 were identified from the recently completed tomato genome sequence (The Tomato Genome Consortium 2012) available on the SGN website. A panel of corresponding restriction enzymes was then used to identify restriction site polymorphisms.

In total, we used 160 CAPS markers (mainly COSII markers) and 40 SCAR markers chosen at 10-15 cM intervals across all twelve tomato chromosomes (Figure 3).

Table 3 shows the primer sequences, restriction enzymes and fragment sizes for a subset of these markers for which some or all of this information was not available on the SGN website at the time of writing, or, if available, differed from the information provided. Two polymorphisms were detected between Tristar and M82, one on chromosome *10* (marker C2_At1g67660) and the other on chromosome *11* (marker C2_At3g44890). However, neither of the Tristar polymorphisms corresponded to the polymorphisms present in LA716 (Figure 4) and neither marker showed linkage to *I-7* in a population segregating for resistance to *Fol* race 3, derived from a cross between Tristar and M82. Although the genome-wide survey did not locate any polymorphic CAPS or SCAR markers linked to *I-7*, it did suggest that *I-7* must be on a small introgressed segment of *S. pennellii* DNA, which is a favourable outcome for plant breeders so far as possible linkage drag of undesirable wild traits is concerned.

Rather than pursue the genome-wide survey further, we instead turned to Illumina next-generation sequencing to identify SNPs in genes linked to *I-7* that could then be converted into CAPS markers. We set up an RNA-seq experiment with several objectives, including identification of SNPs in root transcripts derived from genes in the *S. pennellii* introgression carrying *I-7*. We identified SNPs by using the CLC Genomics Workbench (http://www.clcbio.com) to map RNA-seq reads from mock-inoculated plants onto the tomato reference transcriptome available on the SGN website. Transcripts derived from *S. pennellii* genes were expected to have a higher frequency of SNPs relative to the reference transcriptome than *S. lycopersicum* transcripts. A plot of SNP frequency against gene position on each of the twelve tomato chromosomes revealed two clusters of SNPs occurring at a higher frequency in Tristar. One cluster on chromosome *11* corresponded to the *S. pimpinellifolium* introgression carrying the *I-2* gene, known to be present in Tristar along with the *I* and *I-7* genes. The other was a cluster of 19 transcripts encoded by genes on the long arm of chromosome *8*. SNPs in four of these genes (08g077540, 08g077560, 08g077730 and 08g077780) were used to design CAPS markers (Table 2, Figure 5). These four markers co-segregated with *I-7* in a segregating population derived from a cross between Tristar and M82. Further analysis of the introgression showed it was indeed small, carrying a total of only 29 genes on approximately 210 kb of *S. pennellii* DNA.

