



Commentary

Efficient Protocols for CAPS-Based Mapping in *Arabidopsis*

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Abstract. Positional cloning continues to be an essential method for gene identification and characterisation. The introduction of PCR-based techniques such as Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Length Polymorphisms (SSLP) and Cleaved Amplified Polymorphic Sequences (CAPS) has greatly increased the efficiency of gene mapping in *Arabidopsis*. To develop the CAPS marker approach further, we have altered several critical mapping parameters. Efficiency was improved by using a small volume of dry seed for DNA extraction instead of the commonly used vegetative tissue. Reproducibility of PCR reactions was enhanced by faster and reduced protocols for PCR and restriction enzyme digestion and optimisation of PCR conditions for over 50 CAPS primer pairs. Finally, the density of genetic markers was increased by providing polymorphic information for all CAPS markers in *Arabidopsis* ecotypes Wassilewskija (Ws), Columbia (Col) and Cape Verde Islands (Cvi).

Key words: *Arabidopsis*, CAPS, DNA extraction, ecotype polymorphisms, gene mapping, PCR conditions

Abbreviations: AFLP, amplified fragment length polymorphism; CAPS, cleaved amplified polymorphic sequences; RFLP, restriction fragment length polymorphism; SSLP, simple sequence length polymorphism. Ecotypes for *Arabidopsis thaliana*: B, Bensheim; Col, Columbia; Cvi, Cape Verde Islands; Ler, Landsberg *erecta*; Nd, Niederzenz; No, Nossen; R, RLD; Ws, Wassilewskija.

Introduction

Map-based cloning of *Arabidopsis* genes has been greatly enhanced by completion of the *Arabidopsis* Genome Initiative (for review, see Lukowitz et al., 2000). But, even with the entire sequence in hand, the vast majority of genes still are not experimentally characterised, and their biological functions remain undiscovered. Despite new innovations in reverse genetics-based techniques, the major tool for gene discovery is still the traditional forward genetics approach in which random mutagenesis is followed by phenotypic screening for relevant mutants. Random mutations can be induced by chemical agents or radiation treatment (Meyerowitz

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and Pruitt, 1985), by T-DNA (Errampalli et al., 1991; Feldmann, 1991) or transposon insertion (Speulman et al., 1999; Tissier et al., 1999). Because the inserted sequence is tagged directly to the gene, T-DNA and transposon mutagenesis methods seem to be more convenient. However, it is often required, or at least desirable, to locate tagged mutations to confirm unclear positions, or more importantly to resolve common problems such as multi-copy or backbone insertions (Kononov et al., 1997; Wenck et al., 1997). Chemical and physical mutagenesis methods are still advantageous in that they are inexpensive, easy to handle and, due to their mutational characteristics, superior in their ability to cause point mutations. Single base pair substitutions offer a wider mutational spectrum, including weak-penetrating, recessive or conditional alleles. In addition to chemical or tagged mutagenesis, naturally-occurring genetic variability exists in many traits (for review, see Alonso-Blanco and Koornneef, 2000), which may reflect subtle changes in the DNA sequence (Lukowitz et al., 2000).

Over the last decade, expensive and time-consuming methods involving restriction fragment length polymorphism (RFLP) markers (Chang et al., 1988; Lister and Dean, 1993; Liu et al., 1996; Nam et al., 1989), random amplified polymorphic DNAs (RAPDs) (Reiter et al., 1992) or visible markers (Patton et al., 1991) were used for mapping. These types of markers have been replaced mainly by new PCR-based markers that exploit the polymorphic differences between *Arabidopsis thaliana* ecotypes. The two widely used molecular marker types that amplify polymorphic differences are simple sequence length polymorphisms (SSLP) (Bell and Ecker, 1994) and cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993). SSLP markers are based on sequence length differences, while CAPS and derived CAPS (dCAPS) (Michaels and Amasino, 1998) rely on differences in restriction enzyme digestion patterns of PCR fragments caused by nucleotide polymorphism between ecotypes.

Critical steps in the CAPS marker approach include DNA extraction, PCR conditions, and the number or distribution of polymorphic sites in a variety of ecotypes. Ordinarily, CAPS protocols use the vegetative tissue of F_2 plants or F_3 families as the source of DNA (Bell and Ecker, 1994; Glazebrook et al., 1998; Konieczny and Ausubel, 1993). This damages the plant and requires extensive time and space to grow F_3 populations. To address this problem, we have developed a DNA extraction method for dry seeds.

