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W.J. Drost · D.J. Somers · G. Rakow · J.P. Raney

Identification of a mutant fatty acid elongase allele from zero-percent erucic acid *Sinapis alba*

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Abstract The *fae1* gene codes for KCS (β-keto-acyl-CoA synthase), the candidate enzyme for elongation of oleic acid to eicosenoic acid and erucic acid (C22:1) in various oilseed species. Degenerate primers for the *fae1* gene were used to amplify and clone *fae1* gene homologs in high and zero C22:1 *Sinapis alba*. Under stringent PCR conditions, a polymorphism was revealed between the two genotypes and was mapped as a *fae1* marker in an F_2 population derived from a cross between high and zero C22:1 *S. alba*. The *fae1* marker co-segregated with C22:1 content and the C22:1 phenotypic locus. In addition, a set of 11 RAPD markers for C22:1 in *S. alba* was identified. Cloning and sequencing of the *fae1* alleles in high and zero C22:1 *S. alba* revealed two amino-acid substitutions specific to zero C22:1 *S. alba*. The underlying nucleotide substitution for one of the amino-acid substitutions and an adjacent silent nucleotide substitution were used to design primers for allele-specific amplicons for both the wild-type and zero C22:1 alleles. The two diagnostic PCR tests are reliable selection tools to identify *S. alba* carrying one or both of the wild-type and mutant C22:1 alleles of the KCS gene.

Keywords RAPD · *fae1* · Erucic acid · *Sinapis alba*

Introduction

Yellow mustard (*Sinapis alba*) is currently grown for condiment purposes on the Canadian prairies. *S. alba* produces high-quality mustard seed when grown in hot dry climates and there is interest in developing this species as an oilseed and high-protein crop for western Canada. This requires the selection of plants with low erucic acid contents (C22:1). Zero C22:1 (< 0.1%) *S. alba* has been developed at the AAFC Saskatoon Research Centre for this purpose (Raney et al. 1995).

Selection for C22:1 content in *Brassica* and *S. alba* breeding programs is by gas chromatographic (GC) analysis of fatty acid composition utilizing the half-seed technique (Downey and Harvey 1963; Thies 1971). Due to environmental influences on low C22:1 it is difficult to identify the genotype of seed accumulating 0% and approximately 2% C22:1. C22:1 is accumulated at a lower rate during early seed development and accumulation continues until the seed is fully mature (Rakow and McGregor 1974). Therefore, any interruption of seed development leads to artificial levels of C22:1. Immature seed may be identified as zero-percent C22:1 by GC analysis although the seed is genetically programmed to accumulate higher levels (approximately 2%) of C22:1 which leads to erroneous selection of zero C22:1 plants. Ranges of 0–2% C22:1 in a segregating population make differentiating between homozygotes and heterozygotes difficult. Marker-assisted selection (MAS) for C22:1 content would provide a reliable selection tool that is not influenced by the environment in which the seed is produced and does not affect seedling vigour.

The RAPD procedure has been successfully used to identify molecular markers associated with fatty acid contents in several oilseed species. RAPD markers have been identified for palmitic acid (C16:0) content in *Brassica rapa* (Tanhuanpää et al. 1995a), linolenic acid (C18:3) content in *Brassica napus* (Hu et al. 1995; Tanhuanpää et al. 1995b; Jourdren et al. 1996a; Somers et al. 1998; Rajcan et al. 1999), and C22:1 content in *B. napus* (Ecke et al. 1995; Jourdren et al. 1996b; Rajcan et al. 1999). In this present study, RAPD markers for C22:1 were identified and mapped to a single linkage group corresponding to a single locus controlling the level of C22:1 in *S. alba*.