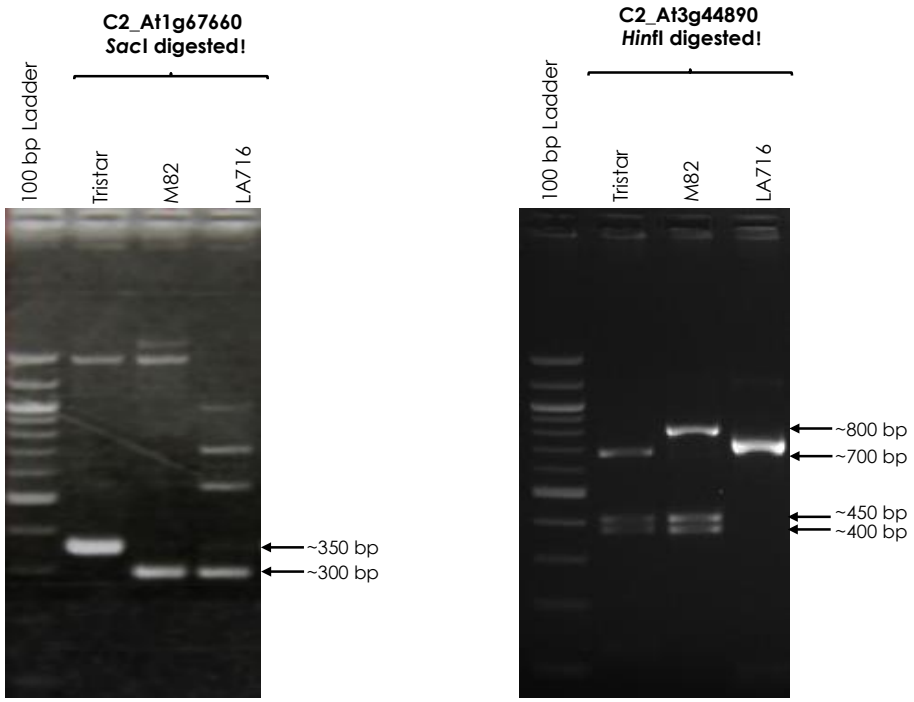

Figure 4. Agarose (2%) gel with CAPS markers C2_At1g67660 and C2_At3g44890 showing polymorphisms between tomato cultivars Tristar and M82, and *S. pennellii* accession LA716. Primer details are provided in Table 3. PCR reactions were performed using Phire Hot Start II DNA polymerase (Thermo Scientific) with an annealing temperature of 54°C.

One of the 19 root-expressed genes was identified as a likely candidate and, at the time of writing, transgenic tomato plants expressing this gene were being generated for testing with *Fol* race 3. Given that the introgression is so small, any of the four CAPS markers could be used for marker-assisted breeding pending conclusive identification of *I-7* and development of a perfect CAPS marker based on the sequence of *I-7* itself.

These findings raise the possibility that *I-7* may be allelic to the *Tfw* (tolerance to Fusarium wilt) gene identified in *S. pennellii* accession LA716 and shown to be located on chromosome *8* near the isozyme marker *Aps-2* (acid phosphatase-2) (Bournival et al., 1989, 1990). The Solyc08g066530 gene located near to the *I-7* region has been annotated as *Aps-1*-like according to the tomato genome web browser (http://solgenomics.net/gbrowse/bin /gbrowse/ITAG2.3_genomic/) and this gene could therefore correspond to *Aps-2*. Moreover, the *Aps-2* isozyme marker is closely linked to the *Got-4* (glutamate oxaloacetate transaminase-4) isozyme marker (Tanksley and Rick 1980). The Solyc08g068330 gene, located even nearer to the *I-7* region than Solyc08g066530, has been annotated as an aspartate amino transferase (= glutamate oxaloacetate transaminase) and this gene may correspond to *Got-4*, a possibility noted previously by Wang et al. (2007), thereby strengthening the circumstantial evidence that *I-7* may be allelic to *Tfw*.

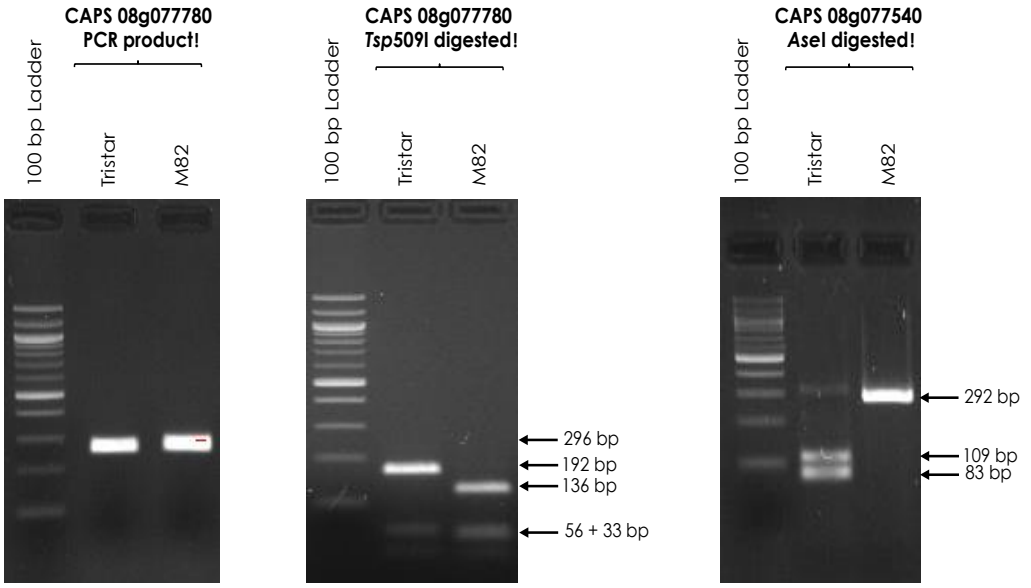

Figure 5. Agarose (2%) gel with CAPS_08g077780 and CAPS_08g077540 markers showing polymorphisms between the tomato cultivars Tristar (carrying the *I-7* gene) and M82 (lacking the *I-7* gene). Primer details are provided in Table 2. PCR reactions were performed using Phire Hot Start II DNA polymerase (Thermo Scientific) with an annealing temperature of 58°C. Note that the CAPS_08g077780 primers generate a 296 bp product with both cultivars (left panel), but both products are digested by *Tsp*509I to show a polymorphic 192 bp digestion product for Tristar and a 136 bp digestion product for M82 (middle panel).

