Inheritance of Mid and High Oleic Acid Content in Ethiopian Mustard

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ABSTRACT

Zero erucic acid Ethiopian mustard (Brassica carinata A. Braun) seed oil has a low oleic acid content (330 g kg$^{-1}$). The mid oleic acid line AB3 (650 g kg$^{-1}$) and the high oleic acid line AB1 (830 g kg$^{-1}$) have been developed. The objective of this research was to study the inheritance of both traits in Ethiopian mustard. Plants of AB1 were reciprocally crossed with plants of the standard line 25X-1, the low linolenic acid line AB4, and AB3. Plants of AB3 were also reciprocally crossed with plants of 25X-1. A genetic study was conducted through the analysis of the fatty acid profile of F$_1$, F$_2$, BC$_1$F$_1$, and F$_3$ seed generations. The results revealed that mid oleic acid content in AB3 was determined by partially recessive alleles at a single locus Ol, whereas high oleic acid content in AB1 was the result of partially recessive alleles at two loci, Ol and OI2. Both loci produced an increment of oleic acid content of similar magnitude, although OI2 alleles had phenotypic expression only in presence of ol alleles in homozygous condition. Segregation patterns for oleic acid content were similar in crosses of AB1 with 25X-1 and AB4, which indicated that loci for high oleic acid content were not related to loci for low linolenic acid content. Recombination of genes controlling high oleic acid and low linolenic acid content resulted in phenotypes with $>$850 g kg$^{-1}$ oleic acid and $<$25 g kg$^{-1}$ linolenic acid. Reduction of linolenic acid content resulted in an increase of linoleic rather than oleic acid content.

Ethiopian mustard is a potential oilseed crop that is considered as an alternative to the other Brassica species for dry areas (Fereres et al., 1983). Even though zero erucic acid types of this species have been developed (Alonso et al., 1991; Getinet et al., 1994; Fernández-Martínez et al., 2001), cultivars with the typical low glucosinolate content of canola crops are not available yet (Alemayehu and Becker, 2002).

Vegetable oils with increased levels of monounsaturated oleic acid in combination with reduced levels of the polyunsaturated linoleic acid and linolenic acid show a higher oxidative stability as well as lower oxidation products (McVetty and Scarth, 2002). Conventional canola (zero erucic, low glucosinolate cultivars of Brassica spp., mainly B. napus L.) oil has a typical oil profile made up of 610 g kg$^{-1}$ oleic acid, 210 g kg$^{-1}$ linoleic acid, and 110 g kg$^{-1}$ linolenic acid as the major fatty acids (Scarth and McVetty, 1999). Canola germplasm with mid ($>$700 g kg$^{-1}$) and high ($>$800 g kg$^{-1}$) oleic acid content has been developed in B. napus (Wong and Swanson, 1991; Auld et al., 1992; Rücker and Röbbelen, 1997; Stoutjesdijk et al., 1999), B. rapa L. (Auld et al., 1992; Tanhuanpää et al., 1996), and B. juncea (L.) Czern. (Stoutjesdijk et al., 1999; Sivaraman et al., 2004). Compared with current canola cultivars, standard zero erucic acid types of Ethiopian mustard are characterized by about a twofold polyunsaturation level in the seed oil, with an average content of 330 g kg$^{-1}$ oleic acid, 370 g kg$^{-1}$ linoleic acid, and 210 g kg$^{-1}$ linolenic acid (Alonso et al., 1991; Getinet et al., 1994; Fernández-Martínez et al., 2001).

Genetic studies on high oleic acid mutants of B. napus concluded that high oleic acid content in the seed oil was mainly explained by alleles with additive effects at a single locus designated HO1 (Schierholt et al., 2001), which was thought to correspond with one copy of the FAD2 (microsomal olate desaturase) gene (Schierholt et al., 2000). Additionally, the authors identified a second locus, HO2, with weak expression in seeds but marked expression in leaves and roots. Möllers (2002) suggested the additional presence of several genes with minor effect in germplasm with a high oleic acid content around 850 g kg$^{-1}$. In B. rapa, Tanhuanpää et al. (1996) found a single QTL affecting oleic acid concentration in a high oleic acid line.