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W.J. Drost · G. Rakow (✉) · J.P. Raney
Agriculture and Agri-Food Canada, Saskatoon Research Centre,
107 Science Place, Saskatoon, Saskatchewan, S7N 0X2, Canada
e-mail: rakowg@em.agr.ca
Tel.: 1-306-956-7235

D.J. Somers
Agriculture and Agri-Food Canada, Cereal Research Centre,
195 Dafoe Road, Winnipeg, Manitoba, R3T 2M9, Canada

The fatty acid elongation 1 (*fae1)* gene is required for the synthesis of very long chain fatty acids (VLCFAs), including C22:1, in *Arabidopsis thaliana* (Kunst et al. 1992). The *fae1* gene encodes β-keto-acyl CoA synthase (KCS), the enzyme which catalyses the first committed step in the oleic acid (C18:1) elongation pathway leading to the VLCFAs eicosenoic acid (C20:1) and erucic acid (C22:1). The *fae1* gene has been cloned in several species that synthesize VLCFAs including *A. thaliana* (James et al. 1995), jojoba (Lassner et al. 1996), *B. napus*, *B. rapa* and *Brassica oleracea* (Fourmann et al. 1998). Amino- and nucleic-acid sequences for *fae1* show a high degree of conservation among these species. Transformation of low C22:1 (LEAR) *B. napus* with the jojoba KCS gene led to the restoration of elongation activity (Lassner et al. 1996) suggesting that the mutations giving rise to the LEAR phenotypes are associated with the *fae1* gene encoding KCS or with KCS regulatory genes (Barret et al. 1998). In amphidiploid *B. napus*, a LEAR phenotype was partly associated with deletions in one of the *fae1* genes, leading to frameshift mutations and premature stop codons (Fourmann et al. 1998). It was hypothesized that these mutations were responsible for the LEAR trait at this locus or that these mutations were secondary mutations in an already non-functional gene.

In the present study, the *fae1* homolog was amplified and sequenced in *S. alba* using consensus primers designed by Fourmann et al. (1998). Unique amino-acid and nucleic-acid sequences for a mutant (zero C22:1) and wild-type *fae1* alleles in *S. alba* were identified and used to design primers for allele-specific amplicons (ASAs). The implications of utilization of these ASAs for MAS in a *S. alba* breeding program are discussed.

Materials and methods

Plant material

A single F_1 plant derived from the cross between the high C22:1 cultivar Sabre (approximately 55% C22:1) (Agriculture Canada 1976) and the zero C22:1 breeding line WD96-2 (< 0.1% C22:1) (Drost et al. 1999) was self-pollinated to produce the C22:1 mapping population consisting of 92 F_2 plants. Parent, F_1, and F_2 plants were grown in controlled conditions and young leaf tissue (4 weeks) was collected from parent and F_2 plants. All plants were analysed for fatty acid composition using the non-destructive half-seed technique (Downey and Harvey 1963) and the gas chromatographic method of Thies (1971) with minor modifications.

For testing of the allele-specific amplicons for zero and wild-type C22:1, the cultivars Sabre and AC Pennant, and the breeding lines WD96-1 and WD96-2 were used. Sabre was bred for high C22:1 levels (approximately 55% C22:1). AC Pennant was bred for yield and agronomic features and has an average C22:1 level of 35%. WD96-1 has low C22:1 levels (average 2%) but is not devoid of C22:1. WD96-2 does not synthesize C22:1 (< 0.1% C22:1).

DNA extraction and PCR

DNA was extracted from 50 mg of ground, freeze-dried leaf tissue in a 1.5-ml microtube by the addition of 1 ml of extraction buffer [0.1 M Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1 M KCl, 0.5% (v/v) SDS] followed by incubation at 80°C for 10 min. Cellular debris was pelleted by centrifugation and the supernatant was transferred to a new microtube containing RNAse and incubated at 37°C for 20 min. The DNA was precipitated with cold isopropanol followed by centrifugation. The supernatant was removed, the DNA pellet was air-dried for 1.5 h, and the DNA was resuspended in 300 µl of water. The DNA was re-precipitated with NaOAc and EtOH. After centrifugation, the supernatant was removed and the DNA pellet was air-dried for 4 h. The DNA was resuspended in 150 µl water by incubation at 50°C and stored at 4°C. The DNA was quantified by fluorimetry using Hoechst 33258 stain and the DNA was diluted with water to 2.5 ng/µl.

Bulked DNA samples for F_2 plants of the zero C22:1 phenotype and high C22:1 phenotype were prepared by combining equal amounts of DNA from each of ten lowest C22:1 F_2 plants and ten highest C22:1 F_2 plants. The bulked segregant analysis (BSA) included DNA from the parental plants (WD96-2 and Sabre) and DNA from zero and high C22:1 bulked samples.