The original CAPS marker protocol (Konieczny and Ausubel, 1993) uses the same PCR conditions for all 18 CAPS markers. This initial collection of CAPS markers has been enlarged by finding related genes (Thorlby et al., 1999) and by mapping of specific regions (Copenhaver et al., 1999) or genes (Dietrich et al., 1997); but the PCR conditions often are unchanged and not optimal for several amplicons, causing inconsistent PCR reactions particularly with poor to moderate quality template DNA. It is obvious that optimised conditions for PCR amplification of each CAPS marker is required.

The ecotype *Ws* was used to generate one of the first T-DNA insertion lines (Feldmann et al., 1989; Feldman, 1991) and is still a common parental ecotype within the T-DNA collections available to the plant community (<http://nasc.nott.ac.uk/>). However, CAPS and SSLP markers originally were designed to detect polymorphisms between *Arabidopsis* ecotypes *Ler* and *Col* (Bell

and Ecker, 1994; Konieczny and Ausubel, 1993). For the Ws ecotype, as well as for the Cvi ecotype, only incomplete restriction enzyme digestion information is available.

We have tested 50 of the most commonly used CAPS markers at various annealing temperatures and other PCR conditions. In addition, polymorphism information is provided for the ecotypes Ws and Cvi for all 50 optimised CAPS primers.

Materials and Methods

DNA extraction from seeds

All manipulations are carried out on ice if not otherwise indicated.

1. Freeze in liquid nitrogen approximately 125 seeds of arabidopsis (2.5-3 mg) in a 1.5 mL microfuge tube. Crush seeds with a plastic pestle (Kontes, USA) using an ordinary electric drill or a small-scale drill (Pellet Pestle® Motor, Kontes, USA). Occasionally, place the tube back into liquid nitrogen to keep the plant material cool. Add 350 μ L extraction buffer (50 mM Tris chloride [pH 8.0], 10 mM sodium EDTA [pH 8.0], 100 mM NaCl, 1% SDS and 10 mM β -mercaptoethanol, the β -mercaptoethanol is added just before extraction) and then grind the seeds into a homogenous slurry using the drill. Rinse the pestle with another 350 μ L extraction buffer into the tube and vortex briefly.
2. Incubate in a water bath for 10-15 min at 65°C, mix after 5 min. Add 220 μ L of the following solution: 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of water (Sambrook et al., 1989); the solution is 3 M in respect to potassium and 5 M with respect to acetate. Mix and put on ice for 30 min. Centrifuge at 9000 *g*-force (10,000 rpm) at 4°C for 10 min.
3. Filter the supernatant through a 4 cm² autoclaved Miracloth square into a new 1.5 mL microfuge tube by pushing the cloth with the pipet tip into the tube and squeezing it with the same tip afterwards. Centrifuge at 9000 *g*-force (10,000 rpm) at 4°C for 5 min. Transfer the supernatant into a new 1.5 mL microfuge tube and add 550 μ L isopropanol (about 0.7 x sample volume), mix gently but thoroughly. Incubate for at least 60 min at room temperature with occasional mixing. Centrifuge at 9000 *g*-force (10,000 rpm) room temperature for 45 min.
4. Discard the supernatant and carefully wash the pellet twice with 1 mL 80% ethanol by mixing gently by hand and then centrifuging 9000 *g*-force (10,000 rpm) at 4°C for 5 min. Dry under vacuum for approximately 3-4 min or briefly air dry. Dissolve pellet in 100 μ L TE (50 mM Tris chloride [pH 8.0] and 10 mM sodium EDTA [pH 8.0]). Add 2 μ L 10 mg/mL RNaseA and incubate at 37°C for 15 min.
5. Add 10 μ L 3 M sodium acetate buffer (adjusted to pH 5.2 with acetic acid [Sambrook et al., 1989]) and mix. Add 200 μ L 100% ethanol to the solution and mix well. Incubate for 30 min at -20°C.

6. Centrifuge at 11,000 *g*-force (11,000 rpm) at room temperature for 10 min. Rinse pellet twice with 1 mL 80% ethanol and dry under vacuum, both as described in Step 4.
7. Dissolve pellet in 25 μ L water or TE at 4°C for 5 min to overnight.
8. The DNA concentration can be determined by measuring the A_{260} of an aliquot from the above 25 μ L, using as a standard $A_{260} = 1.0$ for a solution of 45 μ g/mL in 0.1 M NaCl. The concentration should be approximately 100 μ g/mL for a yield of about 2.5 μ g.
9. Dilute the DNA to 1 μ g/mL in water or TE and store at -20°C.