Sources of increased oleic acid and reduced linolenic acid content were initially developed in high erucic acid Ethiopian mustard. Following chemical mutagenesis, Velasco et al. (1997a) isolated three mutants (N2–1992, N2–3591, and N2–6285) with an increased oleic acid content of about 200 g kg$^{-1}$, compared with 100 g kg$^{-1}$ in the wild type, and a mutant (N2–4961) with a reduced linolenic acid content of about 60 g kg$^{-1}$, compared with 120 g kg$^{-1}$ in the wild type. A second source with similar reduced levels of linolenic acid (HF-186) was developed by Velasco et al. (1997b) through selection from a germplasm accession of this species.

Increased oleic acid content in the mutant N2–3591 was found to be controlled by partially recessive alleles at a single locus designated Ol (Velasco et al., 2003b). Crosses between plants of N2–3591 and HF-186 resulted in F$_2$ transgressive segregants with higher oleic acid content than the N2–3591 parent. It was hypothesized that transgressive segregation was the result of the presence of a second locus for increased oleic acid in the low linolenic acid line HF-186, which only would have a phenotypic effect on oleic acid content if the recessive alleles ol are present in homozygous condition (Velasco et al., 2003a). Further crosses of the transgressive segregants with zero-erucic acid germplasm led to the isolation of a mid oleic acid line (707 ± 24 g kg$^{-1}$) and a high oleic acid line (839 ± 10 g kg$^{-1}$), in both cases with zero erucic acid content (Velasco et al., 2003a). The objective of the present research was to study the inheritance of mid and high oleic acid content in Ethiopian mustard.

MATERIALS AND METHODS

Plant Material

AB1 and AB3 are the abbreviated names for the high oleic, zero erucic acid line AB01323 and the mid oleic, zero erucic acid line AB01169, respectively, developed by Velasco et al.
Genetic Study

Seeds of AB1, AB3, AB4, and 25X-1 were germinated on moistened filter paper in November 2001, and half seeds were analyzed for seed oil fatty acid profile by gas-liquid chromatography (GLC) to ensure that the plants used in the genetic study bred true for this trait. Remaining half seeds were transplanted into pots and grown in an insect-proof screenhouse. Plants of AB1 were reciprocally crossed with plants of AB3, AB4, and 25X-1, and plants of AB3 were reciprocally crossed with plants of 25X-1. Forty-eight F1 half seeds from each reciprocal cross as well as 48 seeds from each parent were analyzed for fatty acid composition. Twelve F1 half seeds from each reciprocal cross and 12 half seeds from each parent were randomly selected and the corresponding plants were grown in the greenhouse in winter 2002–2003. F1 plants from reciprocal crosses were self-pollinated to obtain F2 seeds and also backcrossed to both parents, except for the crosses involving AB3, for which backcrosses were not made. Reciprocal crosses were repeated to obtain F1 seeds under the same environment as the F2 and BC1F1 seeds.

To confirm segregation ratios observed in the F2 generation, a random set of 170 F2 seeds of a population from the cross between 25X-1 and AB1 were germinated, analyzed by the half-seed technique, and the corresponding F2 plants grown in the field in summer 2004. Seventy-two individual F3 seeds from every F2 plant were analyzed for fatty acid composition.

In all cases, microperforated plastic bags were used to prevent cross pollination at flowering. Plants of AB1, AB3, AB4, and 25X-1 were grown as checks in all generations. The number of populations and half seeds analyzed per generation and cross was variable and it is given in the results section. The Chi-square test was used to evaluate proposed segregation ratios. Reciprocal F1 means were compared by independent t tests.

Seed Oil Fatty Acid Analyses by Gas-Liquid Chromatography

The fatty acid composition of the seed oil was determined by simultaneous oil extraction and methyl esterification (Garcés and Mancha, 1993) followed by gas-liquid chromatography (GLC) of fatty acid methyl esters on a PerkinElmer Autosystem gas-liquid chromatograph (PerkinElmer Corporation, Norwalk, CT) equipped with a 2-m long column packed with 3% SP-2310/2% SP-2300 on Chromosorb WAW (Supelco Inc., Bellefonte, PA). A temperature program of 190°C for 10 min, increasing 2°C min⁻¹ up to 220°C was used. The injector and flame ionization detector were held at 275 and 250°C, respectively.