A total of 485 random 10-bp primers from the University of British Columbia were screened by BSA (see www.biotech.ubc.ca/services/naps/primers.html for primer sequences). The PCR reaction consisted of the following reagents: 1U of *Taq* DNA polymerase, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 10 ng of DNA, and 0.2 µM of primer. The PCR amplification protocol was 1.5 min at 95°C, 35 cycles of (20 s at 95°C, 1 min at 36°C, ramp 1°C/s to 72°C, 1.5 min at 72°C), and 7 min at 72°C.

Products of the PCR reactions were separated by agarose-gel electrophoresis using 2% (w/v) agarose gels in 1 × TAE. Electrophoresis was at 100 V for 3 h. The DNA in gels was stained with ethidium bromide and photographed.

Cloning of the *fae1* gene

The *fae1* gene was amplified from *S. alba* using degenerate primers designed on the *A. thaliana* and *B. napus fae1* sequences [Genbank database U29142 (James et al. 1995) and U50771 (Barret et al. 1998)] as described by Fourmann et al. (1998) (Table 1, non-specific). The PCR reaction for amplification of the *fae1* alleles in WD96-2 and Sabre consisted of 1U of *Taq* DNA polymerase, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 10 ng of DNA, and 0.1 µM of each forward and reverse primer in a 20-µl volume. The PCR amplification protocol was 4 min at 94°C, 30 cycles of (1 min at 94°C, 1 min at 53°C, 2 min at 72°C), and 4 min at 72°C. The degenerate primers selectively amplified the Sabre allele (high C22:1) under more-stringent PCR conditions (1.5 mM $MgCl_2$ and primer annealing at 58°C for 1 min).

The *fae1* alleles amplified from Sabre and WD96-2 were ligated into pGEM T Easy (Promega, USA) and sequenced using fluorescent sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, USA.) The Sabre *fae1* allele was sequenced five times and the WD96-2 *fae1* allele was sequenced twice to verify the nucleotide sequences.

The PCR reactions for the WD96-2 and Sabre ASA tests consisted of 1U of *Taq* DNA polymerase, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 ng of DNA, and 0.1 µM of each forward and reverse primer in a 20-µl volume. The PCR amplification protocol for the WD96-2 ASA test was 4 min at 94°C, 29 cycles of (1 min at 94°C, 1 min at 64°C, 2 min at 72°C), and 4 min at 72°C. The PCR amplification protocol for the Sabre ASA test was 4 min at 94°C, 30 cycles of (1 min at 94°C, 1 min at 60°C, 2 min at 72°C), and 4 min at 72°C. Products of the PCR reactions were separated by agarose-gel electrophoresis in the same manner as for the RAPD PCR reactions.

Data analysis

Segregation for C22:1 content in the F_2 population was tested for goodness of fit to 1:2:1 and 1:3 ratios using the chi-squared analysis.

Table 1 Primer sequences designed to amplify *fae1*. * Selective primers

Sequence (5' to 3')[a]	Allele	Fragment length (bp)
CGGAGCTGACGACAARWCNTT TCGATGCAGTGYTSCCANGG	Non-specific[b]	543
GAGCGGCAAAACCGGAGTGAG TGCTTCTATGTATGCCAATTCATACCCA*	WD96-2	361
ACTTCTTTTTTTCRTTACCTTCA ATGTATGCCAATTCGTACCAT*	Sabre (wild-type)[c]	243

[a] R = A + G, W = A + T, N = A + C + T + G, Y = C + T, S = C + G
[b] Non-specific primers are degenerate (Fourmann et al. 1998)
[c] Wild-type primers amplify the *fae1* allele from Sabre, AC Pennant, and WD96-1

Segregation of the C22:1 RAPD markers and of the *fae1* gene was studied in the F_2 population. Scoring of polymorphic bands was by presence or absence only. Linkage analysis of segregation data for the C22:1 locus, RAPD markers and the *fae1* marker was performed using MAPMAKER V3.0 (Lander et al. 1987; Lincoln et al. 1992) using a minimum LOD of 3.0 and a maximum recombination fraction of 0.3. Map distances were expressed as centiMorgans using the Kosambi function (Kosambi 1944).