PCR amplification of arabidopsis CAPS-markers

PCR reactions (20 μ L) included 0.2 μ M of each primer (Research Genetics), 1.5-3.0 mM $MgCl_2$ (optimised for each pair of primers, Table 1), 0.5 unit *Taq* polymerase (Advanced Biotechnologies), 0.2 mM each dNTP (Promega) and 5 μ L 1 μ g/mL DNA template. Primer nucleotide sequences are available at <http://arabidopsis.org/aboutcaps.html>. In general, amplification was initiated at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer-specific annealing at 50-55°C for 30 s (Table 1) and 72°C for 0.5-2 min (depending on PCR product size; use 1 kb/min as the rate of polymerisation) and a final elongation time at 72°C for 7 min. PAT1 primers used denaturation for 45 s, annealing for 45 s and elongation for 2.5 min. PCR products were digested overnight with 1.25 units restriction enzyme per 20 μ L reaction and resulting DNA fragments were fractionated by electrophoresis in 2-4% agarose gels. PCR reactions were performed on Biometra Trio-Thermoblock TB-1, Göttingen, Germany; Techne FGeno2TD, Cambridge, UK and Perkin Elmer Gene Amp PCR-System 2400, Norwalk, US.

Results

DNA extraction from seeds

Seeds provide a source of regenerable plant genotypes as well as DNA. To obtain a rapid protocol for dry seeds, we tested several DNA extraction methods. The standard DNA miniprep protocol from Dellaporta et al. (1983) required several modifications for reliable further downstream PCR applications. For plant materials, we routinely use extraction buffers adapted here for seeds and with lower ionic strength and lower SDS concentrations than those in the original CAPS protocol (Konieczny and Ausubel, 1993). Various alcohols and incubation temperatures also were tested. DNA yield and purity was increased by adding an RNA digestion step and by replacing isopropanol with ethanol in a second precipitation step. In fact, reliable PCR reactions could be obtained from dry seed DNA after the RNA digestion step (Step 4). With these DNA extraction modifications, the PCR-ready DNA yields are approximately 2.5 μ g DNA from 125 seeds and enough to conduct 500 PCR reactions.

Table 1. PCR conditions and ecotype polymorphisms for CAPS markers.

Chr	Marker	Annealing Temperature (°C)	MgCl ₂ (mM)	Fragment Size (kbp)	Restriction Enzyme	Number of Cuts ¹ (size of products in kbp)
I	PAI1	50	1.5	1.4	<i>Dde</i> I	Col=Cvi=Ler, 2 (0.7, 0.4, 0.3); Ws, 3 (0.7, 0.35, 0.3, 0.05)
I	NCC1	53	1.5	1.0	<i>Rsa</i> I	Col, 1 (0.87, 0.05); Ler=C24=Cvi=R=Ws ¹ , 1 (0.92, 0.05)
I	m59	50-55	1.5	0.8	<i>Bst</i> U I-I	Col=Nd=Cvi ¹ , 2 (0.52, 2x 0.12); Ler=Ws ¹ , 1 (0.52, 0.24)
I	g2395	50	1.5	0.3	<i>Xba</i> I	Col=Cvi=B=Nd=Ws, 1 (0.183, 0.153); Ler, 0 (0.336)
I	m235	50	1.5	0.5	<i>Hind</i> III	Col=Nd=Ws ¹ =Cvi ¹ , 1 (0.309, 0.225); Ler=B, 0 (0.534)
I	UFO	55	1.5	1.3	<i>Taq</i> I	Col=R=Ws ¹ =Cvi ¹ , 1 (0.983, 0.316); Ler, 2 (0.6, 0.383, 0.316)
I	7G6	50-55	1.5	0.4	<i>Acc</i> I	Col=Ws ¹ =Cvi ¹ , 2 bands; Ler 1 band
I	GAPB	55	3.0	1.5	<i>Dde</i> I	Col=Cvi=No-0=Ws=R, 3 (0.605, 0.284, 0.225, 0.174); Ler, 4 (0.35, 0.284, 0.255, 0.225, 0.174)
I	GAPB	50	1.5	1.5	<i>Bfa</i> I	Col=Ws ¹ =Cvi ¹ , 2 (1.211, 0.212, 0.058); Ler, 3 (0.85, 0.36, 0.212, 0.058)
I	PAI3	55	1.5	1.5	<i>Mnl</i> I	Col=Ler, 5 (0.28, 0.26, 2x 0.2, 0.12, 0.09); Ws=Cvi ¹ , 6 (0.26, 2x 0.2, 0.15, 0.13, 0.12, 0.09)
I	NPR1	50-55	1.5	0.6	<i>Alu</i> I	Col=Ws ¹ , 0 (0.615); Ler=Cvi ¹ , 1 (0.536, 0.079)
I	g11447	50-55	1.5	0.8	<i>Eco</i> R V	Polymorphic between Col=Ws and Ler, Cvi ¹
I	m305	50	1.5	1.6	<i>Hae</i> III	Polymorphic between Col=Ws=Cvi ¹ and Ler
I	PAB5	55	1.5	0.9	<i>Mbo</i> II	Col=Ws ¹ , 3 (0.360, 0.320, 0.165, 0.04); Cvi=Ler, 2 (0.485, 0.36, 0.04)
I	ADH	55	3.0	1.3	<i>Xba</i> I	Col=Cvi=No=Ws, 0 (1.291); Ler=C24=R, 1(1.097, 0.262)
I	ADH	50	1.5	1.3	<i>Bfa</i> I	Col=Ws ¹ =Cvi ¹ , 2 (1.099, 0.127, 0.065); Ler, 3 (0.849, 0.25, 0.127, 0.065)
I	ADH	55	3.0	1.3	<i>Bfa</i> I	Col=Ws ¹ =Cvi ¹ , 2 (1.099, 0.127, 0.065); Ler, 3 (0.849, 0.25, 0.127, 0.065)
II	m246	50	1.5	1.4	<i>Mae</i> III	Col, 0 (1.354); Ler=C24=Cvi=No=R=Ws, 1 (1.122, 0.232)
II	THY1	50-55	1.5	0.8	<i>Rsa</i> I	Col, 2 bands; Ler=Ws=Cvi ¹ , 3 bands
II	PhyB/HY3	55	3.0	1.1	<i>Xho</i> I	Col, 0 (1.1); Ler=Cvi=Ws, 1 (0.7, 0.4)
II	GPA1	50-52	1.5	1.6	<i>Afl</i> III	Col=C24=Cvi=Ws, 2 (0.705, 0.680, 0.209); Ler=R, 1 (1.385, 0.209)
II	cop1	55	1.5	0.9	<i>Mse</i> I	Col, 3 (0.4, 0.29, 0.12, 0.075); Ler=Ws ¹ =Cvi ¹ , 3 (0.38, 0.325, 0.12, 0.075)