RESULTS

Crosses between AB1 and 25X-1

Seeds of AB1 had a high oleic acid content of 841.0 ± 17.9 g kg⁻¹ (mean ± SD), whereas those of 25X-1 showed a standard oleic acid content of 334.9 ± 46.7 g kg⁻¹. Oleic acid averaged 500.4 ± 39.3 g kg⁻¹ in F1 seeds from the cross AB1 × 25X-1, compared with 462.9 ± 50.4 g kg⁻¹ in the reciprocal cross, which indicated a partial maternal effect on this trait (t = 3.48, p < 0.01). The average oleic acid content of the reciprocal F1 seeds was 481.4 g kg⁻¹, significantly (t = 3.46, p < 0.01) lower than the midparent value (588.0 g kg⁻¹), indicating partial dominance of standard over high oleic acid content. The average oleic acid concentration of F2 seeds was 536.7 g kg⁻¹ in the cross AB1 × 25X-1, which was not significantly (t = 1.13, p > 0.05) different from the average oleic acid concentration of 548.5 g kg⁻¹ in the reciprocal cross. The data revealed absence of cytoplasmic effects.

Oleic acid content of F2 seeds ranged from the lower limit of the 25X-1 parent (around 350 g kg⁻¹) to the upper limit of the AB1 parent (around 870 g kg⁻¹). The F2 population showed discontinuities at around 600, 700, and 800 g kg⁻¹ (Fig. 1). However, as class limits could not be clearly established, F2 seeds derived from 170 F2 plants randomly selected were analyzed for fatty acid profile.

Average oleic acid content of F2:3 families was highly correlated (r = 0.90) with that of the corresponding F2 half seeds. The evaluation of F2:3 families allowed a clear identification of a high oleic acid parent class composed of 11 F2 genotypes, which fit a 15:1 (low + mid: high oleic acid/standard) segregation pattern.

Fig. 1. Histogram of oleic acid content (g kg⁻¹) in an F2 population from a cross between the zero erucic acid Ethiopian mustard lines AB1, with high oleic acid content, and 25X-1, with standard fatty acid profile.
acid) ratio ($\chi^2 = 0.01, p = 0.94$). This corresponded to the segregation of alleles at two recessive or partially recessive loci. Following the nomenclature of Velasco et al. (2003a), the high oleic acid trait is expressed by genotypes with the allelic configuration ololol2ol2. Apart from the nonsegregating high oleic acid class, the analysis of F$_{2:3}$ families revealed the existence of two other nonsegregating classes (Fig. 2). The first class (I) included 40 F$_{2:3}$ families characterized by an average oleic acid content between 277.3 and 468.4 g kg$^{-1}$ and a standard deviation below 55 g kg$^{-1}$. They corresponded to the genotypes OIOL$_{-}$ (4/16 of the total population; $\chi^2 = 0.20, p = 0.66$). The second class (II) included 12 F$_{2:3}$ families characterized by an average oleic acid content between 533.0 and 676.4 g kg$^{-1}$ and a standard deviation below 55 g kg$^{-1}$. They corresponded to the genotypes ololOl2Ol2 (1/16 of the total population; $\chi^2 = 0.04, p = 0.84$). The nonsegregating high oleic acid class is marked as III in Fig. 2. The other F$_{2:3}$ families not included in the three mentioned classes showed segregation for oleic acid content. They corresponded to the genotypes Olol$_{-}$ (8/16 of the total population) and ololOl2ol2 (2/16 of the total population). The oleic acid content of individual seeds from the F$_{2:3}$ families corresponding with genotypes OIOL$_{-}$ (class I), ololOl2Ol2 (class II), and ololOl2ol2 (class III) is presented in Fig. 3. F$_3$ seeds of class I averaged 395.5 g kg$^{-1}$, with a range of variation from 272.5 to 474.8 g kg$^{-1}$. F$_3$ seeds of class II averaged 605.2 g kg$^{-1}$, ranging from 444.4 to 730.2 g kg$^{-1}$. F$_3$ seeds of class III averaged 833.2 g kg$^{-1}$, with a range of variation from 783.6 to 879.0 g kg$^{-1}$.