The DNA and peptide sequence alignments were performed using the Martinez/Needleman-Wunsch DNA Alignment and the Lipman-Pearson Protein Alignment in DNAStar-MegAlign software.

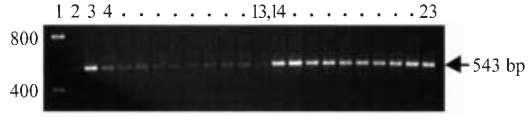

Fig. 1 PCR profile amplified under stringent conditions with degenerate *fae1* primers on *S. alba*. *Lane 1* molecular weight marker, *lane 2* zero C22:1 parent WD96-2, *lane 3* high C22:1 parent Sabre, *lanes 4–13* zero C22:1 F_2 plants, *lanes 14–23* high C22:1 F_2 plants

Results

Fatty acid analysis

The C22:1 frequency distribution of the F_2 population segregated into three classes (< 0.3%, 22–44%, and > 44% C22:1). Single gene control was confirmed by chi-squared analysis for the 1:2:1 ($p = 0.59$) and the 1:3 (< 0.3%:> 22% C22:1) ($p = 1.0$) segregation ratios. F_3 progeny testing of F_2 plants used for bulk formation (ten zero C22:1, ten high C22:1) revealed no F_3 seeds with > 0.1% C22:1 from zero C22:1 F_2 segregant plants, and no F_3 seeds with < 38.0% from high C22:1 F_2 segregant plants.

Bulked segregant and linkage analyses

A total of 485 UBC 10 bp primers were screened by BSA. Ten primers amplified 11 marker polymorphic DNA fragments that were linked to C22:1 content. These markers were tested in the 72 remaining individuals of the mapping population. An additional marker was generated using the *fae1* degenerate primers which selectively amplified a DNA fragment of 543 bp from Sabre template DNA (high C22:1) under stringent conditions (Fig. 1).

The 11 RAPD markers, the *fae1* marker, and C22:1 phenotypic data were mapped to a single linkage group of 22.6 cM by analysis of segregation data using MAPMAKER V3.0 (Fig. 2). The *fae1* marker mapped directly to the C22:1 locus and co-segregated with the C22:1 trait in this mapping population.

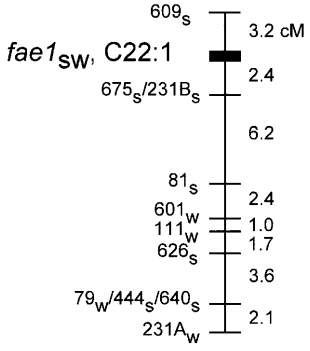

Fig. 2 Linkage map of the *fae1* gene and RAPD markers associated with C22:1 content in *S. alba*. Subscripts W (WD96-2) and S (Sabre) indicate the allele detected with each marker. Map distances are reported in Kosambi centiMorgans. Total length of the linkage group is 22.6 cM

fae1 sequence analysis

The *fae1* degenerate primers amplified a single 543-bp fragment from both parents, and under more-stringent PCR conditions the band was only amplified from Sabre (high C22:1). The *S. alba fae1* gene from WD96-2 and Sabre was confirmed to be the KCS gene by alignment with known KCS gene sequences from *A. thaliana* (genebank accession U29142), *Brassica juncea* (Y11007), *B. napus* (AF054497, AF054498), *B. rapa* (AF054499) and *B. oleracea* (AF054500). Similarity indices of the *fae1* nucleotide sequences of the *Brassica* spp. and *A. thaliana* to those of *S. alba* WD96-2 and Sabre ranged from 86.5 to 95.8. The *fae1* sequences for WD96-2 and Sabre were of identical length (543 nucleotides) and differed only in ten nucleotide substitutions (Fig. 3). The two sequences did not contain introns and, when trans-