Table 1 (con't).

Chr	Marker	Annealing Temperature (°C)	MgCl ₂ (mM)	Fragment Size (kbp)	Restriction Enzyme	Number of Cuts ¹ (size of products in kbp)
II	m429	55	3.0	0.3	<i>Srf</i> I	Col=No, 0 (0.316); Ler=C24=Cvi=R=Ws, 1 (0.216, 0.100)
III	S6	50	1.5	0.9	<i>Hinf</i> I	Col=Cvi ¹ , (major product: 0.29); Nd=Ws ¹ , (major product: 0.2)
III	C6	50	1.5	0.8	<i>Dde</i> I	Col, (major product: 0.45); Nd (major product: 0.65); Ws ¹ =Cvi ¹ , (major product: ca. 0.56)
III	B4	45-52	1.5	0.9	<i>Taq</i> I	Col=Ws ¹ =Cvi ¹ (major product: 0.55); Nd (major product: 0.75)
III	g4711	55-57	1.5	1.5	<i>Hind</i> III	Col=Ws ¹ , 0 (1.5); Ler=Cvi ¹ , 1 (1.0, 0.5)
III	GAPA	55	1.5	0.8	<i>Dde</i> I	Col, 5 (0.42, 0.178, 0.1, 0.033, 0.019, 0.01); Ler=C24=Cvi=No=R=Ws, 6 (0.24, 0.19, 0.178, 0.1, 0.033, 0.019, 0.01)
III	32C9	50-55	1.5	1.0	<i>Dde</i> I	Col, 2 (0.6, 0.3, 0.1); Ler=Ws=Cvi ¹ , 1 (0.7, 0.1)
III	ABI3	50-55	1.5	1.6	<i>Hinf</i> I	Col, 2 (0.8, 0.7, 0.1); Ler=Ws=Cvi ¹ , 1 (1.5, 0.1)
III	GL1	55	1.5	0.5	<i>Taq</i> I	Col, 3 (0.298, 0.1, 0.074, 0.047); Ler=C24, 2 (0.372, 0.1, 0.047); Cvi=Nd=R=Ws ¹ , 1 (0.4, 0.12)
III	ALS	55-57	1.5	1.4	<i>Hae</i> III	Col=Cvi ¹ , 1 (0.952, 0.42); Ler=Ws=Nd, 2 (0.952, 0.22, 0.2)
III	ALS	55-57	1.5	1.4	<i>Rsa</i> I	Col=Cvi ¹ , 5 (0.384, 0.347, 0.267, 0.233, 0.109, 0.032); Ler=Ws=Nd, 4 (0.5, 0.384, 0.347, 0.109, 0.032)
III	NT1	50	1.5	1.8/1.9		No cutting required: Col=Nd=Ws ¹ , 0 (1.8); Ler=Cvi ¹ , 0 (1.9)
III	CDC2A	52	1.5	2.5	<i>Alu</i> I	Col=Cvi=Ws ¹ , 4; Ler, 3
III	BGL1	50	3.0	1.3	<i>Rsa</i> I	Col=Nd, 2 (0.785, 0.34, 0.105); Ler=Cvi=No=0=R=Ws, 1 (0.785, 0.485)
III	BGL1	50	3.0	1.3	<i>Afl</i> III	Col, 4 (0.494, 0.344, 0.258, 0.15, 0.084); Ler=Cvi=R=Ws ¹ , 3 (0.494, 0.434, 0.258, 0.084)
III	BGL1	50	3.0	1.3	<i>Sau</i> 3A I	Col, 0 (1.269); Ler=C24=Cvi=R=Ws ¹ , 1 (0.875, 0.395)
III	TSA1	50-55	1.5	0.4	<i>Alu</i> I	Col, 1 (0.246, 0.134); Ler=Ws ¹ =Cvi ¹ , 0 (0.38)
IV	SC5	55	3.0	0.6	<i>Acc</i> I	Col=Cvi,0 (0.55); Ler=Ws ¹ , 1 (0.3, 0.25)
IV	g4539	50	1.5			
IV	ch42	55	1.5	0.6	<i>Hind</i> III	Col=Cvi=R, 0 (0.6); Ler=Ws, 1 (0.48, 0.12)
IV	g3883-1.4	52	2.25	1.4	<i>Cla</i> I	Col=Cvi, 1 (0.75, 0.65); Ws, 0 (1.4)
IV	g13838-1.4	50	1.5	0.7/1.4		No cutting required: Col=No=Cvi ¹ , 0 (1.4); Ws, 0 (0.7)
IV	g13838-1.4	50	1.5	1.4	<i>Hinf</i> I	Col=Cvi ¹ , 3 (0.45, 0.33, 0.28, 0.2); Ws, 3 (0.45, 0.33, 0.28, 0.16)
IV	RPS2	50	3.0	0.8	<i>Sau</i> 3A I	Col, 1 (0.605, 0.18); Ler=Cvi=Ws ¹ , 2 (0.354, 0.251, 0.18)