Two BC$_1$F$_1$ to AB1 populations were analyzed. They both showed trimodal distributions, in which a low, a mid, and a high oleic acid class could be distinguished (Fig. 4). In both cases, segregation followed 2:1:1 (low:mid:high oleic acid) ratios ($\chi^2 = 4.0, p = 0.13$ in population A, $\chi^2 = 4.0, p = 0.47$ in population B), which corresponded with the theoretical segregation of genotypes Olol$_{-}$, ololOl2ol2, and ololOl2ol2, respectively.

No discrete classes could be identified in two BC$_1$F$_1$ to 25X-1 populations, which included both OIOL$_{-}$ and Olol$_{-}$ genotypes (Fig. 4).

### Crosses between AB3 and 25X-1

Seeds of AB3 had a mid oleic acid content of 665.1 ± 23.6 g kg$^{-1}$, whereas those of 25X-1 showed a standard oleic acid content of 334.9 ± 46.7 g kg$^{-1}$. Oleic acid averaged 451.0 ± 44.6 g kg$^{-1}$ in F$_1$ seeds from the cross 25X-1 × AB3. The number of F$_1$ seeds obtained in the reciprocal cross was too low to enable a statistical comparison of reciprocal F$_1$s. The average oleic acid content of the F$_1$ generation was significantly ($t = 2.79, p < 0.01$) lower than the midparent value (500.0 g kg$^{-1}$), indicating partial dominance of standard over mid oleic acid content.

Oleic acid content in F$_2$ seeds ranged from 262.3 to 773.1 g kg$^{-1}$ with a discontinuity around 620 g kg$^{-1}$ (Fig. 5). The mid oleic acid class represented about one fourth of the total population (172 out of 624 F$_2$ seeds; $\chi^2 = 2.19, p = 0.14$). The data indicated that mid oleic acid in AB3 is controlled by partially recessive alleles at a single locus.
Crosses between AB1 and AB3

Seeds of AB1 had a high oleic acid content of 841.0 ± 17.9 g kg$^{-1}$, whereas those of AB3 showed a mid oleic acid content of 665.1 ± 23.6 g kg$^{-1}$. Oleic acid averaged 763.5 ± 28.3 g kg$^{-1}$ in F1 seeds from the cross AB1 × AB3, compared with 715.4 ± 18.3 g kg$^{-1}$ in the reciprocal cross, which indicated a partial maternal effect on this trait ($t = 5.62$, $p < 0.01$). The average oleic acid content of the F1 generation was 740.6 g kg$^{-1}$, which was not significantly different ($t = 1.36$, $p > 0.05$) from the mid-parent value (753.1 g kg$^{-1}$), indicating absence of dominance effects in this cross. A comparison of the average oleic acid concentration of F2 seeds in reciprocal crosses was not computed, since only one F2 population of each cross was analyzed.

Oleic acid in F2 seeds from the cross AB1 × AB3 (Fig. 6) ranged from 631.7 g kg$^{-1}$, which is around the lower limit of the AB3 parent, to 852.7 g kg$^{-1}$, around the upper limit of the AB1 parent. Even though discrete class limits were not evident, F2 segregation resembled a 1:2:1 segregation ratio, which would correspond to the genotypes $o1o2o1o2$, $o1o2o1o2$ and $o1o2o1o2$, respectively. A similar range of variation was observed in F2 seeds from the cross AB3 × AB1 (Fig. 6). In this case, oleic acid content showed a bimodal distribution in which the high oleic acid class (>790 g kg$^{-1}$) represented about one fourth of the population ($\chi^2 = 2.37$, $p = 0.12$), which confirmed that differences for oleic acid content between AB1 and AB3 are produced by alleles at the Ol2 locus.