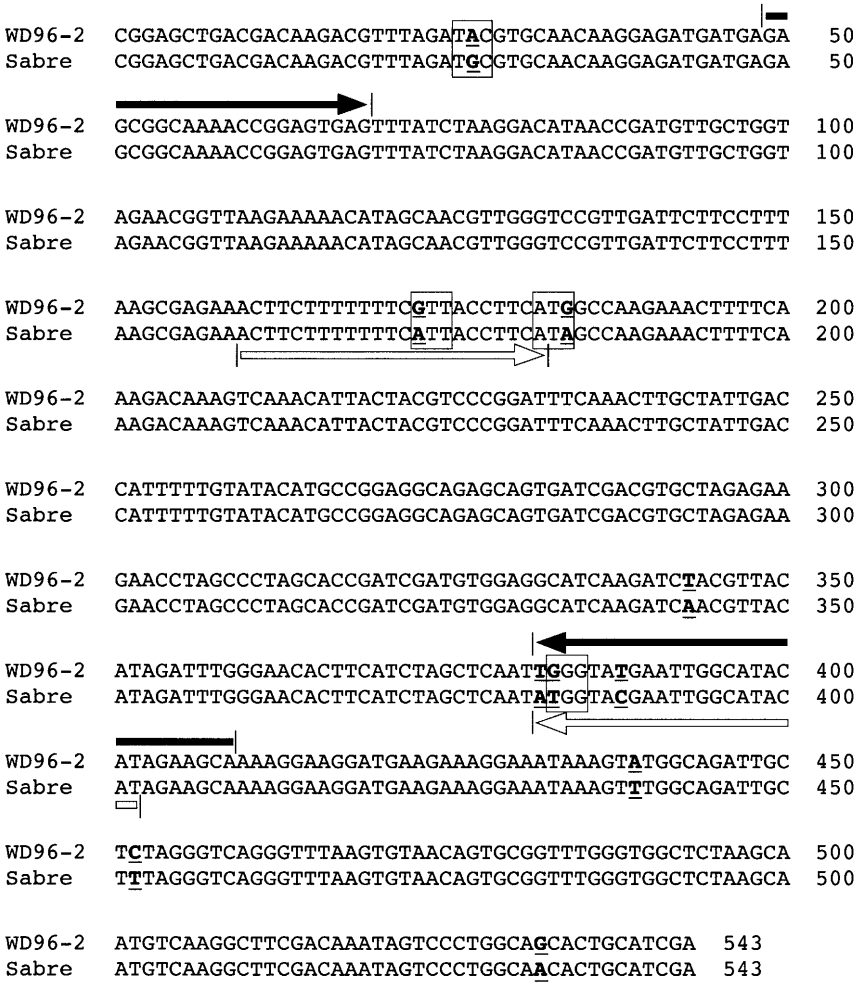

Fig. 3 Alignment of the partial nucleotide sequence (543 bp) of the KCS gene present in the *S. alba* line WD96-2 and cv Sabre. *Underlined nucleotides in bold print* indicate substitutions; *outlined areas* indicate codons with nucleotide differences resulting in amino-acid differences between the WD96-2 and Sabre alleles. *Solid arrows* indicate the primer pair for the amplification of the WD96-2 allele; *open arrows* indicate the primer pair for the amplification of the Sabre allele

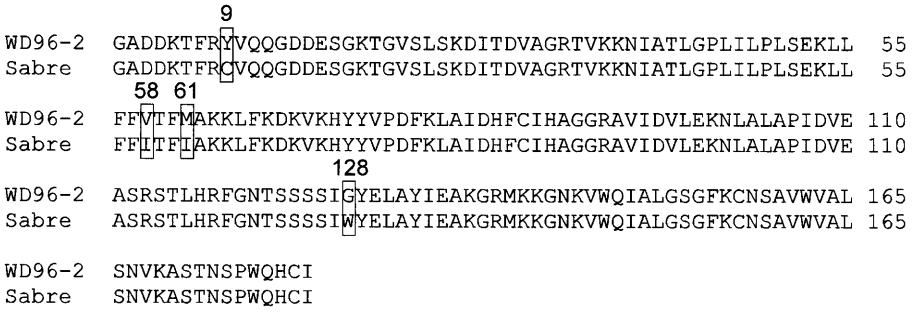

Fig. 4 Alignment of the partial peptide sequence (180 aa) of the KCS gene present in the *S. alba* line WD96-2 and cv Sabre. *Outlined areas* indicate amino-acid differences between the two alleles