Table 1 (continued).

Chr	Marker	Annealing Temperature (°C)	MgCl ₂ (mM)	Fragment Size (kbp)	Restriction Enzyme	Number of Cuts ¹ (size of products in kbp)
IV	prha	50	1.5	1.7	Dde I	Col=Ws ¹ , 3 (0.778, 0.53, 0.348, 0.03); Ler=Cvi=No=R, 4 (0.778, 0.53, 0.3, 0.05, 0.03)
IV	CAT2	52	1.5	0.9	Dde I	Col=Cvi ¹ , 4 (0.393, 0.238, 0.138, 0.124, 0.047); Ler=Ws ¹ , 3 (0.393, 0.262, 0.238, 0.047)
V	PAI2	50	1.5	0.6	Afl III	Cvi=Ws=Ler, 0 (0.644); Col, 1 (0.594, 0.05)
V	N97067	48	1.5	0.2	Hinf I	Col=Ws ¹ =Cvi ¹ , 1 (0.2, 0.02); Ler, 0 (0.22)
V	PAT1	55	3.0	1.9	Dde I	Col, 2 (1.0, 0.6, 0.3); Ler=Cvi=Ws, 1 (1.0, 0.9)
V	PAT1	55	1.5	1.9	Sph I	Col, 0 (1.9); Ler=Cvi=Ws, 1 (1.3, 0.6)
V	R89998	50	1.5	0.5	Rsa I	Col, 2 (0.35, 0.07, 0.03); Ler=Ws ¹ =Cvi ¹ , 1 (0.44, 0.01)
V	NIT4	57	1.5	2.0	Ase I	Col=Cvi ¹ =Ler=Ws ¹ , 1 (1.333, 0.692)
V	NIT4 ²	57	1.5	2.0	Mbo II	Col, 5; Cvi ¹ , 6; Ler=R=Ws, 4
V	NIT4LB1 ²	59	1.5	1.2/1.6	Mbo II/ None	Col, 3 (0.506, 0.443, 0.224, 0.016); Ws ¹ , 2 (0.949, 0.224, 0.016); No cutting required: Col ¹ =Ws ¹ , 0 (1.2); Cvi ¹ , 0 (ca. 1.6)
V	PHYC	50-55	1.5	1.2	Msp I	Col=Ws, 2; Cvi=Ler, 1
V	RBCS-B	50	1.5	1.2	Ssp I	Col, 2 (0.609, 0.286, 0.257); Be=C24=Cvi ¹ =Ler=Ws, 1 (0.866, 0.286)
V	DRF	50	1.5	1.1	BsaA I-C	Col=C24=Cvi=No=Ws, 1 (0.609, 0.534); Ler=R, 2 (0.609, 0.318, 0.216)
V	LTI78	58	1.5	0.9	Rsa I	Col, 3 (different size); Cvi=Ws, 3
V	ILL1	58	1.5	1.3	Taq I	Col, 3; Cvi, 0; Ws, 4
V	17C2	50-55	1.5	1.4	Acc I	Col=Cvi ¹ , 1 (0.9, 0.5); Ler=Ws ¹ , 2 (0.9, 0.3, 0.2)
V	10A10	50	1.5	2.0	BstE II	Col=Ws ¹ =Cvi ¹ , 0 (2.0); Ler, 1 (1.3, 0.7)
V	EG7F2	50-55	1.5	1.2	Xba I	Col, 0 (1.2); Cvi=Ler=Ws ¹ , 1 (0.7, 0.5);
V	ASB2	50	1.5	1.3	Hinf I	Col, 3; Cvi=Ler=Ws ¹ , 4
V	LFY3	50-55	1.5	1.3	Rsa I	Col, 5 (0.708, 0.236, 0.147, 0.126, 0.078, 0.035); Ler=C24=Cvi=No=R=Ws, 4 (0.855, 0.236, 0.126, 0.078, 0.035)
V	g2368	50	1.5	1.4	Hind III	Col=Cvi=Ws ¹ , 0 (1.4); Ler, 1 (1.35, 0.05)