CAPS Markers in the *I* Region of Tomato Chromosome *11*

The *I* gene introgressed from *S. pimpinellifolium* accession PI79532 (Bohn and Tucker, 1939) has been deployed in tomato breeding for so long a time that it is almost ubiquitous among modern tomato cultivars and often tomato breeders may be unaware of its presence. For example, the cultivars Tristar and M82 and the reference transcriptome (derived from the cultivar Heinz 1706) all carry the *I* gene thereby rendering the RNA-seq analysis used for the location of *I-7* incapable of providing any information about the location of *I*. Moreover, breeders may be unconcerned about the presence of *I* because it is no longer effective against *Fol* given that races 2 and 3 have dispensed with the *Avr1* gene (Table 1). However, interest in the *I* gene has been renewed by the finding that the *Fol* Avr1 effector suppresses detection of Avr2 and Avr3 by I-2 and I-3, respectively.

This led to the proposition that durable resistance to *Fol* could be achieved by combining *I* and *I-3*, given that loss of Avr3 would lead to severely reduced pathogenicity and suppression of Avr3 detection by a resurgent Avr1 would lead to recognition by I (Houterman et al., 2008).

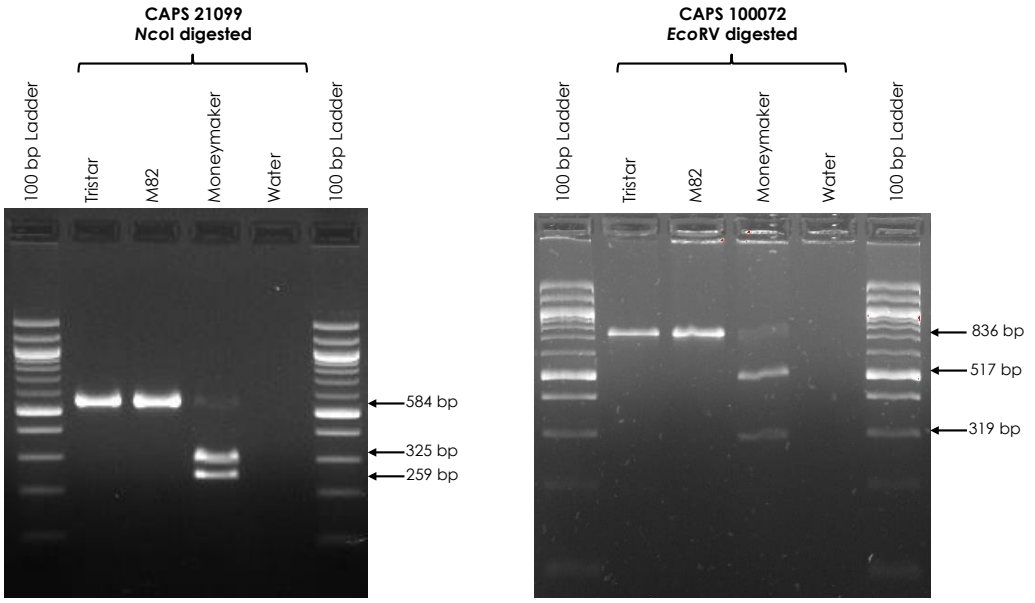

Figure 6. Agarose (2%) gel with CAPS markers 21099 and 100072 showing polymorphisms between tomato cultivars Tristar and M82 (carrying the *I* gene) and Moneymaker (lacking the *I* gene). Primer details are provided in Table 2. PCR reactions were performed using BIOTAQ DNA polymerase (Bioline) with an annealing temperature of 55°C.

