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Development of PCR-based markers from the tomato glutamate oxaloacetate transaminase isozyme gene family as a means of revitalising old isozyme markers and recruiting new ones

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Abstract Sequences annotated as aspartate aminotransferases (synonymous with glutamate oxaloacetate transaminases) in the SOL Genomics Network unigene database were used to design 10 pairs of PCR primers for genetic marker development. These primer pairs generated nine CAPS markers, two SCAR markers and one SSR marker, which were bin-mapped using a set of tomato introgression lines (IL) derived from *Lycopersicon esculentum* cv. M82 and *Lycopersicon pennellii* LA716. Based on their bin locations, these markers are largely dispersed throughout the tomato genome and appear to have tagged all four of the glutamate oxaloacetate transaminase *(Got)* isozyme marker genes placed on the classical genetic map of tomato. Orthologous relationships with Arabidopsis aspartate aminotransferase (*Asp*) genes suggest the existence of at least two additional functional *Got* genes in tomato that have also been tagged by these markers and likewise an additional functional *Asp* gene in Arabidopsis. The *Got-2* isozyme marker has often been used for the marker-assisted breeding of the *I-3* gene for Fusarium wilt resistance introgressed from *L. pennellii* LA716. The *Got-2* CAPS marker that we have developed offers a facile PCR-based alternative to the isozyme marker for the marker-assisted breeding of *I-3*. However, all of the PCR-based markers we have developed have the potential to assist the breeding of linked traits introgressed from wild relatives of tomato.

Keywords Tomato · Glutamate oxaloacetate transaminase · Aspartate aminotransferase · Got-2 · I-3 · Arabidopsis

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Results

Seven distinct tomato (*Lycopersicon esculentum*) unigenes (version: build 3) in the SOL Genomics Network (SGN) database (http://www.sgn.cornell.edu/) encoding putative glutamate oxalaoacetate transaminases were obtained by searching for sequences annotated synonymously as aspartate aminotransferases. Nine pairs of PCR primers (Table 1) were designed in regions of high sequence confidence, using the SGN intron finder (http://www.sgn.cornell.edu/cgi-bin/tools/intron_detection/find_introns.pl) to predict intron

locations and where possible design primers that amplified across one or two introns. Where available, corresponding *L. pennellii* ESTs were also used to guide primer design and the choice of restriction enzyme for conversion of PCR products into cleaved amplified polymorphic sequence (CAPS) markers. In the absence of corresponding *L. pennellii* sequences, a panel of restriction enzymes with sites in the amplified *L. esculentum* sequence were tested to identify restriction site polymorphisms. After amplification, seven primer pairs produced clear single bands and two primer pairs, GOT-C and GOT-H, amplified two fragments each (Table 1). The smaller fragments from these two primer pairs were polymorphic in size between M82 and LA716 and therefore, could be employed as sequence characterised amplified region (SCAR) markers. The remaining nine bands were non-polymorphic between the two parental lines, but digestion with restriction enzymes revealed cleavage polymorphisms in all of them, thus generating nine CAPS markers.

The nine CAPS markers and two SCAR markers were bin-mapped to eight different chromosomal locations (Table 1) using a set of 50 introgression lines (ILs), each containing an introgressed segment of the *L. pennellii* LA716 genome in a *L. esculentum* cv. M82 background (Eshed and Zamir 1994). GOT-F, GOT-G and GOT-H were developed from different parts of unigene sequence U213889 and therefore, amplified the same gene on chromosome *8*. The GOT-C and GOT-H primer pairs each produced two bands of different size and in each case the derived markers did not map to the same regions, suggesting the existence of sequence duplications in different regions. This was further complicated following additional database analysis using U216620, from which GOT-C was developed. The SP6 end sequence of BAC clone SL_MboI0121F21 (http://sgn.cornell.edu/maps/physical/clone_read_info.pl?chrid = 209409) was found to contain an exon identical to part of U216620. An SSR marker was identified and developed from insert sequence at the T7 end of this clone (http://sgn.cornell.edu/maps/physical/clone_read_info.pl?chrid=194433), and subsequently mapped to the *L. pennellii* introgression carried by IL7-4. This was a different location from where the GOT-C CAPS or SCAR markers mapped, suggesting that three copies of GOT-C may exist in the tomato genome. The markers mapping to the introgressions in IL4-2/4-3, IL7-2/7-4, IL7-4 and IL8-1/8-2 corresponded well to the map locations of the classical *Got-1, Got-2, Got-3* and *Got-4* isozyme markers.