¹New polymorphisms for the ecotypes Col, Cvi and Ws. Published data for the Col, Cvi and Ws ecotypes (<http://arabidopsis.org/aboutcaps.html>) were confirmed in this work with the exception of the NIT4 marker digested with Ase I. Ws shares identical digestion patterns with Col, Cvi and Ler. Published data for the B, C24, Ler, Nd, No and R ecotypes were not confirmed.

²For the NIT4 marker the numbers correspond to the larger fragments (ca. 0.150 kbp and above). However, we recommend a different primer set for the NIT4 loci: Forward primer NIT4LB1 5'-TCTTCGCTTCAGGTAATACG-3' and reverse primer NIT4LB1 5'-TCAAAGGCCTATGAAAGAGG-3' (for conditions and fragment sizes see above).

PCR conditions for CAPS markers

The original PCR protocol (Konieczny and Ausubel, 1993) was tested with over 50 CAPS markers from the arabidopsis database (<http://arabidopsis.org/-aboutcaps.html>). We modified the method by reducing the DNA concentration, the number of PCR cycles and the enzyme concentration in the subsequent restriction digestions. The original DNA template of 50 ng was reduced by 95% to 5 ng per PCR reaction. The number of PCR cycles was decreased from 50 to 35 cycles, producing a sufficient PCR product yield for downstream restriction digestion and gel electrophoresis. The time required for a PCR reaction can be further reduced by decreasing the polymerisation time from the 2 min/cycle for all CAPS primers in the original protocol to 30 s/cycle; the polymerisation rate is 1 kb/min because many CAPS primers amplify fragments shorter than 2 kbp. We also found that the restriction enzyme concentration can be reduced to 1.25 units per 20 µL reaction. No negative impact on the reliability of the PCR reactions was observed because of these changes. A flow chart illustrating these modifications is shown in Figure 1.

Our experience indicates that the standard primer annealing temperature, suggested for all primer pairs in the original protocol, often is suboptimal and yields poor PCR reactions. Calculations of the primer annealing temperatures using the primer calculator program (<http://www.williamstone.com/primers/calculator/>) showed that some primer pairs have an annealing temperature difference of up to 10°C. To obtain reliable PCR conditions for each CAPS marker primer pair, conditions for each primer pair at different annealing temperatures and variable MgCl₂ concentrations were tested with seed DNA of the Ws, Col and Cvi ecotypes. The tests were performed using different types of thermal cyclers in order to generalise the applicability of the conditions. The MgCl₂ content and annealing temperatures producing consistent PCR products and strong fragment bands are reported in Table 1. These were chosen for analysis of polymorphisms in other ecotypes.