The *I* gene is located on the short arm of chromosome *11* (Ori et al., 1997; Sela-Buurlage et al., 2001; Scott et al., 2004) and a dominant SCAR marker reporting the presence of *I* has been developed by Arens et al. (2010). To fine map *I*, we sought to develop CAPS markers for the *I* region on chromosome *11*. This was made possible following the publication of the tomato genome sequence (The Tomato Genome Consortium 2012) and the genome-wide identification of SNPs in a range of old and modern tomato cultivars (Sim et al., 2012). By comparing SNPs in the cultivar M82 (which carries *I*) with the corresponding SNPs in the cultivar Moneymaker (which lacks *I)*, we were able to identify a 1 Mb introgression of *S. pimpinellifolium* DNA carrying 93 genes including four candidates for the *I* gene. Using tomato genome sequence spanning these SNPs, we have begun developing CAPS markers from SNPs that correspond to restriction site polymorphisms. Of 114 reliable M82/Moneymaker SNPs reported in this region (Sim et al., 2012), at least 19 correspond to restriction site polymorphisms. Using CAPS markers 21099 and 100072 based on SNPs located at either end of the *I* region (Table 2, Figure 6), we have begun looking for recombinants within the *I* region using an F_2 mapping population obtained from a cross between M82 and Moneymaker. At the same time, we have also commenced the process of generating transgenic plants carrying each of the four candidate genes for testing with *Fol* race 1, which carries the *Avr1* gene. At2 is not ideal for marker-assisted breeding of the *I* gene because it is a dominant marker and more than 450 kb away from the nearest candidate gene. The combination of the two CAPS markers 21099 and 100072 represents a far better choice for marker assisted-breeding of *I* than At2 because they are co-dominant markers that allow maintenance of the *I* introgression intact. Of course, once the *I* gene is identified it will be possible to develop the perfect CAPS marker based on the sequence of the *I* gene itself. To

work towards this goal, we have started developing CAPS markers such as 21040 (Table 2; Figure 7) from SNPs located in the candidate genes themselves where possible.

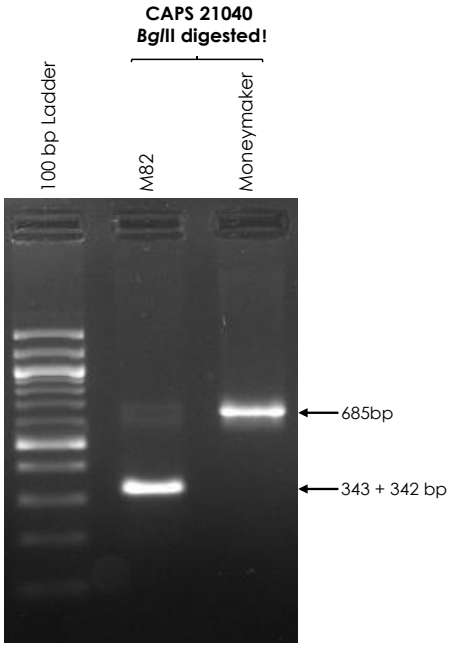

Figure 7. Agarose (2%) gel with CAPS marker 21040 showing PCR product size polymorphisms between tomato cultivars M82 (carrying the *I* gene) and Moneymaker (lacking the *I* gene). Primer details are provided in Table 2. PCR reactions were performed using Phire Hot Start II polymerase (Thermo Scientific) with an annealing temperature of 63°C.

CONCLUSION

When we started developing CAPS markers for use in tomato, the design process was more opportunistic than systematic as we tried to maximise use of scant resources in terms of sequence information available in our region of interest. With the advent of the tomato genome-sequencing project, this quickly changed as new sequence resources became available, culminating in the completion and online publication of the tomato genome sequence. As new wild-tomato genome sequences or cultivar genome sequences are added, it will be possible to design CAPS markers *in silico* with much greater ease and with much more choice with respect to primer sequences, cleavage sites and fragment sizes. For example, band intensity can be problematic if cleavage products are small, but it is now easier to develop CAPs markers with a single central restriction site so that the cleaved bands are almost exactly half the size of the uncleaved band and thus run together as a single band of equal staining intensity compared to the uncleaved band (Figure 7).

As a further example, incomplete cleavage can lead to genotyping errors, but it is now easier to develop CAPS markers that cleave at a non-polymorphic site, which acts as an internal control for cleavage, as well as a polymorphic site, so that both bands show a shift in

size compared to the uncleaved PCR product, thus facilitating unambiguous genotyping (see CAPS_08g077780 in Figure 5).