Discussion

In tomato, many isozymes have been exploited as biochemical markers to construct genetic maps and assist selection in breeding programs. For example, acid phosphatase (Aps-1) proved to be a powerful and reliable screening tool for nematode resistance conferred by the *Mi* gene (Gunther et al. 1988). Focusing on GOT, we have shown that isozyme markers can be developed into simple, reliable DNA markers through mapping sequences annotated with the same enzymatic function as the isozyme marker. The positions of several GOT markers correlate well with the positions of the known *Got* genes on chromosomes *4*, *7* and *8*. Four *Got* loci have been placed on three chromosomes in the classical genetic map of tomato; *Got-1* on chromosome *4*, *Got-2* and *Got-3* on chromosome *7*, and *Got-4* on chromosome *8* (Tanksley et al. 1992). GOT-E based on U216856 mapped to chromosome *4* in the same region as *Got-1* suggesting that U216856 probably corresponds to *Got-1*. Similarly, GOT-B based on U215965 mapped to chromosome *7* in the same region as *Got-2*, suggesting that U215965 probably corresponds to *Got-2*. The *Got-2* isozyme marker is linked tightly to *I-3* (Bournival et al. 1989), a gene for resistance to Fusarium wilt, and has been used for the marker-assisted breeding of *I-3*. In a separate study, we have mapped GOT-B 0.15 cM north of *I-3* (data not shown), strengthening the conclusion that U215965 corresponds to *Got-2*. The GOT-B marker, therefore, provides a useful alternative to *Got-2* isozyme analysis for the marker-assisted selection of lines carrying *I-3* in tomato breeding. The GOT-C SSR marker, derived from a BAC containing sequences identical to U216620, mapped to the same region of chromosome *7* as *Got-3*,

Table 1 Markers developed from SGN sequences related to glutamate oxaloacetate transaminases

Marker name	Sequence ID	Primer sequence (5′ – 3′)	Annealing temperature (°C)[a]	Marker type[b]	IL position	Corresp. isozyme marker	Band sizes (bp)[c]
GOT-A	U214922	F AGGATAAAGAGTGTGAGACAGAAGC R AGAAATTGTCAAAATCTGCTTCATAC	55	CAPS *Dra*I	8-1	*Got-4*	Le: ~580 + 170 Lp: ~580 + 174
GOT-B	U215965	F AGTGGCAGTGAAAAGTCAGTTG R CCAAGTAACCAACATTTCCAGTAG	55	CAPS *Hpa*II	7-2/7-4	*Got-2*	Le: ~440 + 210 Lp: ~650
GOT-C	U216620	F CTCATGTTGTTCGTGCTCCTG R CTCGCGTTGCTGGGTCATAG	45	SCAR	2-4/2-5		Le: ~170 Lp: ~180
				CAPS *Dra*I	11-2/11-3		Le: ~310 Lp: ~195 + 95 + 20
	SL_MboI 0121F21_T7	F TAATCATAATGTTCGATTAACAGTG R GGAGTAAAAAGAACGGAGACATAG	55	SSR $(AT)_9(AC)_4$	7-4	*Got-3*	Le: 206 (~310) Lp: ~216 (~320 + 170 + 110)
GOT-D	U221060	F AGAGATGATAAAGGAAAACCAGTAAC R TATGAGCGCAAGGATGAAGTAAG	55	CAPS *Hha*I	10-1		Le: ~900 + 620 + 300 + 280 Lp: ~900 + 620 + 580
GOT-E	U216856	F GCAGTTTGTTCTCCAGGGG R GCTGGTATGAGTTGCTGGTG	55	CAPS *Hin*dIII	4-2/4-3	*Got-1*	Le: ~600 + 300 + 200 Lp: ~800 + 300
GOT-F	U213889	F TCACTGGGTTGGCAGACTTC R ATTTTTGTGTGGTTTCCCC	55	CAPS *Nde*I	8-2		Le: ~530 + 410 Lp: ~940
GOT-G	U213889	F GGATTGCTACCCTTCTTTGAT R TACAAGTTTCTGTCTTTGAGGAT	55	CAPS *Hin*fI	8-2		Le: ~400 + 210 + 170 + 90 Lp: ~400 + 390 + 80
GOT-H	U213889	F ACTAAAATGCACACACAACAATCAC R CCAACTCCCAAGTTCAACTTC	50	SCAR	12-2		Le: no band Lp: ~130
				CAPS *Taq*I	8-2		Le: ~400 + 380 Lp:~390 + 390
GOT-I	U221509	F TTTGTAAGGAAGCGGGATGT R GACACTTCCATCATCCATTTA	55	CAPS *Apo*I	3-5		Le: ~900 + 560 + 340 + 210 Lp: ~900 + 800 + 570 + 550 + 340