Polymorphic sites in other ecotypes

The original 18 CAPS markers were devised for detection of sequence differences between the Ler and Col ecotypes (Konieczny and Ausubel, 1993). Other CAPS markers are now available; however, the digestion patterns for several commonly used ecotypes are not available for many of the CAPS markers. The main objective here was to determine the patterns for the ecotypes Ws, Col and Cvi. The information for CAPS markers in Table 1 will make these ecotypes more available for mapping arabidopsis genes. Table 2 contains pairwise comparisons of polymorphic differences in CAPS markers for various Col, Cvi, Ler and Ws crossing combinations. Mapping with Col x Ler crosses provides the largest percentage of applicable CAPS markers (95%), followed by Col x Cvi, Col x Ws, Ler x Ws and Ler x Cvi combinatorial pairs (40-60%), which is still approximately two-fold the number of usable CAPS markers for the Ws x Cvi combination (33%). The three ecotypes Col, Cvi and Ws together share 27% identical patterns. The Col ecotype shows the highest number of diverse alleles in relation to all other ecotypes.

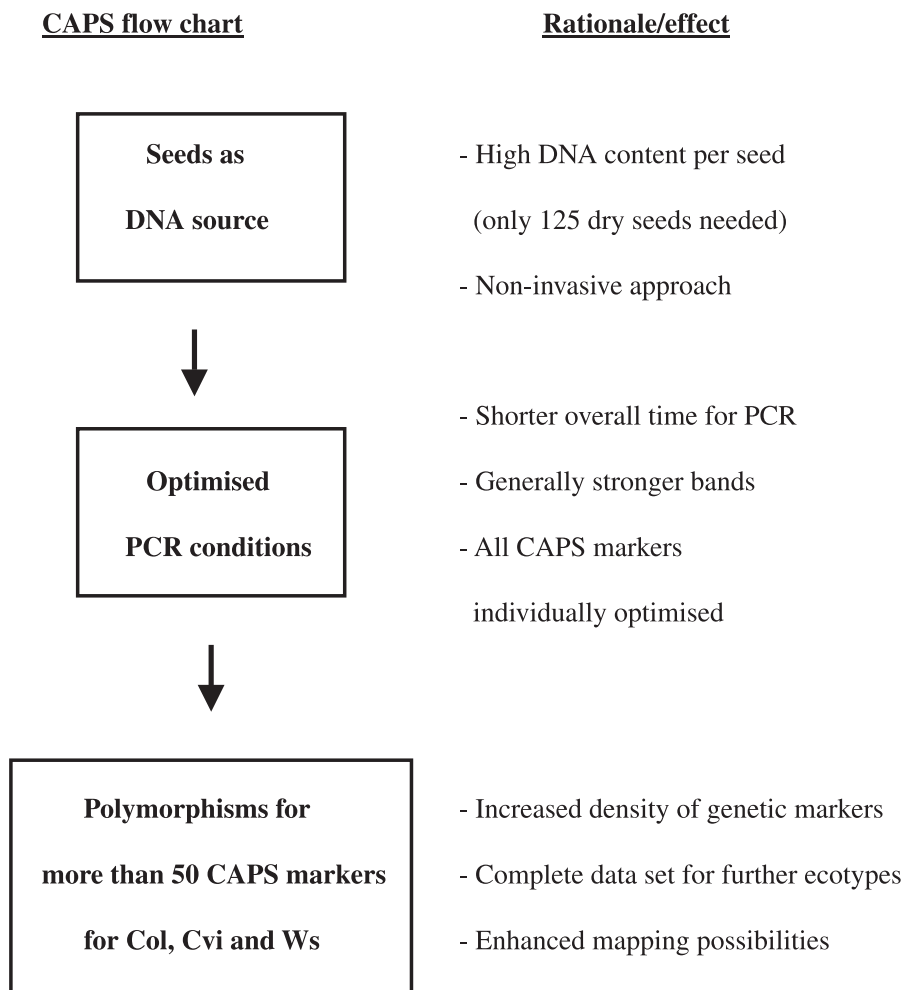


Figure 1. Schematic diagram of our improvements for CAPS based mapping.

Discussion

The recent breakthrough in arabidopsis sequencing will support, but does not replace, positional cloning of genes (Lukowitz et al., 2000). Gene mapping is an indirect approach where the positional window is narrowed by progressively closer markers. Mapping with visible markers (Koornneef and Hanhart, 1983; Koornneef et al., 1983) has changed dramatically since the introduction of SSCP and CAPS markers (Bell and Ecker, 1994; Konieczny and Ausubel, 1993). In principle, a few crosses between contrasting ecotypes are sufficient to create an unlimited number of polymorphic DNA markers among F_2 individuals. In contrast to RFLP markers (Chang et al. 1988), SSCP and CAPS markers have the advantage of being based on PCR techniques and can therefore be visualised by electrophoresis rather than DNA blot hybridisation. Precise mapping requires a high density of markers and a

Table 2. Pairwise comparisons of polymorphic sites in CAPS markers for representative ecotypes.

