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Expressed sequence markers for genetic analysis of bulb onion (*Allium cepa* L.)

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Abstract Sequencing of cDNA clones previously screened for ability to reveal RFLPs in bulb onion has been completed and a further 128 ESTs from 111 clones have been deposited in public databases. A putative function was assigned to 66% (84/128) of ESTs by BLASTX searches against public databases and FASTA comparisons were used to determine similarity among clones, including those which detected linked RFLP loci. Cleavage amplified polymorphisms (CAPs) and single-stranded conformation polymorphisms (SSCP) were evaluated as strategies for converting onion expressed sequence tags (ESTs) into PCR-based assays for gene mapping. We screened 14 ESTs with 8 to 12 restriction enzymes and detected two CAPs, which mapped in the 'Brigham Yellow Globe' (BYG15–23)×'Ailsa Craig' (AC43) mapping population. A wider survey of CAPs for ESTs among eight bulb onion populations with six frequently cutting restriction enzymes detected variation, but too little to be practical for routine gene mapping. By contrast, non-radioactive SSCP of amplicons from 3′ UTRs of ESTs was found to detect useful levels of variation within bulb onion germplasm. In addition to SSCPs, homo- and hetero-duplex polymorphisms (duplex polymorphisms) were also frequently observed on the same gels. Of a total of 31 ESTs surveyed, 26 exhibited SSCP/duplex variation among bulb onion populations. SSCP/duplex polymorphisms in 11 ESTs were mapped in the 'BYG15–23'×'AC43' family and, of these, ten were linked to an RFLP locus revealed by the original cDNA. The SSCP/duplex assays of five additional ESTs showed Mendelian segregations in the 'Colossal Grano'×'Pukekohe Longkeeper' (P12) F_2 population. Two of these markers were linked, as predicted from linkage of their corresponding RFLPs in the 'BYG15–23'×'AC43' family. Ninety two percent (12/13) of EST PCR products that amplified in *Allium roylei* exhibited marked differences in SSCP patterns from bulb onion. ESTs for invertase and sucrose-sucrose fructosyltransferase were mapped by SSCP and an ATP sulfurylase gene cloned by RT-PCR revealed SSCP/duplex polymorphism within bulb onion. These results demonstrate that SSCP/duplex is an efficient and economical technique for exploiting onion EST information for gene mapping in onion.

Keywords Expressed sequence tag · Single-stranded conformation polymorphisms · Heteroduplex analysis · *Allium cepa*

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Introduction

The sequences of cDNAs from economically significant and model plant species are accumulating at a massive rate and are now a key resource for gene discovery and mapping (Marra 1998; Somerville 1999). A major challenge in minor crops with complex genomes is how best to use these growing numbers of expressed sequence tags (ESTs) as markers for genetic mapping. The advantages of EST markers are that the resulting transcriptional map provides a preliminary description of the organisation of expressed genes (Jermstad et al. 1998) and insights about genome evolution. By combining transcriptional maps with marker techniques that target other genomic regions, notably transposable elements (Ellis et al. 1998), a clearer picture of plant genome structure and evolution should emerge.

Onion is an outcrossing, biennial diploid (2n=2*x*=16) plant with a very large (32 pg/2 n) genome and a low GC

content (Jones 1990). It is the second most-valuable vegetable in the world, following only tomato. Despite progress in other crops, there is relatively little public DNA sequence information for onion and other *Allium* vegetables. We previously developed a genetic linkage map for bulb onion based primarily on RFLP (King et al. 1998a) and sequenced some of the cDNA clones revealing RFLPs. RFLP mapping revealed duplications and the highest level of dominant RFLPs yet reported in plants, suggesting that gene duplication may contribute to genome size increases in onion.

Although RFLPs are valuable for elucidating gene family structure and genomic distribution they are not practical for routine mapping or breeding of onion. In particular the large genome size makes RFLPs technically demanding, requires substantial quantities of good quality genomic DNA and uses radioactivity. The RFLP patterns detected by most cDNAs in onion and other plants with large diploid genomes such as pine (Devey et al. 1999) are relatively complex. Polymorphisms can be in pseudogenes as well as expressed sequences. To date PCR-based markers used in *Allium* species include AFLPs (van Heusden et al. 2000a), SSRs (Fischer and Bachmann 2000) and RAPDs (Bradeen and Havey 1995a). Van Heusden et al. (2000b) described the conversion of onion cDNA sequences to CAPs for mapping in an interspecific cross of *Allium cepa*×*Allium roylei*. Identifying efficient strategies for conversion of cDNA sequences to PCR-based assays for genetic analysis in *Allium* species is desirable to facilitate exploitation of the onion RFLP framework map and other *Allium* EST data. In large genomes, such as onion and pine, ESTs will be particularly important for QTL mapping, because markers such as AFLPs, SSRs and RAPDs may predominantly target non-coding regions. However there are some important considerations that must be addressed in developing PCR-based EST markers in large plant genomes where duplication and/or polyploidy complicate interpretation.

Due to the practical difficulties involved in developing sufficiently large onion mapping populations, the use of candidate-gene and other cDNA-based markers to target gene-rich regions of the genome will be a vital component of any QTL mapping strategy in onion. The biochemical pathways that produce the distinctive carbohydrate and sulfur components of onions and other edible *Allium* species are now becoming better understood at the molecular level (Hell 1997), although there are still several steps in the biosynthesis of flavour precursors that are poorly characterised (Lancaster and Boland 1988). The use of sequences encoding genes in these pathways offers one strategy to develop markers for candidate genes that may condition the genetic variation for important bulb traits.

Here, we describe the sequencing of cDNAs revealing RFLPs in bulb onion germplasm. We developed PCR-based CAPs (cleaved amplified polymorphisms) and SSCPs (single-stranded conformation polymorphisms) to provide more convenient markers for mapping and to complement AFLP, SSR, and other PCR-based markers. We also report preliminary attempts to map candidate genes of bulb quality.

Material and methods

Plant material and DNA isolation

The origin and generation of the segregating family (designated BxA) from the cross between lines 'Brigham Yellow Globe (BYG) 15–23´ and 'Ailsa Craig (AC) 43´ have been described previously (Bradeen and Havey 1995a; King et al. 1998a). Genomic DNA from F_3 massed families was purified by CsCl gradient centrifugation (King et al. 1998a).

An F_2 family (designated C×P12) was obtained from the cross between plants from open-pollinated 'Pukekohe Longkeeper (P12)' and 'Colossal Grano' (Sunseeds). A single hybrid bulb was self-pollinated using blowflies. Individual F_2 plants were grown hydroponically under high (4.0 meq l^{-1}) S fertility in a greenhouse (Randle 1992). Leaf tissue (1–1.5 g) was collected from young plants in 100 mm×120 mm polythene bags, crushed with a roller and DNA was extracted with hot CTAB buffer (Timmerman et al. 1993).