[a] PCR reactions were performed in a PTC-200 Peltier Thermal Cycler using 10 µl PCR reaction mixtures each containing 1 × PCR buffer (SIGMA), about 25 ng genomic DNA, 0.2 mM of each dNTP, 0.2 µM of each primer and 0.5 U REDTaq (SIGMA). The PCR amplification conditions comprised one cycle of 94 °C for 5 min; 35 cycles of 94°C for 1 min, 1 min at the annealing temperature specific for the primer combination and 72°C for 1 min; and one cycle of 72°C for 5 min

[b] The restriction enzymes used to generate the CAPS markers and the repeat sequence underlying the SSR marker are also indicated

[c] Le = *Lycopersicon esculentum* cv. M82; Lp = *Lycopersicon pennellii* LA716

Table 2 Correspondence between glutamate oxaloacetate transaminase (*Got*) genes in tomato and aspartate aminotransferase (*Asp*) genes in Arabidopsis

Tomato				Arabidopsis		
Name	Unigene	Chr.	Predicted Localization[a]	Name	AGI number	Localization[b]
Got-1	U216856	*4*	Chloroplasts[c]	–	At2g22250	Chloroplasts
Got-2	U215965	*7*	Mitochondria	*Asp1*	At2g30970	Mitochondria
Got-3	U216620	*7*	Cytosol	*Asp2*	At5g19550	Cytosol
Got-4	U214922	*8*	Chloroplasts[c]	*Asp5*	At4g31990	Chloroplasts
–	U213889	*8*	Peroxisomes	*Asp3*	At5g11520	Peroxisomes
–	–	–	–	*Asp4*	At1g62800	Cytosol

Predicted orthologues are shown on the same row of the table

[a] Localization predicted by TargetP and Predotar (http://au.expasy.org/tools/) except for U213889, which is predicted to be targeted to the peroxisome because it carries the type 2 peroxisome targeting signal R(IL)xxxxxHL (Reumann 2004)

[b] Localization according to Liepman and Olsen (2004) except for At2g22250, which is predicted to be targeted to the chloroplast by TargetP and Predotar (http://au.expasy.org/tools/)

[c] Moore and Sink (1988) report Got-1 and Got-4 isozyme activities in chloroplast fractions

suggesting that U216620 probably corresponds to *Got-3*. GOT-A based on U214922, and GOT-F, GOT-G and GOT-H-CAPS based on U213889, all mapped to chromosome *8* in the same region as *Got-4*, but the position of *Got-4* relative to the IL has been approximated from a different mapping population, so it was not possible to determine from the available positional data whether U214922 or U213889 more likely corresponded to *Got-4*. However, Got-4 isozyme activity has been localised to the chloroplast (Moore and Sink 1988) and U214922 encodes a protein with a chloroplast transit peptide, whereas U213889 encodes a protein with a peroxisomal targeting signal (Table 2). This suggests that GOT-A (U214922) probably corresponds to *Got-4*.

Reciprocal blast searches indicate that tomato U216856 (*Got-1*), U215965 (*Got-2*) and U214922 (*Got-4*) are probably orthologues of Arabidopsis At2g22250, *Asp1* (At2g30970) and *Asp5*(At4g31990), respectively, (Tables 2 and 3). At2g22250 has not hitherto been clearly identified as an *Asp* gene, although Liepman and Olsen (2004) note that it encodes a protein with a high degree of sequence similarity to an aspartate aminotransferase from *Thermus thermophilus*. Our data suggest that At2g22250 does indeed encode an aspartate aminotransferase. Similarly, tomato unigenes U216620 (*Got-3*) and U213889 are highly homologous to *Asp2* (At5g19550), *Asp3* (At5g11520) and *Asp4* (At1g62800) suggesting additional orthologous relationships

Table 3 Amino acid identity between proteins encoded by glutamate oxaloacetate transaminase (*Got*) genes in tomato and aspartate aminotransferase (*Asp*) genes in Arabidopsis

