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Increasing erucic acid content in rapeseed (*Brassica napus* L.)

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Abstract
High erucic acid rapeseed (HEAR) cultivars are regaining interest for industrial purposes because erucic acid (22:1) and its derivatives are important renewable raw materials for the oleochemical industry. For oleochemical use, it is desirable to increase 22:1 content from now 50-60% to 80% and more. This would significantly reduce processing costs and would increase the market prospects of HEAR oil. In the present experiment, the transgenic winter rapeseed genotype 361.2B (60% 22:1, 19% 18:2+18:3) over expressing the *fae*1-gene in combination with a LPAAT *pls*C-gene from *Limnanthes douglasii* was crossed with the high erucic acid winter rapeseed line 6575-1 with a low content of polyunsaturated fatty acids (HELP; 50% 22:1, 7% polyunsaturated fatty acids) with the hypothesis that this would result in an increased 22:1 content in the seed oil. 220 F_2 plants derived from this cross were grown in the greenhouse and bagged at flowering. F_3 seeds were harvested and analysed for fatty acid composition and trierucoylglycerol (trierucin) content. Recombinant F_2 plants with 68-72% 22:1, 9-14% polyunsaturated fatty acids and with trierucin content up to 25% were identified, indicating a 5-10% increase in 22:1 content compared to parent 361.2B. Results will be confirmed by testing F_3 plants from selected F_2 plants in the greenhouse. Additional steps required to achieve 80% or more 22:1 in the seed oil are discussed.

Key words: Erucic acid, LPAAT, *fae*1, *pls*C, trierucin, PUFA, fatty acids

Introduction

Erucic acid (22:1) is associated mainly with plants of the family *Brassicaceae*. From the last decade, high erucic acid rapeseed (HEAR) cultivars have regained interest for industrial purposes because erucic acid and its derivatives are important renewable raw materials for the oleochemical industry. It is used for the production of plastics, lubricants, slip and coating agents, soaps, printing inks, surfactants, etc. A substantial increase of 22:1 content could significantly reduce processing costs and would increase the market prospects of HEAR oil. In conventional HEAR oil, 22:1 is exclusively esterified at the *sn*-1 and *sn*-3 positions of the glycerol backbone, thus limiting its content to 67%. To overcome this limitation, the fatty acid elongase *fae*1-gene has been over expressed in combination with the LPAAT *pls*C-gene from *Limnanthes douglasii*, which enables the insertion of 22:1 in the *sn*-2 position of the glycerol backbone. But this has not led to a major increase in 22:1 content (Han *et al.* 2001). One explanation for this result was the insufficient availability of oleoyl-CoA as a substrate for fatty acid chain elongation, due to its competitive desaturation and irreversible incorporation into storage lipids. To test the first assumption, conventional HEAR was crossed to rapeseed with a reduced content of polyunsaturated fatty acids (PUFA, 18:2+18:3). In selected recombinant F_3-plants, 22:1 content remained unchanged (50% 22:1), although the PUFA content decreased from 23% to 7% (Sasongko and Möllers 2005). Therefore, the present study was undertaken to cross *fae*1-*pls*C-overexpressing transgenic rapeseed plants (Wilmer *et al.* 2003) with high erucic acid and low polyunsaturated fatty acid (HELP) plant material (Sasongko and Möllers 2005) to increase the erucic acid content beyond 70% in the seed oil.

Materials and Methods

The transgenic winter rapeseed genotype 361.2B (provided by J. Wilmer, Biogemma Ltd.) carrying two copies of *fae*1-*pls*C-gene (linked on one T-DNA) and having 60% 22:1 was crossed with the non-transgenic F_4-line 6575-1 (HELP; 50% 22:1, 27% 18:1 and 7% 18:2+18:3) derived from a cross between the high erucic winter rapeseed cv. Maplus and a low erucic, low polyunsaturated fatty acid winter rapeseed line (Sasongko and Möllers 2005). F_1 plants were grown in the greenhouse and at flowering selfed to obtain F_2 seeds. 220 F_2 plants and 8 plants from each of the parent were grown in the greenhouse. At flowering plants were bagged to secure self pollination. DNA was isolated from 57 randomly selected F_2 plants and PCR was performed with *pls*C-specific primers to follow the segregation pattern of the *fae*1-*pls*C-transgene. Finally F_3 seeds were harvested and analysed for their fatty acid composition and trierucoylglycerol (trierucin) content by GLC as described by Möllers *et al.* (1997) taking 100mg seed sample from each plant.