Seed of the bulb onion cultivar 'Jumbo' and of *A. roylei* (van Heusden et al. 2000a) were kindly provided by A.W. van Heusden (Plant Research International, Netherlands). Other onion lines surveyed were the male sterile inbred W202 A (Goldman 1996) and three open-pollinated populations from the Crop and Food Research onion breeding programme: 'Texas Grano 482', 'Gladallan Brown' and 'Heian Keykei'. Seed was germinated in the dark and DNA isolated from seedlings by CTAB extraction (Timmerman et al. 1993).

cDNA preparation and sequencing

cDNA libraries

Two cDNA libraries used to identify RFLPs were described previously (King et al. 1998a). The first was synthesised from unknown tissues and cloned into an unknown pUC-like vector by the former Native Plants, Inc. (NPI), Salt Lake City, Utah, and provided by Pioneer Hi-Bred Inc. (Johnston, Iowa). Eighty four of these clones (designated with the prefix API) revealed RFLPs among bulb-onion germplasms, of which 32 revealed RFLPs segregating in B×A (King et al. 1998b). A further eight clones were provided by Gilroy Foods (Gilroy, Calif.) and given the prefix AGI. The second library was constructed in lambda GT-10 from seedling-leaf RNA (clones designated with AOB/AJB/AJK prefixes; Bark and Havey 1995). A third cDNA library previously prepared in Lambda-Zap II from sprouting onion bulb RNA (Clark et al. 1998) was subjected to mass excision and clones from this were given the prefix OJ.

Cloning of ATP sulfurylase

Total RNA was isolated from roots of bulb onion cv 'Pukekohe Longkeeper' grown hydroponically under low (0.25 meq $l^{-1)}$ S fertility (Randle 1992). RT reactions (40 µl) were performed at 42°C for 45 min in the presence of 2 µg of total RNA, 1×first-strand buffer as supplied by the manufacturer, dNTPs to 1 mM each, 100 µM of Oligo$DT_{12–18}$ primer, 1 mM of DTT, 40 units of RNasin (Promega) and 200 units of SuperScript II (Gibco-BRL). The primers ATP-SULF5´ (5´CTGAGAAATCCTGTNCAYAAYGG 3´) and ATP-SULF3´ (5´TGCGTCTTGTCRTANGCNGC 3´) were designed from highly conserved regions of the amino-acid sequence of plant ATP sulfurylases (Bolchi et al. 1999) using the COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) strategy (Rose et al. 1998). Touchdown PCR was

performed over 50 cycles of 94°C for 30 s, 60°C for 30 s reducing to 50°C over 20 cycles, and 72°C for 45 s. A 450-bp PCR product was cloned, sequenced and used to screen a bulb onion root cDNA library as previously described (Lancaster et al. 2000).

cDNA sequencing

Plasmid vectors were purified using QIAprep Spin Miniprep Kits (QIAGEN). Inserts from lambda vectors were PCR-amplified and purified using Qiagen PCR Spin Purification Kits (Qiagen). The cDNA inserts were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer-Applied Biosystems) with appropriate primers, purified by ethanol-precipitation and analysed on the ABI377 Sequencer.

Sequences were edited using Sequencer 3.0 (Gene Codes) and homology searches were carried out using BLASTX (Altschul et al. 1990). For assessment of redundancy in libraries used for RFLP mapping, sequences were individually compared against a library consisting of all existing Genbank onion sequences and all new sequences not yet submitted using FASTA3 (Pearson 1990). Sequences were submitted to GENBANK EST databases.

PCR primer design and conditions

Primers were designed using 'Primer 3' software (Rozen et al. 1998). Initially primer pairs were chosen to amplify products of 500 bp or longer for CAPs assays using the program default conditions. For SSCP, design focussed on the 3´ UTR region of cDNAs. A 3´ primer with a T_m of 55°C to 60°C and up to 28 bp in length was designed manually as close to the poly-A tail as possible. 'Primer 3' was then used to design a compatible primer in the 3´ coding region of the cDNA to give a PCR product of 200–400 bp. The primer sets and amplification conditions are given in Table 1.

PCR reactions were performed in 96-well polycarbonate plates in a total volume of 15 µl. Reactions contained 0.1 µM of primers, 200 µM of dNTPs, 10–30 ng of template DNA, 0.6 U of *Taq* polymerase (Roche) in the manufacturer's 1×reaction buffer containing 1.5 mM of Mg^{++} and with addition of further Mg^{++} for some primer sets. Cycling was performed under mineral oil on a Hybaid Omnigene PCR machine. Cycling conditions were 3 min at 95°C followed by 40 cycles of 1 min 95°C/1 min of 55 or 60°C/1 min, 72°C. During optimisation, 5 µl of PCR reactions were analysed by electrophoresis on 2% NuSieve (FMC BioProducts) agarose gels and stained with ethidium bromide.

SSCP analysis

Denaturation of PCR products was performed in 48-well 20 µl polypropylene plates (Nunc GeneModule cat#232298). The PCR reaction (2 µl) was mixed with 9 µl of SSCP buffer (95% formamide, 0.02 M NaOH, 0.05% bromophenol blue), sealed with tape, heated at 95°C for 4 min on a Hybaid Omnigene in situ hybridisation block and chilled on ice/water slurry (Fukuoka 1994; Nataraj et al. 1999). Gel electrophoresis was carried out in a Bio-Rad Protean II apparatus on 16-cm long 1-mm thick 6% acrylamide gels cross-linked with 1:15 piperazine diacrylamide (PDA; BioRad) in 0.6×TBE running buffer. Use of notched spacer plates permitted analysis of 96 samples at a time. Gels were equilibrated and pre-run at 80 V for 30 min in a 4°C cold room. A double-stranded DNA marker (1-kb DNA ladder Life Technologies; in a non-denaturing glycerol buffer containing 0.05% xylene cyanole) and denatured samples were loaded at room temperature using a 10-µl Hamilton micro syringe. Gels were returned to the cold room and run overnight at 4°C with 80 V constant current until xylene cyanole dye had run 16 cm or more. Gels were then stained in 1:10,000 Sybr Green II dye (Roche) in 0.6×TBE and visualised on a UV transilluminator.

CAPs-analysis

PCR products (10 µl) were mixed in 48-well 20 µl polypropylene plates with 0.25 µl of restriction enzyme diluted to 5 µl in 1×PCR buffer and sealed with tape. Plates were incubated at 37°C for 2 h. Restriction products were fractionated on 3% NuSieve (FMC BioProducts) agarose gels in 1×TBE at 100 V and stained with ethidium bromide.