Ecotypes	Ws ¹	Col ¹	Cvi ¹
Ler	43% ² (56) ³	95% ² (56) ³	46% ² (55) ³
Ws	x	63% ² (64) ³	33% ² (64) ³
Col	x	x	53% ² (64) ³

¹Identical CAPS patterns for the ecotypes Ws, Col and Cvi = 27% (64)³

²Percentage of polymorphic differences in CAPS markers for crossings between the arabidopsis ecotypes Col, Cvi, Ler and Ws.

³The total number of determined CAPS markers for a crossing of two ecotypes indicated in parentheses. A few CAPS markers can utilise two (or in one case, three) different restriction enzymes for an identical site (see Table 1). In these coincidences, each CAPS marker polymorphism detected by one of these restriction enzymes is counted separately.

large number of individuals in the mapping population. Managing mapping populations can be very time and space consuming, especially if further analysis of F₂ individuals is required for determination of their genotypes.

There are several potential advantages to the use of F₃ seeds as the source of the F₂ plant DNA rather than commonly used rosette leaves of the F₂ plant. It is nondestructive of the F₂ plant and it can store the parental genotype by pooling F₃ individuals as seeds. Unlike frozen leaves, seeds do not require freezers or freeze-dryers for DNA preservation and, of course, they can be grown into plants if a requirement exists for more DNA or further analysis of the F₂ genotype. In some circumstances, extracting DNA from pooled seeds may eliminate the time and space required for growing a subsequent number of plants solely for DNA preparation.

Compared with other plant tissues, seeds have high concentrations of protein, oil, other storage compounds, secondary cell walls and lignins in the seed coat that may inhibit PCR reactions. Several modifications of the widely applicable method of Dellaporta et al. (1983) have been described for extracting DNA from small tissue samples for later PCR applications. In particular, the methods of Neff et al. (1998) can be applied to fresh and dried seedlings, cotyledons, young and old leaves, flowers, siliques and imbibed seeds. We found it necessary to try other modifications (Michaels et al., 1994; Glazebrook et al., 1998) and to use a step to remove RNA. The RNA digestion step proved to be essential, and the subsequent ethanol precipitation step helped to avoid occasional inhibition of PCR reactions. About 125 seeds are recommended for obtaining reasonable amounts of DNA in high purity, but, in principle, fewer seeds may be used.

In our mapping efforts, most of the standard conditions for PCR reactions and restriction enzyme digestions were scaled down. Most importantly, the amount of DNA template was reduced to 5% because this was sufficient and excessive quantities of DNA (or impurities in the DNA) can inhibit PCR reactions. Reducing the number of PCR cycles to 35 shortens the time requirement for a PCR reaction and increases its accuracy by minimising possible false PCR products due to primer mismatching. The PCR products of many CAPS markers are less than 2.0 kbp. Consequently, the PCR elongation time can be scaled down

based on an estimated synthesis rate of 1.0 kbp/min. Depending on length, sequence composition and secondary structures, the primers for PCR reactions are likely to differ in their required annealing temperatures. The MgCl_2 concentration is another critical PCR parameter. In our experiments, the standard annealing temperatures and the MgCl_2 concentration often resulted in unreliable PCR reactions with either no PCR products or products of insufficient quality. Calculating the primer annealing temperatures was of limited help, however, because forward and reverse primers of some primer pairs have annealing temperature differences of up to 10°C. This provided the motivation to test the optimal annealing temperature for the given CAPS marker primers (Table 1).

The CAPS markers have been developed primarily for the ecotypes Ler and Col. Until now, other ecotypes like Ws, used by Feldmann (1991) to create one of the first large T-DNA tagged collections, have had only a limited application for mapping projects. Occasionally, the restriction digestion patterns in Ws and other ecotypes have been added, especially by the Koornneef group for Cvi (<http://arabidopsis.org/aboutcaps.html>), but they are far from complete. It is desirable to include more ecotypes in the mapping procedure, particularly to exploit the natural variations in *arabidopsis* ecotypes (Alonso-Blanco and Koornneef, 2000). The results reported here increase the possibility for mapping with different ecotypes by providing the complete digestion patterns for over 50 CAPS markers for the Ws, Col and Cvi ecotypes.

The number of polymorphic differences is highly variable among various *arabidopsis* ecotypes. Choice of ecotypes for mutagenesis and for mapping populations should take into consideration the unequal distribution of polymorphic variations. For Ler x Col crossings, almost every tested CAPS marker can be utilised; but for other examined ecotype pairs, only half of the CAPS markers are useful, and, for the Ws x Cvi crossing, a mere third are available. Our results demonstrate that the Col ecotype displays the most deviant genotype as measured by CAPS markers, making this ecotype a preferable choice for mapping. The above information can be used for efficient positional mapping of *arabidopsis* genes.

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