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PERMANENT GENETIC RESOURCES

Consensus primers of *cyp73* genes discriminate willow species and hybrids (*Salix*, Salicaceae)

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Abstract

Consensus primers, based on exon sequences of the *cyp73* gene family coding for cinnamate 4-hydroxylase (C4H) of the lignin biosynthesis pathway, were designed for the tetraploid willow species *Salix alba* and *Salix fragilis*. Diagnostic alleles at species level were observed among introns of three *cyp73* genes and allowed unambiguous detection of the first generation and introgressed hybrids in populations. Progeny analysis of a female *S. alba* with a male introgressed hybrid confirmed the codominant inheritance of each intron. Sequences of the diagnostic alleles of both species were similar to those found in the hybrids.

Keywords: cinnamate 4-hydroxylase, consensus primers, *cyp73* genes, hybridization, *Salix*

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Salix alba L. (white willow), *Salix fragilis* L. (crack willow) and their hybrid *S. x rubens* Schrank are widespread along rivers in Europe. Natural hybridization and introgression occur in this tetraploid species complex. Hybrids were detected at the local scale with amplified fragment length polymorphism (AFLP; Beismann *et al*. 1997) and random amplified polymorphic DNA (RAPD; Triest *et al*. 2000). However, dominant markers did not allow to distinguish first generation hybrids from introgressed ones and might show homoplasy at large geographical scale. Therefore, we developed consensus primers to obtain species-specific markers for discrimination among *S. alba*, *S. fragilis*, their interspecific hybrid F1 and introgressed hybrids, which is important for breeding programmes and ecological research. *S. alba* differs from *S. fragilis* in the higher bending moment of the twig base (Beismann *et al*. 1997), possibly due to lignin concentration in their cells. *Cyp73* genes code for cinnamate 4-hydroxylase (C4H), a key-enzyme in lignin biosynthesis (Bell-Lelong *et al*. 1997). In the related genus *Populus*, three genes (*cyp73a, -b, -c*) each consist of three exons and two introns (Kawai *et al*. 1996). We designed consensus primers within the conserved exons to amplify the more variable intervening introns of *cyp 73* gene sequences.

Exon sequences of the *cyp73a*, *cyp73b*, *cyp73c* gene family of *Populus kitakamiensis* (Accession nos D82812, D82813, D82814) and *cyp73* sequences of other plant species (Accession nos AF302495, U47293, AF378333, AF255014, AF286647, AF088847, D87520) were downloaded (http://www.ncbi.nlm.nih.gov/entrez/) and aligned (DNAMAN version 4.0.1.1; Lynnon BioSoft). Two primers were designed with 90–100% of similarity within the exon of the aligned *cyp73a, -b, -c* sequences and results in a polymerase chain reaction (PCR) product that spans the intervening intron: C4H1 (F: 5′-GGGGAGAGGAGCAGATTGGCTC-3′; R: 5′-CGTTGTCCTCGTTGATCTCTCC-3′) from the first and the second exon and spanning intron 1; C4H2 (F: 5′-GGAGAGATCAACGAGGACAACG-3′; R: 5′-CAGGATGGTTCACAAGCTCAGC-3′) from the second and third exon and spanning intron 2.

Genomic DNA was extracted from young leaves (DNeasy® Plant Mini Kit, QIAGEN). Initial screening to detect diagnostic alleles at species level was done with 15 reference trees (Table 1 A) from a hybrid zone along the Neumagen River (Germany), previously genotyped with AFLP (Beismann *et al*. 1997). The total PCR amplification volume was 50 μL containing about 100 ng genomic DNA, 20 mM Tris HCl pH 8.4, 50 mM KCl, 0.2 mM each dNTP, 0.2 μM each primer, 3 mM $MgCl_2$ and 1.25 U *Taq* DNA Polymerase. PCR conditions were 2 min at 94 °C; 30 cycles of 40 s at 92 °C; 1 min at 60 °C for C4H1 and 57 °C for C4H2 primers; 2 min at 72 °C; one cycle of 5 min at 72 °C. The amplification products were separated and visualized on 5% polyacrylamide gels using DNA fragment analyser GelScan 2000 (Corbett Research). C4H1 primers gave similar banding