Segregation analysis

Segregation of markers was confirmed in subsets of 12 progeny from B×A and/or C×P12 populations and then scored for 46 segregating progeny from each population. Goodness of fit to expected F_2 segregation ratios and linkage analysis were performed using Joinmap 2.0 software (Stam and van Ooijen 1995).

Results

cDNA sequencing and library composition

Sequencing of all clones detecting RFLP in bulb onion (Bark et al. 1994; Bradeen et al. 1995b; King et al. 1998a, b) revealed significant redundancy, as expected when using non-normalised libraries. Representatives of highly expressed gene families including chlorophyll A/B binding proteins were common in both libraries, indicating that the NPI library was also prepared from leaf tissue (Table 2). However 35% of the cDNAs showed no significant homology, nor did they or match *Arabidopsis* genes of unknown function. Sequence comparison by FASTA showed significant sequence similarity among clones of several gene families that revealed linked RFLPs (Table 3). Two pairs of elongation-factor clones (API15/API92 and AOB302/AOB151) and a pair of RuBISCO clones (API65/AOB210) that revealed linked RFLPs differed only in the length and number of bands detected. Other clones from such gene families were less similar (approximately 70% identity) suggesting that they represent distinct transcripts from divergent loci.

Development of PCR-based markers from ESTs

CAPs markers

We attempted to develop CAPs markers from mapped and randomly sequenced cDNAs. We amplified PCR fragments of 14 ESTs from 'AC43' and 'BYG15–23' and screened these with 8–10 restriction enzymes with four-base recognition sequences. No significant size variation was observed on agarose gels between PCR products from different lines, and polymorphism was detected in only two ESTs. The CAPs detected in API27 co-segregated with the original RFLP. The clone OJ4 (AA508907) is a thaumatin-like protein homologue and revealed 8–10 bands when used to probe Southern blots (data not shown). Thaumatin-like proteins detect high levels of RFLP in wheat (Mingeot and Jacquemin 1997). The locus detected by the OJ4 CAPs assay was linked to a pair of

Table 1 Expressed sequence tag (EST) primer sequences and the number of putative alleles detected among bulb-onion lines as estimated from variable SSCPs and duplex band number

EST name[a]	Genbank accesion number	EST homology	Forward and reverse primer sequences (5′–3′) and locations	Estimated number of *A. cepa* alleles	*A. cepa/roylei* polymorphism	PCR product size (bp)[b]	PCR conditions[c]
ACE6066	AJ006066	Sucrose sucrose 1-fructosyl-transferase	TGATTCAACGCGTGTTTTTC 3′ CDS AACACAGTAACAAATTCGACCATTC 3′ UTR	2	No	330	60
AJB37	AA451574	No match	TCCTATAACCCAGATTGAAGA ATAATGGAAACTGTGCAAGG	2	Did not amplify	320	55
AJK295	AA451599	ACC oxidase	TACAAAAGTGTGTGGCACCG 3′ CDS GCAAACCCAAACAGTTTTCC 3′ UTR	2	Yes	470	60
ALCLECTINA	L12171.1	Mannose-specific lectin	ACTGTCGATGGAGGCTCTGT 3′ CDS CAGAATGCTGGTGGTCATCTTATC 3′ UTR	3	Yes	274	60
ALCALLI	M98267	Alliinase	GCATGGGTGAAGTGTGAATG 3′ CDS GAAAGATACTCCTTACTACCATTGC 3′ UTR	1	Yes	337	60
AOB116	AA451588	Ubiquitin	GTTGGAGGATGGAAGAACTC 3′ CDS CCCATTATAAACATAAAACCATAC 3′ UTR	2	Yes	260	55
AOB151 AOB302	AA451586; AA451587	Elongation factor	TGTTGCTGTTGGGGTTATCA 3′ CDS GAAGACGGAATCGAAACAAAAC 3′ UTR	4	Did not amplify	319 +extra bands	60
AOB156	AA451565	Glutathione-S-transferase	TCATGTTGATTACGACAGACA 5′ UTR TGGGATTTGACTTCAGAAAC 5′ CDS	2	Yes	290	55
AOB162	AA451589	Histone	AGAGGAAAGGGCGGAAAAG 3′ CDS TGGAAAAACCAAATCGTTGA 3′ UTR	1	Yes	404	60
AOB249	AA451570	Alliinase	GGTATGGCCATCACACATTG 3′ CDS TGTCGTAGTTGTACCCAGACG 3′ CDS	3	Did not amplify	354 (500)	55 (2.5 mM Mg^{++})
AOB262 AOB186	AA451571;A A451571	Ubiquitin	GCTCATCTTTGCTGGGAAAC 3′ CDS AGAACCAGGTGGAGGGTTG 3′ UTR	1	Yes	316	60
API10	AA451543	Ubiquitin	GAAGGAGCAGTGGAGTCCTG 3′ CDS AGGGATACACTGGGTATAGGGAAG 3′ UTR	3	Yes	260	60
API14	AA451575	No match	AGTGTCCAAGCTGTCAAGTT 3′ CDS ATGACTTTTGTACTGGTCCG 3′ UTR	2	Yes	363 (800)	60
API18	AA451544;A A451545	Heat-shock protein	TGGCTGCACTGAATCAAGAA 3′ CDS CTGCTCAATAATCAATATCACC 3′ UTR	2	Yes	341	55
API21	AA451546	Match to ESTs	TCCTCCATCGACTCTCCAAC 3′ CDS GCAAAAGATCCAAAGCGAAG 3′ UTR	2	Yes	502	60
API27	AA451547	Match to ESTs	GACAAGACCATCACTAAGCTCT 3′ CDS AGTACTCCTCAGTCCTGCCT 3′ UTR	2	Not determined	360 (510)	55
API32	AA451548	No match	AAAACCAAATCGTTTGTGCC 3′ CDS AAACCAAAGCAGGCAATTAAC 3′ UTR	1	Yes	394	60
API40	AA451549	Tubulin	TTGCAGTTAACCTGATCCCC 3′ CDS CGTGACACCACAATTATCGC 3′ UTR	2	Did not amplify	787	60

Table 1 (continued)