Results and Discussion

The F_2 population (F_3 seeds) showed a continuous variation for erucic acid (22:1) content in the seed oil, ranging from 45% to 72% (Fig. 1). There were no separable classes as expected for a polygenic inherited trait. There were a considerable number of F_2 plants having a higher 22:1 content compared to the parent line 361.2B. In conventional high erucic acid rapeseed the 22:1 content is inherited by two genes and in crosses with low erucic acid genotypes, a 1:4:6:4:1 segregation can be expected in F_2. However, the contribution of the two genes and their alleles to the total 22:1 content may be different. In addition to this, the quantitative variation in 22:1 content in the present F_2 population is caused by the segregation of two transgene copies (see below) and likely by the segregation of genes responsible for the low PUFA (18:2+18:3)-content. The low PUFA content in parent 6575-1 is caused by a mutation in the oleic acid desaturase *fad*2-gene which causes a reduction of 18:2 content by around 15% and by 2 to 3 other unknown genes having minor effects.

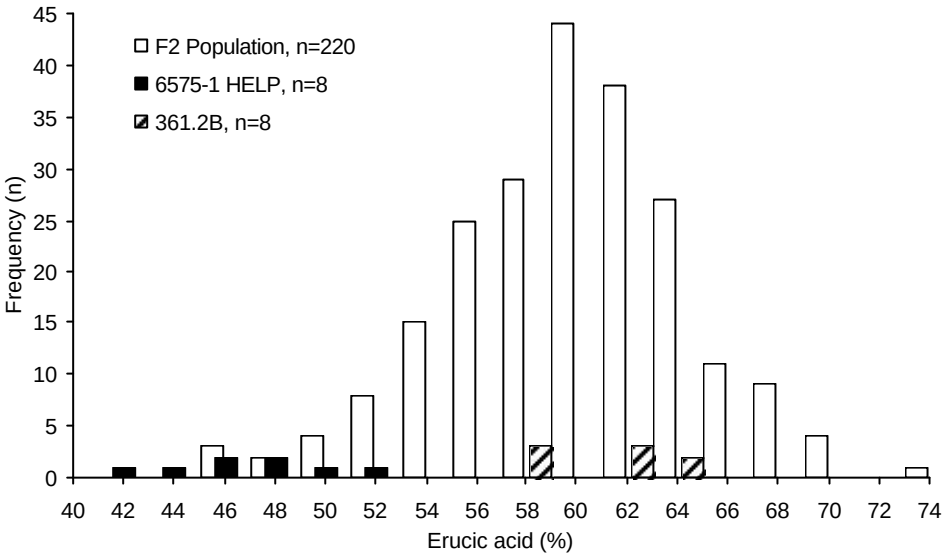

Fig. 1. Frequency distribution of the erucic acid (22:1) content in F_2 population (F_3 seeds) derived from a cross between transgenic high erucic acid (361.2B) and non-transgenic high erucic, low polyunsaturated fatty acid (HELP) rapeseed along with the parents.

The scatter plot in Fig. 2a shows that among the F_2 population there are some F_2 plants (F_3 seeds) that have a higher 22:1 and a reduced PUFA content compared to the transgenic parent 361.2B. These F_2 plants had an oleic acid (18:1) and eicosenoic acid (20:1) content which were about equal to the transgenic parent 361.2B (Fig. 2b+c), suggesting that the reduction in PUFA content has led to an increased 22:1 content in the range of 5-10%. In some of the high erucic acid segregants, the PUFA-content was as low as in the HELP parent line 6575-1, indicating homozygosity for the genes causing low PUFA-content. Erucic acid content was negatively correlated with the contents of polyunsaturated fatty acids ($r = -0.22^{**}$), oleic acid ($r = -0.61^{**}$) and eicosenoic acid ($r = -0.64^{**}$). The analysis of the trierucin revealed a positive correlation between erucic acid and trierucin ($r = 0.58^{**}$; Fig. 2d); in individual F_2 plants up to 25% trierucin was detected in the seed oil. The capability of forming trierucin is linked to the transgenic character of plants, expressing the *fae*1-*pls*C-transgene. Differences among the F_2 plants in trierucin content are caused by the presence of either one or both of the transgenes in the homozygous or hemizygous state. Beside trierucin (C69), the triglyceride species C67 consisting of one molecule eicosenoic acid and two molecules erucic acid was found (not shown).