		Arabidopsis					
		At2g22250	At2g30970	At5g19550	At4g31990	At5g11520	At1g62800
Tomato[a]	U216856	**82%**	ND	ND	ND	ND	ND
	U215965	ND	**84%**	49%	47%	51%	48%
	U216620	ND	53%	**84%**	54%	81%	74%
	U214922	ND	48%	54%	**81%**	52%	51%
	U213889[b]	ND	52%	83%	56%	**86%**	73%

Predicted orthologous relationships are highlighted in bold type

[a] Blastp searches of the Arabidopsis protein database were carried out using the SGN blast search tool (http://www.sgn.cornell.edu/tools/blast/) with translations of the tomato unigenes as query sequences. Similar identities were obtained using Arabidopsis proteins as query sequences in tblastn searches of the SGN tomato unigene database. ND = no detectable homology

[b] The blastp search was carried out with the first 50 amino acids containing the peroxisome targeting signal removed

(Table 3). The proteins encoded by U216620 and U213889 show highest matches to the proteins encoded by *Asp2* and *Asp3*, respectively, in reciprocal blast searches and neither matches the protein encoded by *Asp4* as well as they do those encoded by *Asp2* and *Asp3* (Table 3). Moreover, the proteins encoded by U213889 and *Asp3* have 44 and 46 amino acid extensions at their amino termini compared to those encoded by U216620 and *Asp2*, respectively. These amino terminal extensions are homologous to one another and contain the type 2 peroxisomal targeting signal R(IL)xxxxxHL (Reumann 2004). This suggests that U216620 and U213889 are probably orthologues of *Asp2* and *Asp3*, respectively, (Tables 2 and 3). These correspondences to functional aspartate aminotransferase genes in Arabidopsis suggest that the tomato genome harbours at least two additional *Got* loci corresponding to *Asp3* and *Asp4*. However, the tomato genome may harbour up to four additional *Got* loci corresponding to the GOT-C (U216620) and GOT-H (U213889) SCAR and CAPS markers. It is possible that one of the GOT-C (U216620) or GOT-H (U213889) markers corresponds to *Asp4*. However, it remains uncertain whether the GOT-D and GOT-I markers correspond to *Got* genes or genes encoding aminotransferases with different substrate specificity. Nevertheless, development of these markers has enabled functional genomic comparisons to be made between tomato and Arabidopsis with functional implications about homologous sequences in both genomes. One interesting outcome of this comparison is the observation that the tomato *Got-1* and Arabidopsis At2g22250 genes appear to be unrelated to the other tomato *Got* or Arabidopsis *Asp* genes (Table 3). Rather than suggesting convergent evolution, this observation might instead indicate that the Got isozyme assay does not discriminate between differences in aminotransferase function between Got-1 and Got-4, which are both located in the chloroplast, and/or that *Got-1* is a gene of endosymbiotic origin.

The development of PCR-based markers from multigene families, exemplified here by the development of 12 new PCR-based markers from the tomato GOT gene family, has the potential to complement PCR-based markers based on unique gene sequences, because many genes are members of multigene families e.g., 65% of the genes in Arabidopsis are members of multigene families (Arabidopsis Genome Initiative 2000), and unique gene sequences may therefore, be distributed sparsely in some regions of the plant genome. The conversion of old isozyme markers into new PCR-based markers will also enable better integration of classical genetic mapping data based on isozyme and morphological markers into recent genetic maps based on molecular markers. Moreover, markers developed from multigene families have the potential to assist genome-sequencing projects by anchoring BAC ends to individual chromosomes, which might otherwise be problematic for BAC ends containing members of multigene families e.g., the Got-3 SSR marker anchors BAC clone SL_MboI0121F21 to tomato chromosome *7*, which may be useful for the tomato chromosome *7* sequencing project.

The Got isozymes have been useful genetic markers in tomato because of the variation in electrophoretic mobility they display between tomato and its wild relatives. Although not necessarily amplifying the coding subregions responsible for these polymorphisms, the intron-spanning SCAR and CAPS markers that we have developed are also likely to detect variation between related tomato species and may be useful in introgression breeding in tomato e.g., in the introduction of new alleles of the *I-3* gene for Fusarium wilt resistance that have novel resistance specificities. Given that SSR markers are often used to reveal polymorphisms between tomato varieties, the GOT-C SSR marker may also be useful for intra-specific breeding in addition to introgression breeding. As with any marker, the GOT markers reported here will need to be adapted to the specific objectives of the tomato breeder. The GOT markers serve to anchor their associated unigene sequences to specific subregions of the tomato genome and these in turn can be used to underpin the development of new markers tailor-made for marker-assisted breeding of traits of interest in these subregions.

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