EST name[a]	Genbank accesion number	EST homology	Forward and reverse primer sequences (5′–3′) and locations	Estimated number of *A. cepa* alleles	*A. cepa/roylei* polymorphism	PCR product size (bp)[b]	PCR conditions[c]
API43	AA451576	Proline and glycine-rich protein	GGAGGTGAAAAGGATGTGGAG 3′ CDS AGCAGCAGCAATAGGGTAGC 3′ UTR	3	Yes	552	60
API53	AA451579	No match	GATAAAAGTCTTGTTGCCTCA GAAAATGGAAATAACGTTGC	4	Yes	328+800	55
APi61	AA45180	RuBISCO small subunit	G/ATACTGGACAATGTGGAAGC 3′ CDS CTAAAAGAAAGCAACAATCTGAC 3′ UTR	3	Yes	316	55
API65 AOB210	AA451551; AA451552	RuBISCO small subunit	G/A TACTGGACAATGTGGAAGC 3′ CDS AAAGTTGCAACACTTGGAGT 3′ UTR	2	Not determined	301	55
API76	AA451555	High-affinity potassium transporter	AGAGCAAAGAGTGGTTCAAGC CATCATCAAAACTCTTCATCGAG	2	Yes	263	60
API86	AA451556	Aldolase	CGAGCACCATCAAGAAAT CATTCTTCATACCATGTCCA	2	Not determined	268	55
API89	AA451558	Invertase	GAGAGTTCGCTCAAGGAGGA CACTAATTACAATTTGATTTTCATC	2	Did not amplify	335	55 (4.5 mM mg^{++})
ATPS	AF212154	ATP sulfurylase	CCAAGATGAGATCCCTAGCAA 3′ CDS CTACAAACAAGTCTGCATTCCAC 3′ UTR	2	Did not amplify	312	55
OJ39	AA508912	Basic peroxidase	AACATCCTCGACAACAAGGG 3′ CDS AAACAAATCCAACATGATTATTCC 3′ UTR	2	Not determined	395	60
OJ4	AA508907	Thaumatin-like protein	AACCCTAGCCTTCTACAACAAATG 3′ CDS CGCATGTTAATAGCCAGATTGTAC 3′ CDS	2	Not determined	360	60
OJ8	U58207	Aquaporin	GTACACTGTTTATGCAACG 3′ CDS CTCCAAAACATGAAACCG 3′ UTR	1	No	450	60

[a] Where two EST names are given these clones varied only in length

[b] Size of PCR product expected from initial design is given, as well as observed size (in brackets) where this differed

[c] PCR conditions are given as annealing temperature and Mg^{++} concentration when differing from 1.5 mM

Table 2 Homology of ESTs from cDNA libraries used as source of probes for the onion RFLP map. ESTs from clones revealing mapped RFLPs are highlighted in bold. Unless shown, expectations for BLASTX matches were smaller than 10^{-6}. Clones sequenced earlier were described in King et al. (1998)

No match		Match to unknown genes		Photosynthetic proteins		
AGI156	BE205622	AJB31	BE205628	**AJB57R**	BE205631	Chlorophyll A/B binding protein
AJB45F	BE205629	**AOB107**	BE205646	**AOB105R**	BE205644	Chlorophyll A/B binding protein
AJB61	BE205632	AOB118	BE205648	AOB141	BE205652	Chlorophyll A/B binding protein
AOB114R	BE205647	AOB157R	BE205656	**AOB187**	BE205665	Chlorophyll A/B binding protein
AOB146	BE205653	AOB161	BE205660	AOB218	BE205668	Chlorophyll A/B binding protein
AOB15	BE205637	AOB191	BE205666	API24	BE205557	Chlorophyll A/B binding protein
AOB160F	BE205658	**AOB200**	BE205667	API34R	BE205566	Chlorophyll A/B binding protein
AOB165	BE205661	AOB256	BE205669	API35	BE205567	Chlorophyll A/B binding protein
AOB263	BE205671	AOB98	BE205643	API49	BE205580	Chlorophyll A/B binding protein
AOB271F	BE205672	API17F	BE205552	**API55F/R**	BE205583/4	Chlorophyll A/B binding protein
AOB76	BE205640	**API20**	BE205554	API58F	BE205588	Chlorophyll A/B binding protein
AOB8	BE205636	**API46**	BE205576	API74	BE205599	Chlorophyll A/B binding protein
AOB81	BE205641	API57R	BE205587	API93	BE205606	Chlorophyll A/B binding protein
AOB82	BE205642	API68	BE205595	API97	BE205608	Chlorophyll A/B binding protein
API11R	BE205547	API83F	BE205616	API99	BE205609	Chlorophyll A/B binding protein
API19F	BE205553	API90	BE205604	API82	BE205602	Ferredoxin precursor
API22	BE205555	API96	BE205607	AJB47	BE205630	RuBISCO small subunit
API25F/R	BE205558/9			**API62**	BE205592	RuBISCO chaperonin
API26R	BE205561			API33	BE205565	Plastocyanin
API36	BE205568			API69R	BE205597	Photosystem II 10-k protein
API39	BE205570			API52	BE205582	Photosystem I subunit X
API48R	BE205579			API50	BE205581	Photosystem II protein X
API57F	BE205586			API83R	BE205617	RuBISCO activase
API58R	BE205589			API56	BE205585	photosystem I chain XI
API80R	BE205615					
API85	BE205618					
Other Matches						
API87	BE205619	3-methyl-2-oxobutanoate hydroxy-methyl-transferase		API60	BE205591	Methionine aminopeptidase
API78	BE205612	6-phosphogluconolactonase		**API23**	BE205556	Nascent polypeptide associated complex alpha chain
API79F/R	BE205613/4	ACC oxidase		**API59**	BE205590	Nascent polypeptide associated complex alpha chain
API42F/R	BE205572/3	Alliinase		AOB130	BE205651	Pectinesterase
API48F	BE205578	Aluminum-induced protein		AGI106	BE205626	Peptidylprolyl isomerase
API84	BE205603	Aquaporin		AOB106	BE205645	Peptidylprolyl isomerase
API13	BE205549	Aquaporin		**API81**	BE205601	Peroxidase
AOB155	BE205655	Branched-chain amino acid aminotransferase		AGI128R/F	BE2056245	Phosphoglucomutase
API26F	BE205560	Catalase (4×10^{-5})		AOB122F	BE205649	Polyubiquitin
AOB182	BE205664	Catalase		AJB114R	BE205633	Ribosomal protein L27
AOB159	BE205657	Cysteine proteinase inhibitor		AOB169	BE205663	Ribosomal protein L34
API88	BE205620	DNA-binding protein		AGI101R	BE205627	Ribosomal protein P2b
API69F	BE205596	Elongation factor		API28	BE205562	Ribosomal protein S2
API15	BE205550	Elongation factor		AOB69	BE205639	Ribosomal protein S8
API92	BE205605	Elongation factor		API67R	BE205594	RNA helicase
API63F	BE213512	Elongation factor		API45	BE205575	S-adenosylmethionine decarboxylase
AOB64	BE205638	Glycine hydroxymethyltransferase		**API66**	BE205593	Sucrose transport protein
API16	BE205551	Glutaredoxin		AOB160R	BE205659	Thioredoxin (4×10^{-4})
API72	BE205598	Golgi transport protein		**API47**	BE205577	Triose-phosphate isomerase
API37	BE205569	Heat shock protein		**API63R**	BE213513	Triose phosphate translocator
AOB147	BE205654	Histone deacetylase		**AOB260**	BE205670	Trypsin inhibitor (3×10^{-3})
AOB167	BE205662	Lipid transfer protein		AGI151	BE205623	Ubiquinol-cytochrome *c* reductase
AGI178	BE205621	Isoaspartyl methyltransferase		**AJK267R**	BE205635	Ubiquinol-cytochrome-*c* reductase
API12	BE205548	Membrane protein		API44	BE205574	Ubiquitin-conjugating Enzyme
AOB122R	BE205650	Membrane protein		API77R/F	BE205610/11	Zinc-finger protein
API41	BE205571	Membrane protein		API75	BE205600	β-Glucosidase
AJK248F	BE205634	Metallothionein		**API31F/R**	BE205563/4	β-Xylosidase