Four out of 57 randomly selected F_2 plants were negative in the PCR reaction for the *pls*C transgene (see an example in Fig. 3), confirming that the transgenic parent 361.2B had two transgene copies which led to a 15:1 segregation in F_2. This result was also found when looking at the segregation of the trierucin phenotype (data not shown). All F_2 plants which were positive in PCR for the *pls*C-gene contained trierucin, whereas those which were negative in PCR did not have trierucin. The fatty acid composition of some selected high erucic acid F_2 plants (F_3 seeds) is shown in Table 1. The total content of monounsaturated fatty acids (MUFA) is in the range of the HELP-parent.

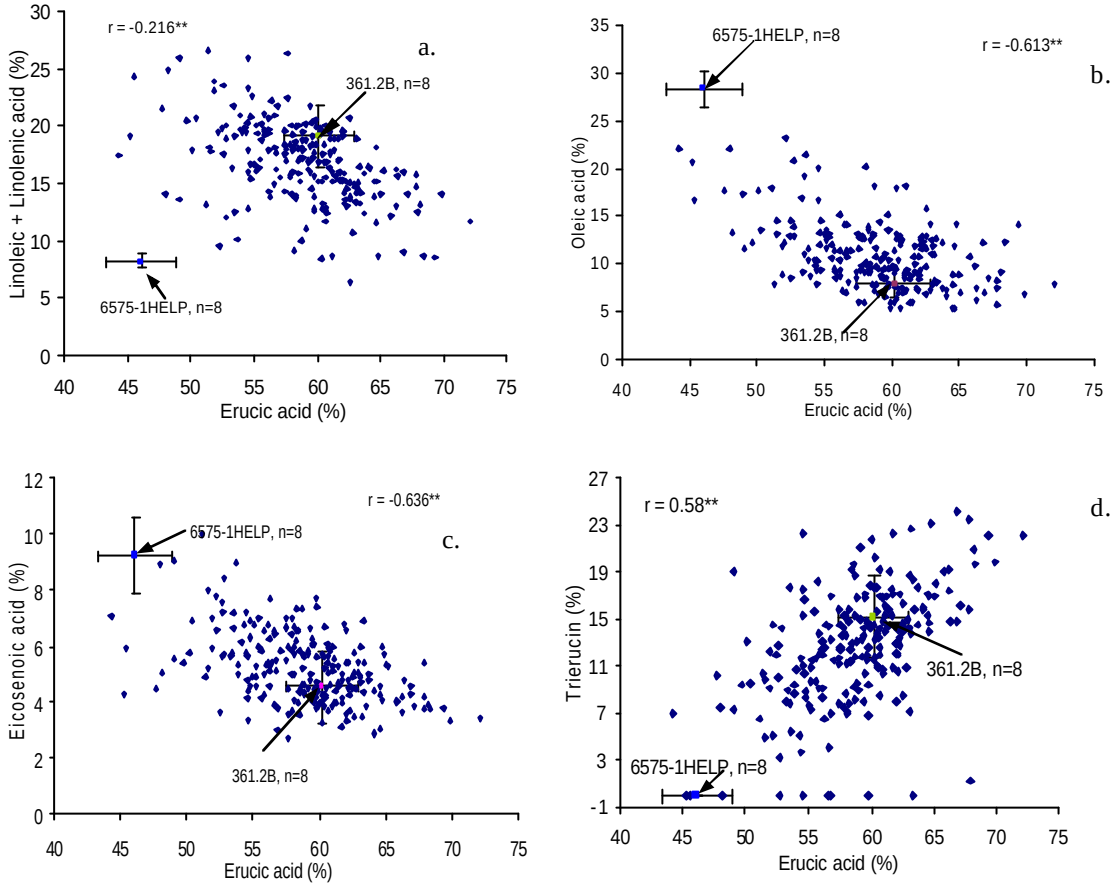

Fig. 2a-d. Fatty acid compositions and trierucin content of F_2 plants (F_3 seeds) derived from a cross between transgenic high erucic acid (361.2B) and non-transgenic high erucic acid rapeseed with a low 18:2+18:3 content (HELP). ** indicates significance at P= 0.01 probability.