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Table 1 Diagnostic alleles of three introns of *cyp73* genes in *Salix alba* and *Salix fragilis*: (A) 15 trees from a hybrid zone; (B) 85 progeny individuals of a full-sib controlled cross

Sample number	Altitude (m a.s.l.)	Sex	Identity C4H2	*cyp73I*	*cyp73II*	*cyp73III*
A. Reference samples						
1	626	Male	f	*cc*	*mm*	*yy*
2	623	Male	f	*cc*	*mm*	*yy*
3	621	Male	f	*cc*	*mm*	*yy*
4	566	Male	f	*cc*	*mm*	*yy*
5	566	Male	f	*cc*	*mm*	*yy*
6	566	Female	h	*ac*	*mp*	*yz*
7	454	Male	f	*cc*	*mm*	*yy*
8	454	Female	fh	*ac*	*mp*	*yy*
9	454	Male	ah	*ac*	*mp*	*zz*
10	454	Male	f	*cc*	*mm*	*yy*
11	237	Female	h	*ac*	*mp*	*yz*
12	218	Male	h	*ac*	*mp*	*yz*
13	218	Female	a	*aa*	*pp*	*zz*
14	218	Female	h	*ac*	*mp*	*yz*
15	218	Female	a	*aa*	*pp*	*zz*
B. Progeny testing						
V82.258	IBW	Female	a	*ab*	*np*	*zz*
V82.200	IBW	Male	fh	*ac*	*mp*	*xy*
Possible genotypes				*ac:ab:bc:aa*	*mn:np:mp:pp*	*xz:yz*
Observed genotype frequencies				25:25:21:14	13:20:25:27	37:42
Expected genotype frequencies				21:21:21:21	21:21:21:21	42:42
Chi-square (*P* value)				3.800 ($P = 0.284$)	5.495 ($P = 0.139$)	0.717 ($P = 0.396$)

m a.s.l, metres above sea level.

patterns for all samples (not shown), whereas fragment length polymorphism was detected with C4H2 (Fig. 1A, Table 1A). The reference samples showed five multilocus genotypes, named a, f, h, ah, fh: (a) = *S. alba* with three alleles at ~755 bp, ~920 bp and ~975 bp; (f) = *S. fragilis* with three alleles at ~660 bp, ~1025 bp and ~1170 bp; (h) = a putative first generation hybrid containing six alleles (three of each species); in this experiment (ah) = an introgressed hybrid missing one *S. fragilis* allele and (fh) = an introgressed hybrid missing one *S. alba* allele.

Second, PCR amplification using C4H2 primers and genomic DNA of 85 progenies (Table 1B) of a female *S. alba* (a) with a male introgressed *S. fragilis* (fh), a posteriori identified as an introgressed hybrid (full-sibs performed in 1997) was analysed to confirm codominant disomic inheritance of the markers in these allotetraploids (Barcaccia *et al*. 2003). Three introns were assumed (*cyp73I, -II* and *-III*) and similar sized bands were considered as the same allele. The female *S. alba* parent used in the full-sib controlled cross was heterozygous for *cyp73-I* (allele *a* at 755 bp; *b* at ~710 bp) and *cyp73-II* (allele *n* at ~940 bp; *p* at 920 bp). The male parent was heterozygous for *cyp73-III* (allele *x* at ~1050 bp; *y* at ~1025 bp) (Fig. 1B, Table 1B). Progeny analysis confirmed that the three genes each had three alleles. *Cyp73-I* consisted of alleles *a* (~755 bp), *b* (~710 bp) and *c* (~660 bp); *cyp73-II* of alleles *m* (~1170 bp), *n* (~940 bp) and *p* (~920 bp); and *cyp73-III* of alleles *x* (~1050 bp), *y* (~1025 bp) and *z* (~975 bp). The progeny contained four genotypes of *cyp73-I* (*ac, ab, bc* and *aa*), four of *cyp73-II* (*mn, np, mp* and *pp*) and two of *cyp73-III* (*xz* and *yz*). Multiple alleles were designated to a gene if the ratio of progeny genotype frequencies followed Mendelian segregation. A chi-square test ($P > 0.05$) confirmed codominance of alleles in each homologous gene (Table 1B).