Table 3 Selected pairwise comparisons of nucleotide and RFLP banding similarity for clones that detected linked RFLPs in the onion linkage map. Mapped clones showing low nucleotide similarity were not included

Gene family	EST and genbank accession #	RFLP linkage[a]	EST and genbank accession #	RFLP linkage[a]	Nucleotide % identity and overlap	RFLP banding similarity[b]
Chlorophyll A–B binding proteins	AOB213R AA451569	G/G	AJK265F AA451563	C/C	71% 456 nt	More bands in AJK265, some in common
			API55R BE205583/4	A	64% 196 nt	Different patterns
Ubiquitin	AOB262F AA451571	B/B	AOB186R AA451597	B	78% 329 nt	Identical patterns
			AOB116 AA451588	B	77% 321 nt	Some bands in common
Elongation factors	API92 BE205605	E	API15 BE205555	E	99% 887 nt	Identical patterns
			API63F BE213512	B	71% 449 nt	Different patterns
	AOB302 AA451573	A/G	AOB151F AA451587.1	A	99% 446 nt	More bands in AOB302
RuBISCO small subunits	API65–2 AA451552	B	AOB210R AA451567	A/B/I	99% 388 nt	Many bands revealed, more by API65
			API61 AA451580	A/J	77% 370nt	Many bands revealed, more by API65, some may be in common
Histones	AOB162R AA451589	A	AOB114 AA451601	U	74% 236 nt	AOB162 reveals many bands, AOB114 only one band. Difficult to say if they are the same.
			AOB77 A451590	A	78% 189 nt	Numerous bands of similar sizes revealed by both clones, more by AOB162.

[a] Linkage group designation as in King et al. (1998a)
[b] Details of RFLP screening in Bark and Havey (1995)

Table 4 Discrimination of eight onion populations by CAPs with five ESTs

EST	Restriction enzyme[a]	Populations[b]						
		'BYG15–23'	'Texas Grano 482'	'Pukekohe Long-keeper'	'Colossal Grano'	'Gladallan Brown'	W202A	'Heian Keykei'
OJ4	*Hinf*1	1	1	0	0	0	1	1
OJ4	*Alu*1	0	0	0	0	0	0	0
API21	*Mbo*II	0	0	0	1	0	1	0
OJ39	*Mbo*II	1	0	0	1	0	1	0
OJ39	*Rsa*1	0	1	0	1	1	1	1
API10	*Nla*III	1	0	0	0	0	0	0
API10	*Rsa*1	0	0	0	0	1	0	1
API43	*Mse*1	1	0	1	1	0	1	1
API43	*Dpn*II	1	0	1	1	0	1	1

[a] PCR products were digested with *Alu*1, *Ase*1, *Mnl*1, *Mbo*II, *Nla*III, *Rsa*1, *Mse*1 and *Dpn*II. Only informative enzymes are shown
[b] Observed restriction patterns were classified as putative alleles compared to 'AC43' pattern which was designated as '0'

RFLP loci detected by the clone API81. Although CAPs has been used successfully for other crop plants (Gilpin et al. 1997; Tragoonrung et al. 1992) similar inefficiency has been reported in pine by Plomion et al. (1999).

We attempted to improve marker-conversion efficiency by identifying better combinations of primer design strategy and restriction digestion. Where possible, primers pairs were chosen to amplify between the 3′ end of the coding sequence and across most of the 3′ UTR. Analysis of the composition of 3′- UTRs from 22 onion ESTs showed a 33% G+C content over 3,647 bp and relatively few sites for commonly used restriction enzymes. Enzymes that cut more frequently (*Mse*1 25 sites; *MnL*1 13 sites; M*bo*II 9 sites; *Nla*III 21 sites; *Rsa* 1 11 sites; *Dpn* I 7 sites) were surveyed over a wider range of onion germplasm with five ESTs. This indicated that although

some polymorphisms were present their frequencies were too low for mapping (Table 4). For an interspecific bulb onion×*A. roylei* cross, CAPs were revealed when PCR products were screened with up to 30 restriction enzymes (van Heusden 2000a; S. van Heusden, personal communication).

SSCP markers

A parallel survey carried out on the same lines showed that polymorphisms could be readily detected for most ESTs within bulb onion germplasm by a simple non-radioactive SSCP procedure. Polymorphisms in most ESTs were reproducibly resolved on 6% acrylamide gels run at 4°C without further optimisation of temperature and/or glycerol content (Fukuoka et al. 1994; Plomion et al. 1999). PCR products of 200 bp or less were run on 9% acrylamide gels. Superior resolution of SSCPs in low-GC% PCR products at 4°C was reported by (Nataraj et al. 1999; Plomion et al. 1999). Sybr Green II detection makes SSCP a practical marker for routine use as it provides much higher sensitivity than ethidium bromide without the complexity of silver staining. Similar results were obtained both with PCR products amplified from genomic mini-prep and CsCl-purified template DNAs, suggesting that the method is not unduly sensitive to template quality. SSCPs were successfully detected in PCR products of 200 to 800 bp, confirming that PCR product size is not a critical factor, as observed by (Plomion et al. 1999).

Out of 34 ESTs surveyed, 29 showed variation in banding among the eight bulb onion populations. Examples of banding variation among population samples are shown in Figs. 1 (a and b) and 2 (a-i, b-i, c-i). Single-stranded band number varied from the two to eight, corresponding to sense and antisense strands of one to four sequence variants. Reproducible and polymorphic dsDNA bands were also observed on SSCP gels of many ESTs. These included both slight mobility differences in homoduplex dsDNA bands and heteroduplex bands, with mobility intermediate between ssDNA and homoduplex bands. Markers based on resolution of polymorphic dsDNA bands on acrylamide gels have been termed 'duplex polymorphisms' by Hauser et al. (1998). Duplex polymorphisms complemented SSCP for identification of heterozygotes and/or homozygotes, and in some cases were more informative than the SSCPs (plate 1). The buffer and conditions used in this study for denaturation and resolution of PCR products were not optimised for heteroduplex formation and it is possible that better conditions could be identified for simultaneous SSCP/duplex analysis.