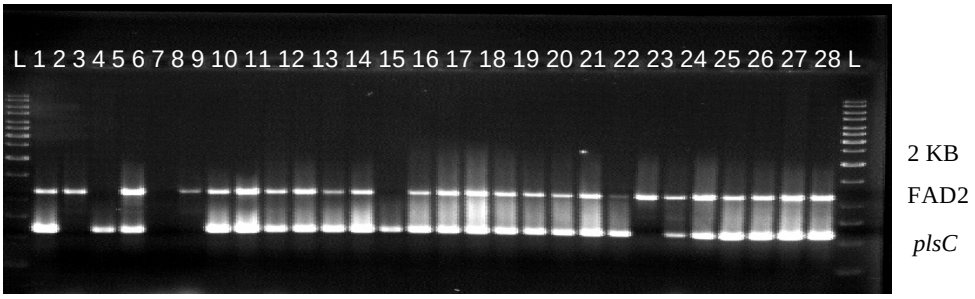

Fig.3. Multiplex-PCR of a 603bp fragment of the *pls*C gene together with internal control amplification of the 1.1kb *fad*2 gene sequence using DNA extracted from 26 F_2 plants segregating for the *pls*C gene (Lanes 3-28 F_2, lanes 1 and 2: 361.2B and 6575-1 as control)

Table 1. Fatty acid composition of selected F_2 plants (100mg F_3 seeds) with high erucic acid content along with the parental lines having the highest erucic acid content

Genotype	16:0+18:0	18:1	18:2+18:3	20:1	22:1	MUFA
IV-A-4	2.12	7.93	11.67	3.43	72.10	83.46
III-H-10	3.00	6.98	14.01	3.31	69.84	80.13
IV-A-10	2.47	14.18	8.57	3.79	69.35	87.32
III-G-7	3.47	12.41	8.63	3.74	68.38	84.53
II-C-10	2.67	9.31	13.03	3.90	68.15	81.36
6575-1HELP	3.63	26.42	7.98	9.25	50.06	85.73
361.2B	2.91	7.73	16.38	5.99	63.20	76.92

MUFA = monounsaturated fatty acids (18:1+20:1+22:1)

Conclusions

The around 70% 22:1 detected in selected recombinant F_2 plants compare favourably to the 22:1 contents of both parental lines, cultivated together at the same time in the greenhouse. Following field experiments, Sasongko and Möllers (2005) reported for line 6575-1 erucic acid contents of 50%, which is 4% higher than the mean of 6575-1 (n=8) . This indicates that with the material developed in the present study up to 75% erucic acid in the seed oil can be expected in field experiments. This would represent a significant improvement in comparison to the 50% erucic acid normally obtained from conventional HEAR cultivars in commercial field production.

To find out whether the ß-ketoacyl-CoA synthase activity of the *fae*1-gene is still limiting, crosses with the transgenic high erucic acid lines expressing the *fae*1-*pls*C-transgene of Han *et al*. (2001) are currently performed. Further increases in erucic acid content can also be expected from progress in reducing the remaining PUFA-content from now 8% to values of 2-3%. However, this has so far not been achieved by mutagenesis or transgenic approaches. Oleoyl-CoA is elongated in the cytoplasm to 20:1 and 22:1 by subsequent condensation with malonyl-CoA. Malonyl-CoA is produced from acetyl-CoA by the acetyl-CoA carboxylase (ACCase). Acetyl-CoA itself is deliberated from citrate by cytoplasmic ATP-citrate lyase. Increased erucic acid contents may be expected from over expression of these two genes in transgenic rapeseed. However, cytoplasmic acetyl-CoA is also a precursor for a range of secondary compounds in rapeseed, e.g. phytosterols and sinapate esters (Fatland *et al*. 2005). Amar *et al*. (2007) reported a close negative correlation between erucic acid, phytosterols and sinapate esters, respectively, in a winter rapeseed DH population segregating for erucic acid. This indicates that availability of acetyl-CoA may be limited and that genetic combination of HELP rapeseed with material having a low phytosterol and sinapate ester content may have a positive effect on erucic acid content. It seems that a number of additional small steps are necessary to realize erucic contents of 80% and above.

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