lated in-frame revealed four amino-acid changes due to four of the nucleotide substitutions (Figs. 3, 4). The amino-acid changes at positions 58 and 61 were conserved changes, whereas changes at position 9 and 128 were non-conserved. When compared to the *Brassica* spp. and *A. thaliana* FAE1 peptide sequences, two of the amino-acid changes were specific for the WD96-2 allele (Fig. 4, position 9, 128) and the two other changes were specific for the Sabre allele (Fig. 4, position 58, 61). The underlying nucleotide substitution for the amino-acid change at residue 128 (Fig. 3, nucleotide 383) and an adjacent silent nucleotide substitution (Fig. 3, nucleotide 382) were used in the development of ASAs for the WD96-2 and Sabre alleles. The amino-acid change at residue 128 was the result of a single change (point mutation) at nucleotide 383 of the WD96-2 KCS sequence. Although the nucleotide substitution at position 382 does not affect the amino-acid sequence, it was still used in the ASA primer designs at the direct 3' end of the selective primers and therefore this nucleotide substitution also contributes to the allele specificity of the selective primers. The pair of PCR primers synthesized based on the WD96-2 KCS allele sequence included the selective primer for the WD96-2 allele (Fig. 3, 5′ to 3′: nt 409–382) and a non-selective primer (Fig. 3, 5′-3′: nt 48–69) (Table 1, WD96-2). With these PCR primers and

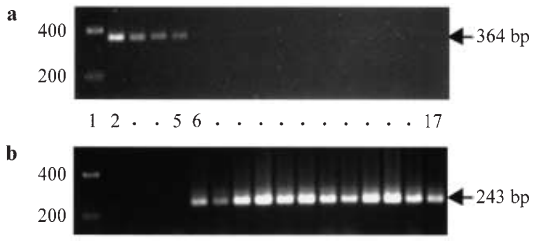

Fig. 5 PCR profile produced by amplification of the KCS allele present in **a** zero C22:1 line WD96-2 (Table 1, WD96-2 primers) and **b** wild-type C22:1 accessions (Table 1, Sabre primers). *Lane 1* molecular weight marker, *lanes 2–5* WD96-2 accessions, *lanes 6–8* Sabre accessions, *lanes 9–14* WD96-1 accessions, *lanes 15–17:* AC Pennant accessions

under stringent conditions, the WD96-2 allele could be specifically amplified. The zero C22:1 ASA was tested against Sabre (approximately 55% C22:1), AC Pennant (35% C22:1) and WD96-1 (2% C22:1), and only amplified the 0% C22:1 allele derived from WD96-2 (Fig. 5a). In addition, a pair of PCR primers was synthesized based on the Sabre KCS allele sequence to specifically amplify the *S. alba* Sabre allele. This included a selective primer for the Sabre allele (Fig. 3, 5′-3′: nt 402–382) and a non-selective primer (Fig. 3, 5′-3′: nt 160–182) (Table 1, Sabre). With these primers, and under stringent conditions, the Sabre allele was specifically amplified. This PCR reaction was also tested against WD96-2, AC Pennant and WD96-1. The PCR primers amplified the wild-type alleles found in Sabre, AC Pennant and WD96-1, but not the mutant WD96-2 allele (Fig. 5b).

The WD96-2 and Sabre ASA tests for C22:1 were also tested in the F_2 population of the cross between Sabre and WD96-2. The tests correctly identified zero C22:1 homozygotes (band present for WD96-2 ASA, band absent for Sabre ASA), high C22:1 homozygotes (band absent for WD96-2 ASA, band present for Sabre ASA), and intermediate C22:1 heterozygotes (bands present for WD96-2 and Sabre ASAs).