From 31 EST primer sets evaluated 19 showed clear banding differences between parental populations of B×A and/or C×P12. Three of these did not segregate in the F_2 populations. Ten of the eleven SSCP markers mapped to a single locus on or adjacent to the RFLPs detected by the original cDNA (Table 5). Most SSCP and duplex polymorphisms segregated in the same manner as the RFLPs, though some showed dominant segregations when the original RFLP was co-dominant. The primer set for the EST API53 amplified two fragments and showed a complex SSCP pattern that segregated as two closely linked loci with a single crossover. Six SSCPs for mapped cDNAs showed Mendelian segregation in the C×P12 F_2 population (Table 6). The SSCPs for the cDNAs API86 and API10, which revealed linked RFLPs on linkage group I in B×A, also showed linkage in CxP12 (0.17 recombination, LOD 4.72).

Some ESTs were also screened for polymorphisms with parents of the interspecific cross *A. cepa*×*A. roylei* (van Heusden et al. 2000a). Out of 16 primer sets screened, three did not amplify a product from *A. roylei*. Of the remaining 13 only one (aquaporin homologue OJ8) did not show major differences in SSCP patterns between *A. roylei* and *A. cepa* (plate 2). This indicates

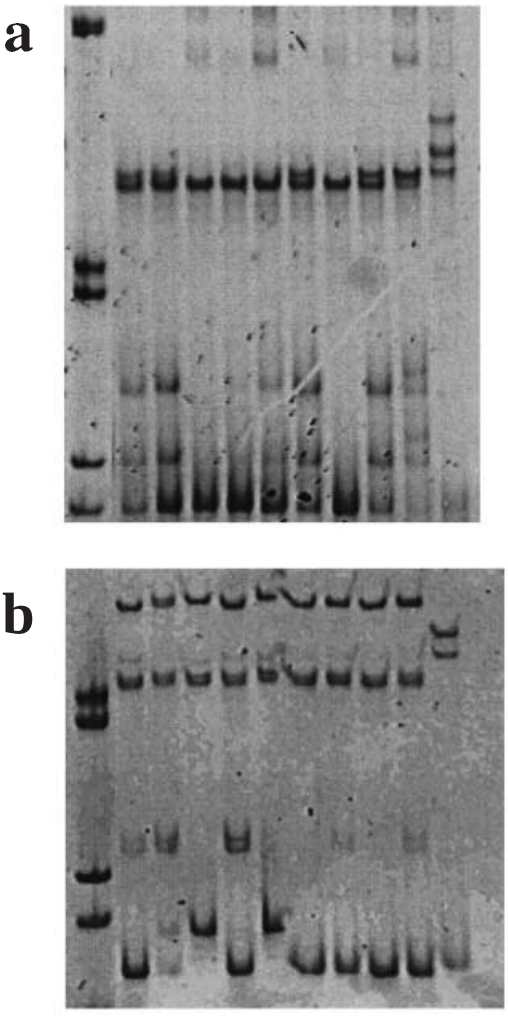

Fig. 1a, b SSCP/duplex banding variation among *A. cepa* populations (*lanes 2–10*) and *A. roylei* (*lane 11*) revealed by ESTs (**a**) ATP sulfurylase (AF212154) and (**b**) Glutathione-S-transferase AOB156 (AA451565). The order of populations in *lanes 2–10* is: 'ÁC43', BYG-15–23', 'Texas Grano 482', 'Pukekohe Longkeeper', 'Colossal Grano','Gladallan Brown', W202 A, 'Heian Keykei'. *Lane 1* is a double-stranded marker (doublet at 500 bp)

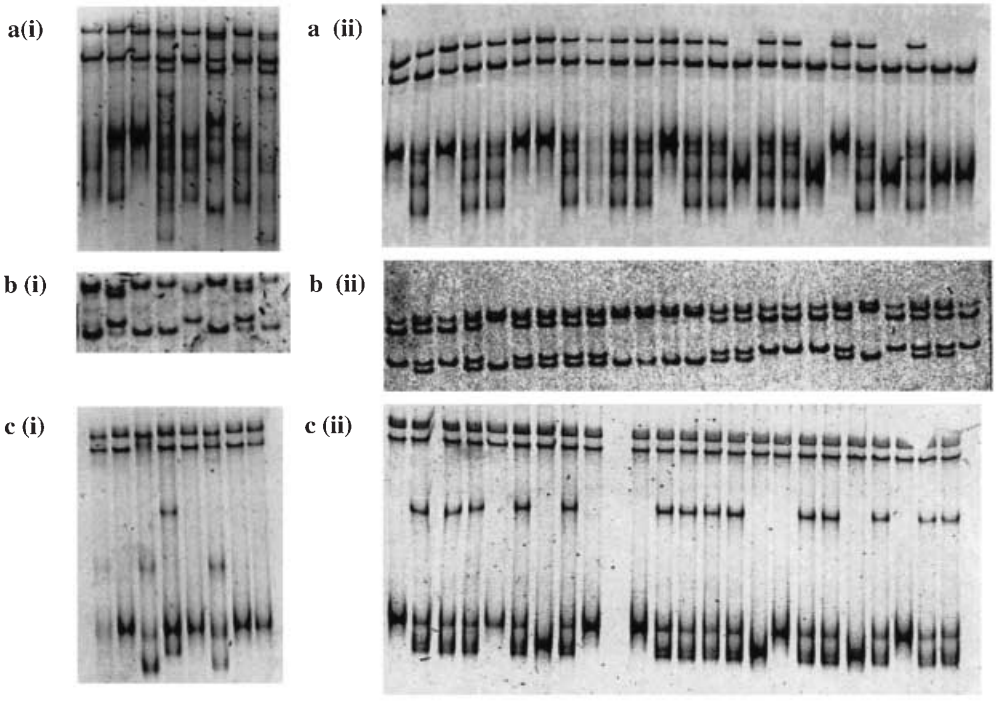

Fig. 2a–c Variation in SSCP/duplex banding revealed by the ESTs API10 (**a**), API86 (**b**) and API43 (**c**) among samples from open-pollinated onion populations (**i**) and between F_2 individuals from 'Colossal Grano'× 'Pukekohe Longkeeper' (**ii**). Order of populations in lanes of (**i**) as for Fig. 1 *lanes 2–10*