Finally, 12 PCR fragments corresponding to the diagnostic alleles named *a*, *p* and *z* of *S. alba* (sample a13); *c*, *m* and *y* of *S. fragilis* (sample f3) and the similar-sized six fragments of the putative first generation hybrid *S. x. rubens* (sample h6) were cloned, sequenced and analysed to confirm homology within each gene. The fragments were excised from 1% low melting agarose-gel, purified with Gel-purification Kit (QIAGEN) and then ligated into pUC18 *Sma*I/BAP with Rapid ligation kit (Roche). The ligated DNA was transformed into *E. coli* DH5α competent cells. Plasmid DNA was isolated from positive colonies using GenElute Plasmid Miniprep kit (Sigma). Inserted fragments were confirmed by PCR and digestion before selecting and sequencing with the automated Applied Biosystem 3100 Genetic Analyser using the BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems) and M13 primers. Sequences were analysed with DNAMAN (version 4.0.1.1). Sequences of six alleles of the hybrid were > 98%

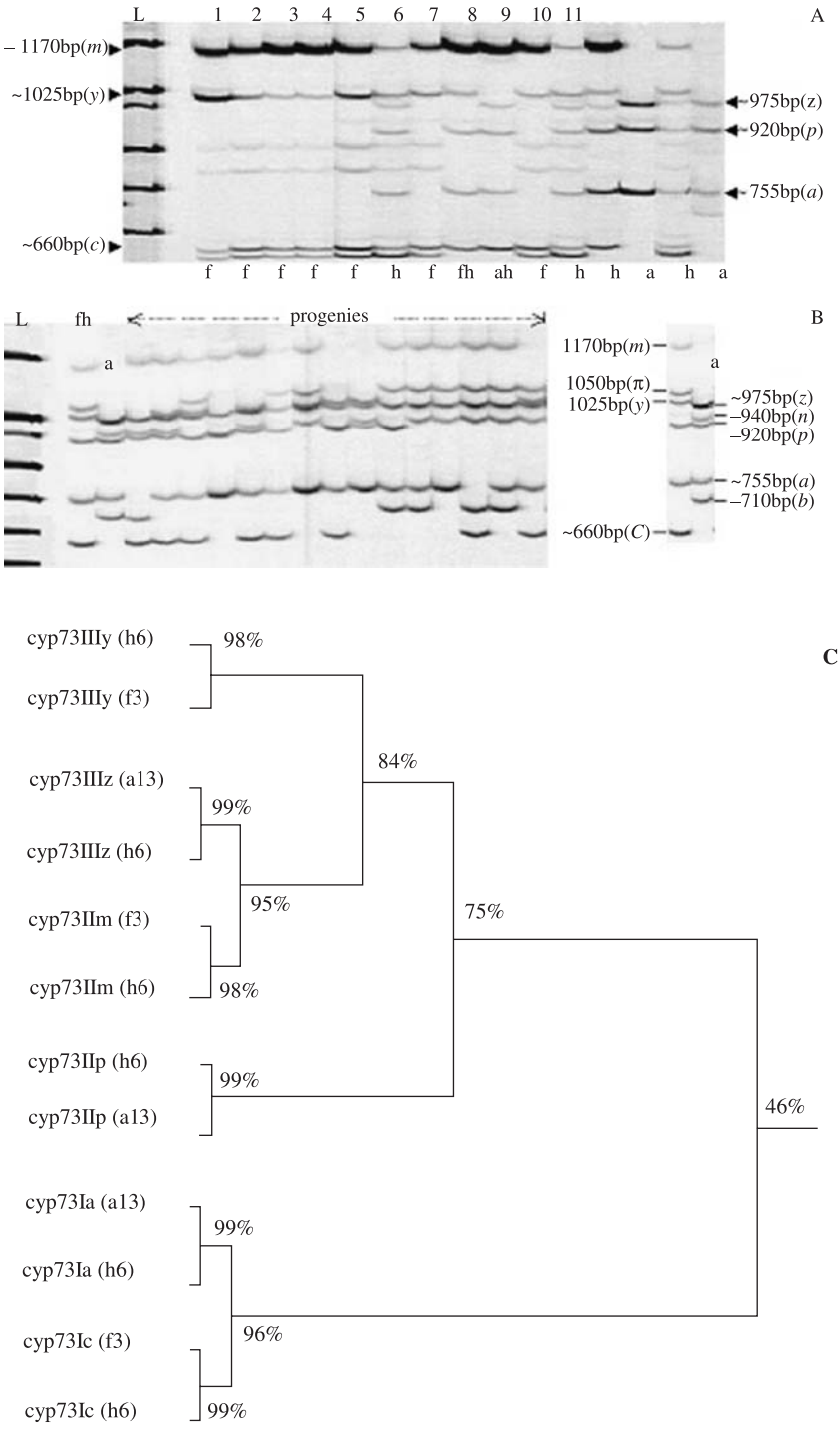

Fig. 1 C4H2 multilocus banding patterns with: (A) diagnostic alleles of *Salix alba* and *Salix fragilis*, first generation and introgressed hybrids (see text); (B) diagnostic alleles of both parental types, a male introgressed *S. fragilis* (fh) and a female *S. alba* (A) and segregation of their progeny; L-GeneRuler (Fermentas); (C) Homology tree of diagnostic allele sequences of *S. alba* (sample a13), *S. fragilis* (sample f3) and hybrid (sample h6); *cyp73I, -II, -III* = names of genes; a, c, m, p, y, z = alleles; % = homology between sequences.

similar to the corresponding alleles of *S. alba* and *S. fragilis* (Fig. 1C). Deletions were present in *cyp73I* for *S. fragilis* (39 and 47 bp); in *cyp73II* for *S. alba* (10, 15, 162, 30 and 29 bp) and in *cyp73III* for *S. alba* (18 bp).