Discussion

The BSA was very efficient and successful in identifying a set of 11 RAPD markers associated with C22:1 content in *S. alba*. A 543-bp DNA fragment was amplified in *S. alba* utilizing the *fae1* degenerate primers known to amplify the 3′ half of the *fae1* gene in *B. napus*, *B. rapa*, *B. oleracea* and *A. thaliana*. The *fae1* gene was amplified in WD96-2 (zero C22:1) and Sabre (high C22:1) with low stringency PCR conditions. Stringent PCR conditions (1.5 mM $MgCl_2$, 58°C annealing temperature) eliminated amplification of the band in WD96-2 and thus provided an additional molecular marker for C22:1 content. Since the *fae1* marker for the candidate gene controlling C22:1 content co-segregated with the C22:1 locus (Fig. 2), this marker could act as a precise diagnostic test for the presence of the allele conferring C22:1 production.

The identification of a single linkage group controlling C22:1 content in *S. alba* is consistent with genetic segregation data indicating single gene control (Drost et al. 1999). The high marker density over this 22.6-cM interval may be attributed to the high degree of polymorphism between the parental lines and to the large number of primers tested.

The structure and function of a specific enzyme are dependent on the interaction of side-chains of the amino-acids that make up that enzyme. Changes in the physical shape or polarity of the side chains may alter the structure and function of the enzyme, particularly if the changes occur in the active site of the enzyme. In this study, the non-conserved amino-acid substitutions included (changes from Sabre to the WD96-2 *fae1* allele): Cysteine (non-polar, S-containing) to Tyrosine (polar, ring structure) (position 9, Fig. 4), and Tryptophan (non-polar, double-ring structure) to Glycine (non-polar, simple structure) (position 128, Fig. 4). Although the nucleotide and peptide sequences of the *fae1* gene are known in several species, little is known about the structure of KCS or of the sequence of the active site. However, it is possible that one or both of the non-conserved amino-acid changes led to changes in the structure of KCS and to the absence of C22:1 in WD96-2. Based on this hypothesis, specific primers were designed on the nucleotide sequences for the second non-conserved change (position 128, Fig. 4). Alternatively, it is possible that the changes represent mutations in an already non-functional gene since the cloned sequences for the *S. alba fae1* gene represent only part of the gene. Furthermore, the mutation giving rise to the zero C22:1 *S. alba* may affect KCS regulatory genes rather than the *fae1* gene encoding KCS. This is plausible only if the regulatory gene is very tightly linked to the *fae1* gene so that no recombination would occur between the phenotypically mapped C22:1 locus and the *fae1* gene. In *B. napus*, two *fae1* alleles were sequenced from a low C22:1 genotype and one of them (*fae1.2*) was found to contain deletions, resulting in frameshift mutations and premature stop codons that may have been responsible for the low C22:1 trait at this locus (Fourmann et al. 1998). The zero C22:1 *fae1* allele in *S. alba* is distinctly different from the low C22:1 *fae1.2* allele in *B. napus* in that only amino-acid substitutions and no changes in the reading frame were observed.

The sequence information from the *S. alba fae1* alleles was utilized for the development of ASAs for the WD96-2 and Sabre alleles. The ASAs will provide a very efficient molecular marker system for C22:1 content in *S. alba*. Screening a segregating population with both of these markers will allow identification of zero C22:1 homozygotes, intermediate C22:1 heterozygotes, and high C22:1 homozygotes.

The PCR test and primer pair designed on the Sabre sequence identified genotypes that synthesize C22:1 and do not carry the WD96-2 mutation. This test does not, however, distinguish between genotypes that synthesize C22:1 and does not predict the level of C22:1 that is

synthesized by these genotypes. The ASA test using the Sabre (wild-type) primers (Table 1) identifies the wild-type KCS allele only. Specific mutations associated with extremely high levels of C22:1 (i.e., in Sabre), intermediate levels of C22:1 (i.e., in AC Pennant), and low levels of C22:1 (i.e., in WD96-1) may be located in different parts of the gene that were not identified in this study.

The PCR test and the primer pair that amplifies the WD96-2 allele provide a precise method to determine the genotype of accessions of *S. alba* with respect to the KCS gene and thus the C22:1 levels. This test is not influenced by the quality of the seed, the environment in which the seed is produced or the environment in which the seedling is grown. This PCR test provides a robust, high throughput, diagnostic test for all oilseed *S. alba* carrying this 0% C22:1 allele of the KCS gene.

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