Table 5 Linkage of CAPs and SSCP/duplex markers with RFLP loci in the 'BYG15-23'×'AC43' family. The linkage group of the PCR-based assay is shown with the location of other RFLP loci revealed by the cDNA clone indicated in brackets

EST	Linkage groups[a]	Marker[a, b]	Recombination %	LOD	RFLP segregation mode	Segregation mode of PCR-based assays
API53	A(G)	API53J-E5–3/2.2	9.7	5.3	Dominant	Two tightly linked dominant SSCP
		AJK242-E5–9/3.5	4.1	7.2		
API18	A	API18H-E5	10.3	7.1	Co-dominant	Co-dominant SSCP/duplex
API61	A(J)	D12–0.53	7.0	7.2	Dominant	Dominant SSCP
		API61F-E1–3.0	0.0	1.95		
AJB37	B(U)	AJB37-E1–5.0	2.4	8.6	Co-dominant	Dominant SSCP
API27	B	API27-E5–20.0/7.0	0.0	17.9	Co-dominant	Co-dominant *Rsa*1 CAPS and dominant SSCP
API21	B	API21B-E5–4.5/4.1	0.0	18.4	Co-dominant	Co-dominant SSCP/duplex
ACE66	G	AB14–0.70	5.3	7.9	Not mapped	Co-dominant SSCP
OJ4	M	API81E-H3–6.7Z	2.3	9.2	Not mapped	Dominant *Hpa*II CAPS
AOB249	B(I/U)[c]	AOB116-E1–3/3.5	6.8	6.5	Both Dominant	Dominant SSCP
API76	H	API76H-E5–12/10	3.5	12.2	Co-dominant	Co-dominant SSCP/duplex
API14	F (F)	API14C-H3–4/4.5Z	0.0	8.1	Both co-dominant	Dominant SSCP
API89	D	API89-E1–5.0/4.3	2.3	14.4	Co-dominant	Codominant SSCP
API10	I	API10E-H3–7/8	0.0	17.7	Co-dominant	Dominant SSCP + co-dominant duplex

[a] Nomenclature of linkage groups and RFLP loci as King et al. (1998)
[b] Recombination between marker and RFLP detected by original cDNA is given, as well as other markers where these are more tightly linked
[c] SSCP assay mapped locus unlinked with RFLP loci

that SSCP will be an efficient means to map ESTs in interspecific *Allium* crosses.

SSCP markers for candidate genes

In addition to attempting PCR conversion of mapped cDNAs, an important goal was to assess the effectiveness of using other ESTs as genetic markers that had not been pre-selected for their ability to reveal RFLPs. Several candidate genes affecting bulb quality were identified by sequencing, selected from public databases, or cloned and evaluated as SSCP-based markers.

Table 6 Segregation of SSCP/duplex assays in the 'Pukekohe Longkeeper P12'×'Colossal Grano' F_2 population

EST	Linkage group[a]	SSCP/duplex segregation mode[b]	Segregation ratio	χ^2
AJK295	B	Co-dominant SSCP+duplex	8:26:12	1.48
API27	B	Dominant SSCP	16:47	0.15
API43	D/D	Co-dominant DSCP	14:21:10	0.91
API10	I	Dominant SSCP+co-dominant duplex	9:23:14	1.09
API86	I	Co-dominant SSCP	13:23:10	0.39
Lectin	Not previously mapped	Co-dominant SSCP+duplex	16:18:12	2.87

[a] Linkage group designation as in King et al. (1998)
[b] All RFLPs revealed by the original cDNAs exhibited co-dominant segregation

Sulfur metabolism

The cDNA clone AOB249 (AA451570) was isolated from a seedling leaf library and encodes an alliinase, the CS-lyases that act on alkyl cysteine sulfoxides to generate the characteristic odours of *Allium* species (Lancaster and Boland 1988). This gene has 55% amino-acid homology to the alliinase expressed in onion bulbs (Clark et al. 1998; Van Damme et al. 1992). We isolated the full-length gene from onion roots and completed characterisation of the biochemistry and expression of this novel alliinase (AF126049; Lancaster et al. 2000). We designed primers to amplify a segment of the coding region and mapped a dominant SSCP in B×A to linkage group B. This did not show linkage with either of the two RFLP loci revealed by AOB249 in B×A, in contrast to the co-segregation of other SSCP assays with their RFLP loci. AOB249 reveals six to seven bands on Southern blots and it is possible that some loci were not polymorphic for RFLP. Conversion of the bulb alliinase sequence (M98267) using the same strategy did not reveal polymorphism within *A. cepa*, although *A.cepa/A.roylei* polymorphism was observed. A further cDNA clone encoding a bulb-type alliinase was identified during sequencing but exhibited identical RFLPs to the alliinase clone (Van Damme et al. 1992) and was not pursued further. The cDNA AOB156 (GB AA451565) is homologous with type II glutathione-S-transferases (GSTs), a diverse gene family involved in xenobiotic de-toxification and secondary product biosynthesis in plants (Marrs 1996). GST-type activities are involved in the synthesis of S-methyl cysteine and related *Allium* flavour precursors (Maw 1982; Lancaster and Boland 1988). The assay for this EST revealed two SSCP alleles in *A. cepa*, clear *A.cepa*/*A.roylei* polymorphism and heteroduplex bands in populations exhibiting both SSCP alleles. The two mapping populations were each fixed for alternate alleles. We are currently cloning other GSTs from onion to develop a more-detailed understanding of this gene family in onion.

ATP sulfurylase catalyses the activation of sulfate during assimilation into cysteine (Hell 1997). We amplified a partial fragment of this gene from onion root RNA by an RT-PCR strategy based on homology with monocot sequences (Bolchi et al. 1999) and used this as a probe to isolate two full-length clones from a Liberia root cDNA. These encoded ORFs of 461 and 464 amino acids and showed 79% nucleotide and 77% amino-acid identity with the maize cDNA. An SSCP assay based on the 3´UTR of the gene indicated putative allelic variation within *A. cepa*, but it did not segregate in either population (Fig. 1a).

Carbohydrate biosynthesis

The clone API89 (AA451558) is highly homologous (>95% nucleotide identity) with invertases, which are involved in turnover of the carbohydrate polymers (fructans) that compose the dry matter of onion bulbs (Darbyshire and Steer 1986). This EST was successfully converted to an SSCP which mapped in B×A to the same location on linkage group D where the RFLP API89-E1–5.0/4.3 also mapped. API89 is a partial clone of the full-length cDNA Act1 (GB AJ006067) isolated by Vijn et al. (1997, 1998). The sequence of the sucrose-sucrose 1-fructosyltransferase (1-SST) clone ACN2 (GB AJ006067) isolated by these workers was used to design an SSCP assay that showed linkage in B×A to markers on linkage group G (Table 5).