Crosses between AB1 and AB4

AB1 was developed from a transgressive segregant that resulted from a cross between a line with increased oleic acid content, N2–3591, and a line with low linolenic acid content, HF-186 (Velasco et al., 2003a). The authors hypothesized that the high oleic acid content of AB1 might be partially produced by the effect of alleles for low linolenic acid content from HF-186. Since AB4 contains all the genes for low linolenic acid content present in HF-186, crosses between AB1 and AB4 were made to confirm the existence of two genes involved in the high oleic acid trait in populations with no segregation of genes related to the low linolenic acid trait of HF-186. Since AB4 contains all the genes for low linolenic acid content present in HF-186, crosses between AB1 and AB4 were made to confirm the existence of two genes involved in the high oleic acid trait in populations with no segregation of genes related to the low linolenic acid trait of HF-186.

Seeds of AB1 had 841 ± 17.9 g kg$^{-1}$ oleic acid and 46.9 ± 12.5 g kg$^{-1}$ linolenic acid, compared with 378.6 ± 31.1 g kg$^{-1}$ oleic acid and 19.8 ± 6.7 g kg$^{-1}$ linolenic acid in AB4 seeds. F1 seeds showed an intermediate oleic acid content closer to that of the AB4 parent (493.7 ± 29.2 g kg$^{-1}$), which confirmed the results obtained in the
crosses between AB1 and 25X-1. F1 seeds exhibited a greater linolenic acid content than AB1 (77.5 ± 22.9 g kg⁻¹), which was a consequence of the strong reduction of oleic acid content in this generation.

F2 populations showed a similar segregation pattern to F2 populations from the cross between AB1 and 25X-1, i.e., they showed trimodal distributions consisting of a low oleic, a mid oleic, and a high oleic acid class (Fig. 7). Even though the boundaries between the low and the mid oleic acid classes could not be clearly distinguished, the high oleic acid class was well defined. High oleic acid phenotypes represented about one sixteenth of the total populations, including 37 phenotypes out of 755 (χ² = 2.35, p = 0.12). Such a 15:1 (low:mid:high oleic) ratio confirmed that high oleic acid content in AB1 is produced by alleles at two loci that are not involved in the genetic control of low linolenic acid content, as the latter, if present in AB1, are not segregating in this cross.

These results were further confirmed through the analysis of a BC1F1 to AB1 population (Fig. 8), which showed a trimodal distribution composed of a low oleic acid (n = 108), a mid oleic acid (n = 50), and a high oleic acid class (n = 63), which did not differ significantly from the expected 2:1:1 ratio (χ² = 1.64, p = 0.44).

From a breeding point of view, it was interesting to check whether a recombination of the genes for high oleic acid content present in AB1 with the genes for low linolenic acid content present in AB4, some of them probably also present in AB1 (Velasco et al., 2003a), might result in a further increase of oleic acid content. However, such an increase was not observed. The phenotype with the maximum oleic acid content in F2 populations from crosses between AB1 and 25X-1 had 866.5 g kg⁻¹ oleic acid, 27.1 g kg⁻¹ linoleic acid, and 49.4 g kg⁻¹ linolenic acid, whereas the phenotype with the maximum oleic acid content in F2 populations from crosses between AB1 and AB4 grown in the same environment had 869.2 g kg⁻¹ oleic acid, 40.6 g kg⁻¹ linoleic acid, and 23.3 g kg⁻¹ linolenic acid, which indicated that reduction of linolenic acid content in high oleic acid phenotypes mainly resulted in an increase of linoleic acid rather than oleic acid content.

**DISCUSSION**

The results of the present research revealed the existence of two loci (Ol and Ol2) involved in the accumulation of oleic acid in Ethiopian mustard seed oil. Modified alleles at both loci are partially recessive. The ol alleles in homozygous condition at the Ol locus produced an increment of about 210 g kg⁻¹ oleic acid in the conditions of the present research. The ol2 alleles only
have a phenotypic effect on oleic acid content when they are combined with ol alleles in homozygous condition. In this case, they produce in homozygous condition an additional increment of oleic acid content of similar magnitude to that produced by ol alleles. The identification of homozygous genotypes in the F3 generation showed that the genotypes OlOlOlOl produced around 395 g kg\(^{-1}\) oleic acid, which was increased to 605 g kg\(^{-1}\) in genotypes ololOlOl, and to 833 g kg\(^{-1}\) in genotypes olololOl. Additionally, the similar segregation patterns and levels of oleic acid found in crosses of AB1 with 25X-1 and AB4 indicated that the high oleic acid trait is determined by alleles at both Ol and Ol2 loci, regardless of alleles involved in reduced levels of linolenic acid.