We conclude that alleles of the *cyp73* intron regions allow unambiguous detection of first generation and introgressed hybrids. Banding patterns of both species and hybrids remain consistent at large geographical distance when using C4H2 markers on > 1000 *Salix* samples collected throughout Europe and these markers could be cross-amplified in other willow taxa (*S. x babylonica*, *S. triandra*, *S. viminalis*, *S. caprea* and *S. purpurea*).

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References

Barcaccia G, Meneghetti S, Albertini E, Triest L, Lucchin M (2003) Linkage mapping in tetraploid willows: segregation of molecular markers and estimation of linkage phases support an allotetraploid structure for *Salix alba* × *Salix fragilis* interspecific hybrids. *Heredity*, **90**, 169–180.

Beismann H, Barker JHA, Karp A, Speck T (1997) AFLP analysis sheds light on distribution of two *Salix* species and their hybrid along a natural gradient. *Molecular Ecology*, **6**, 989–993.

Bell-Lelong DA, Cusumano JC, Meyer K, Chapple C (1997) Cinnamate-4-hydroxylase expression in *Arabidopsis*. Regulation in response to development and the environment. *Plant Physiology*, **113**, 729–738.

Kawai S, Mori A, Shiokawa T, Kajita S, Katayama Y, Morohoshi N (1996) Isolation and analysis of cinnamic acid 4-hydroxylase homologous genes from a hybrid aspen, *Populus kitakamiensis*. *Bioscience, Biotechnology, and Biochemistry*, **60**, 1586–1597.

Triest L, De Greef B, De Bondt R, Van Slycken J (2000) RAPD of controlled crosses and clones from the field suggests that hybrids are rare in the *Salix alba–Salix fragilis* complex. *Heredity*, **84**, 555–563.