Other genes

Aquaporins are a class of membrane proteins involved in dessication tolerance and may play a role in the water relations of bulb tissues. Aquaporin homologs were identified by sequencing from a sprouting bulb library (OJ8) and in libraries used for mapping (API31, API41, API84). The OJ8 SSCP assay did not reveal any genetic variation among the lines screened. Lectins are a distictive and abundant component of *Allium* tissues. The sequence of a mannose-specific lectin (L12171.1) that is a member of a gene family of 4–6 members (Van Damme et al. 1992) was used to develop an SSCP assay that showed Mendelian segregation in the C×P12 population.

Discussion

A concern during construction of the onion RFLP map was the risk of obtaining a biased model of genome organisation by preferentially selecting tandemly duplicated clones that gave stronger signals on autoradiograms. Sequencing and comparison of homology among the

clones employed in previous studies does not suggest that the sample was markedly biased in favour of housekeeping genes. The patterns of gene-family complexity and duplication revealed by RFLP mapping in onion are similar to those in pines (Kinlaw and Neale 1997), suggesting that gene-family amplification is associated with genome enlargement in angiosperms and gymnosperms.

Our results demonstrate that SSCP is a practical means to convert onion ESTs into PCR-based markers that reveal genetic variation among commercial bulb onion germplasm. By contrast CAPs were inefficient for detecting variation. The levels of SSCP variation observed among the set of lines surveyed suggests that it is practical for mapping candidate genes and anchor loci in *Allium.* However, this study was focussed primarily on a sample of cDNAs already shown to detect RFLPs in onion. The efficiency of SSCP as a means to convert ESTs into markers was also reported for zebrafish (Fornzler et al. 1998) and rice (Fukuoka et al. 1994). Our confirmation of the practicality of SSCP and the impracticality of CAPs agrees with Plomion et al. (1999).

SSCP and duplex analysis are practical for low- to moderate-throughput EST-based genotyping because they do not rely on specialised equipment or knowledge of allele sequence variation. They are a useful complement to single nucleotide polymorphisms (Alcala et al. 1997) and other allele-specific PCR assays that require allele sequence data and optimisation but are more suitable for high-throughput applications. During development of allele-specific assays for complex genomes of outcrossing species such as onion, SSCP could provide a means to analyse PCR-products so that lines or individuals with distinct, putatively homozygous genotypes can be selected for allele sequencing. Alleles could also be resolved by SSCP from heterozygous samples and sequenced individually.

Experience with SSCP has shown that, by use of multiple conditions, over 90% of mutations can be detected in a given sequence, and when combined with heteroduplex analysis this can approach 100% (Nataraj 1999). In similar studies where plant cDNA and genomic sequences have been used to develop SSCP markers, genotyping has been based solely on SSCP (Bodenes et al. 1996;Slabaugh et al. 1997;Plomion et al. 1999), in contrast to the combination of SSCP and DSCP we have employed. Our novel observation of frequent and informative duplex polymorphisms on SSCP gels may be due to our use of shorter gel runs, a higher concentration of PCR product and SYBR Green II for detection. In a highly heterozygous species such as onion the ability to identify heterozygous populations or individuals is a valuable feature of these assays. The combination of SSCP and duplex analysis also increases the probability of resolving products of different loci, which is critical for mapping in the onion genome where duplications are common (King et al. 1998a). Failure to resolve such products could lead to spurious segregation data. This is of particular concern in *Allium* mapping, where family sizes are relatively small.

The molecular bases of SSCPs are point mutations and short insertion-deletions (Bodenes et al. 1996). The number of bands in an SSCP/duplex assay is a function of the number of loci and alleles amplified by the PCR, the frequency of such mutations in these sequences and how well they can be resolved by the electrophoretic system. We would expect that some dominant SSCP/duplex polymorphisms are the result of mutation in the primer annealing site(s) leading to non-amplification of one allele, though some will be due to dominant gene duplications (King et al. 1998a). Most SSCP/duplex assays showed banding complexity consistent with allelic products from one locus. Although gene family numbers are higher in onion than in smaller genomes, we expect that additional copies revealed by RFLP are pseudogenes or copies with diverged functionality and that PCR amplifies the transcribed sequence. A notable exception may be representatives of the highly expressed gene families such as RuBISCO and elongation factors. These families are much more likely to include similar, transcriptionally active copies that enable higher levels of transcripts.

In most cases where we designed PCR primers from EST sequences we adhered to a defined strategy of targeting 3´ UTR regions. There are few cases of development of PCR assays from ESTs where an explicit PCR primer design strategy was used such as that reported by Harry et al. (1998). Although it is preferable to design intron-spanning primer sets to maximise the probability of finding polymorphisms, the 3´ UTR region can be targeted without a knowledge of intron structure. The 3´ UTR of protein-coding genes show rates of nucleotide substitution slightly lower than synonymous substitutions in coding regions, suggesting they are under some functional constraint (Graur and Li 2000). Many primer sets designed in this way also amplify products from *A. roylei.* These will provide convenient cDNA-based markers for interspecific mapping and introgression studies. It would be desirable to critically compare this strategy with others, such as designing longer, intron-spanning PCR products, and to develop more general guidelines for exploiting EST data for molecular-marker applications by SSCP or other mutation-scanning techniques.

The interpretation of SSCP banding patterns when comparing open-pollinated onion lines requires caution similar to the use of RFLP fingerprints, and the use of genetically characterised markers is clearly preferable. The complexity of SSCP/duplex banding observed in this study was not excessive, and mapping of 10 out of 11 EST markers to expected genomic locations suggests that duplication may not complicate PCR-conversion unduly.

Further experience with these assays in other mapping populations is required to confirm that they can be used as framework markers to reliably identify linkage groups on the RFLP map. Our observations confirm that, as with RFLPs (Bark and Havey 1995), genetic interpretation of SSCP banding requires segregation analysis

and that heterozygosity complicates comparison among open-pollinated onion populations. However, because heterozygosity can be directly observed in many SSCP/duplex assays these may be more suitable than RFLP for a comparison of populations.

cDNA-AFLP techniques will also be a valuable means to characterise gene family evolution and distribution in onion and other large genomes such as pine (Cato et al. 2000). This approach has been used to place some cDNA sequences from the onion RFLP map on the interspecific *cepa-roylei* cross using gene primers designed for 3´UTR regions (S. Cato, personal communication). By the use of gene primers that are designed to conserved regions it may be feasible to map the genome distribution of family members in order to further extend knowledge of gene family duplication and its role in *Allium* genome evolution.

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