Ethiopian mustard germplasm with increased oleic acid content was first developed in high erucic acid background through chemical mutagenesis (Velasco et al., 1997a). The study of the inheritance of increased oleic acid content in one of these mutants, N2–3591 (196 g kg\(^{-1}\) compared with 64 g kg\(^{-1}\) in the wild type), concluded that the trait was produced by partially recessive alleles at a single locus (Velasco et al., 2003b). In a further research, crosses involving plants of N2–3591 and HF-186, a low linolenic acid line with a standard oleic acid content of 86 g kg\(^{-1}\) (Velasco et al., 1997b), resulted in transgressive segregants with a high oleic acid content of 310 g kg\(^{-1}\) (Velasco et al., 2003a). The authors hypothesized that transgressive segregation for oleic acid was the result of the recombination of partially recessive alleles at the Ol locus in N2–3591 and alleles at a second locus present in HF-186, which would not have a phenotypic effect on oleic acid content in absence of ol alleles. That preliminary hypothesis has been confirmed in the present research.

Genetic studies on the high oleic acid trait in B. napus mutants (around 750 g kg\(^{-1}\) compared with 600 g kg\(^{-1}\) in the wild type) concluded the existence of two genes involved in the control of the trait (Schierholt et al., 2001). One of the genes, HO1, had a major effect on rising oleic acid content (about 150 g kg\(^{-1}\) ), whereas the second gene, HO2, had only a very minor effect on seed oil oleic acid content (about 16 g kg\(^{-1}\) ). The HO1 locus was associated with the microsomal FAD2 desaturase (Schierholt et al., 2000). The HO2 locus was hypothesized to be related to the plastidial FAD6 desaturase, since it had expression not only in the seeds but also in roots and leaves (Schierholt et al., 2001). Since B. napus germplasm with oleic acid content above 850 g kg\(^{-1}\) was obtained by continued selection from the original mutants, Möllers (2002) suggested the additional presence of several genes with minor effect in that germplasm. The results obtained in the present research on Ethiopian mustard clearly differ from previous results in B. napus. Even though the effect on oleic acid content of alleles at the HO1 locus of B. napus seems to be very similar to the effect of the alleles at the Ol locus reported in the present research, our results suggested that high oleic acid levels around 850 g kg\(^{-1}\) in B. carinata are produced by a second gene, Ol2, with a major effect on this trait and of similar magnitude to the effect of Ol, and not by several genes with minor effects, as suggested for B. napus.

Genetic recombination of genes for high oleic acid content from AB1 with genes for low linolenic acid from AB4 did result in a fatty acid profile combining a high oleic acid (above 850 g kg\(^{-1}\) ) with a very low linolenic acid content (around 20 g kg\(^{-1}\) ). However, the reduction of linolenic acid did not result in a concomitant increase of oleic acid but rather an increase in linoleic acid content. These results are in complete agreement with those reported by Möllers (2002) in B. napus, where similar levels of oleic acid and linolenic acid to those obtained in the present research were achieved after recombination of high oleic and low linolenic acid genotypes.

Through a differential isolation of ololOl2Ol2 and ololol2Ol2 genotypes, it was possible to develop a mid oleic (AB3) and a high oleic acid (AB1) Ethiopian mustard line, respectively (Velasco et al., 2003a). This opens up the possibility of producing two different types of Ethiopian mustard oils with increased oleic acid content. Mid oleic acid oils are preferred for applications requiring a combination of oxidative stability and sensory properties of the end products (McVetty and Scarth, 2002), whereas high oleic acid oils are desired in applications demanding a very high oxidative stability (Yodice, 1990). The simple genetic control of both traits in Ethiopian mustard will facilitate their incorporation to elite lines if...
this species is finally transformed into a canola crop for dry areas. A major advantage of the availability of both mid and high oleic acid oil types in Ethiopian mustard is that this species has a low intercrossing rate with \textit{B. napus} (Getinet et al., 1997), which will enable the coexistence of both crops in common areas without need for cultivation under isolation if one of them is used for producing a mid or a high oleic acid oil.

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