Table 4. SCAR markers mapping to one end (starting at 7.6 Mb and extending to 10.1 Mb) of a large *S. pimpinellifolium* introgression (starting at 7.6 Mb and ending at 46.4 Mb) on the long arm of chromosome *11* in the tomato cultivar M82

Marker	Forward primer (5'-3')	Reverse primer (5'-3')	Band size (bp)	
			M82	MM*
SCAR76	GAAACGTCTGGGAAGGATGA	CGAGCACATGAATTGTGTTTC	354	461
SCAR79	CCAACAATCAAATGCACCAG	TTTGGTCGAACTGTACCATCC	801	679
SCAR83	GGGACTGGGCAGTGATAAAA	CAAGTCGACGCTACTGACCA	879	760
SCAR86.1	TGTTGGCGGTAGTGATGAGA	TCACCAATATTAGGCCCCTTT	314	583
SCAR89	GACGAATTGCATTTGGTAATGA	CAATCGCCTTAACCGACAAT	653	475
SCAR96.1	GCCCATTTAGGATCAACAGG	GGTCAAGACCCAACACGATT	244	522
SCAR99	GCCAAGGATCCTCAAGTGTT	GTTAGCACCCAAAGGTCCAA	680	583
SCAR101	CCCAAGGGTTTTCAAATCAA	TGTCGCAGGAAAATTGAGACT	533	777

* MM = the tomato cultivar Moneymaker.

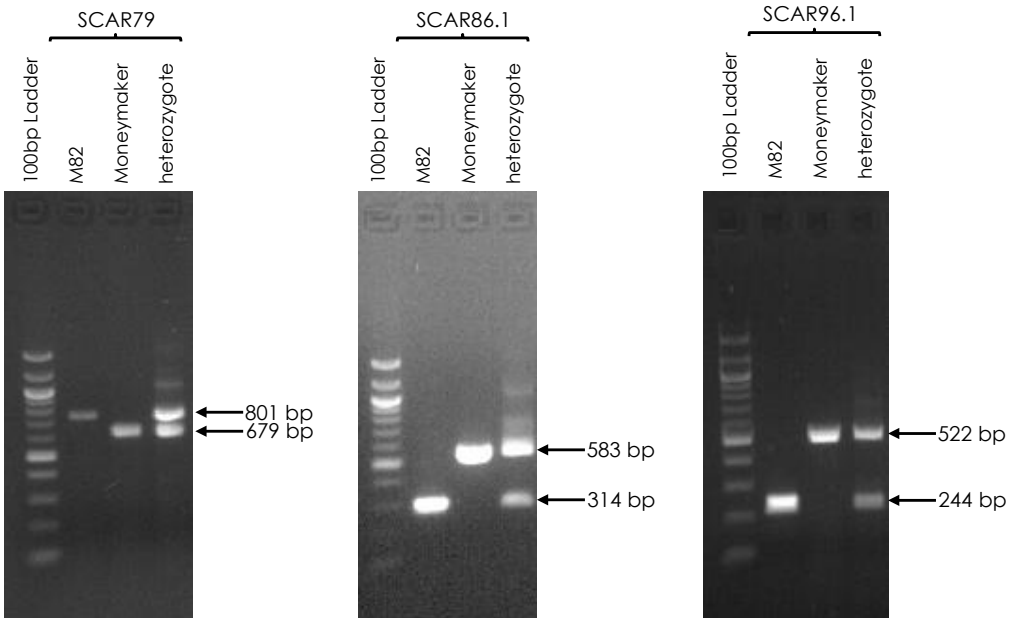

Figure 8. Agarose (2%) gel with SCAR79, SCAR86 and SCAR96.1 markers showing PCR product size polymorphisms between tomato cultivars M82 (carrying an *S. pimpinellifolium* introgression on the long arm of chromosome *11*) and Moneymaker. Primer details are provided in Table 4. PCR reactions were performed using REDTaq DNA polymerase (Sigma) with an annealing temperature of 52°C.

There is also an increased opportunity to use indels (insertions or deletions) in genomic sequence alignments to develop SCAR markers. A pre-publication draft of the *S. pimpinellifolium* genome sequence has been produced by D. Ware, W. R. McCombie, and Z. B. Lippman at the Cold Spring Harbor Laboratory and made available on the SGN website. We have used alignments between *S. lycopersicum* and *S. pimpinellifolium* genomic sequences to develop SCAR markers detecting part of a second larger *S. pimpinellifolium* introgression (starting at 7.6 Mb and ending at 46.4 Mb) on chromosome *11* in the tomato cultivar M82 (Sim et al., 2012; Table 4; Figure 8). This analysis reinforced the fact that SCAR markers are easier and cheaper to work with than CAPS markers because they require no restriction enzymes, but also confirmed that they will not replace CAPS markers because indels occur at a lower frequency than restriction site polymorphisms and often the indels are not large enough for development into SCAR markers. SCAR markers will be useful for crude mapping in tomato but CAPS markers will remain the markers of choice for fine mapping.

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