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Increased levels of erucic acid in *Brassica carinata* by co-suppression and antisense repression of the endogenous *FAD2* gene

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Abstract

Erucic acid and its derivatives represent important industrial feedstock compounds, and there is an increasing demand for the production of high erucate oils in this regard. Our goal therefore, is to develop high erucic acid (HEA) *Brassicaceae* lines with increased proportions of erucic acid and very long-chain fatty acids (VLCFAs). We proposed that oleate availability may be a rate-limiting factor in the biosynthesis of erucic acid. We have tried to address this question by manipulating the expression of the endogenous *FAD2* gene in *B. carinata* using co-supression and antisense approaches. Both methods resulted in transgenic lines exhibiting decreased proportions of polyunsaturated C_{18} fatty acids (18:2 + 18:3) and concomitant and significantly increased proportions of 18:1, 22:1 and total VLCFAs. Co-suppressed *FAD2 B. carinata* lines exhibited 3–18% decreases in 18:2, 22–49% decreases in 18:3 and significantly increased proportions of 18:1 (36–99%), 22:1 (12–27%) and VLCFAs (6–15%). Transgenic *B. carinata* lines developed using an antisense *FAD2* approach exhibited decreased proportions of 18:2 and 18:3 (9–39% and 33–48%, respectively) and significantly increased proportions of 18:1 (54–130%), 22:1 (5–19%) and VLCFAs (6–21%). The possibility of using these approaches to produce prototype transgenic germplasm of the *Brassicaceae* accumulating seed oils with improved proportions of erucic and other VLCFAs is discussed.

Keywords: Antisense repression; *Brassica carinata*; Co-suppression; *FAD2*; High erucic acid; Transformation

1. Introduction

One of the strategic goals of our research is to modify high erucic acid (HEA) germplasm of the *Brassicaceae* to increase the content of erucic acid (22:1 Δ13) and other strategic very long-chain fatty acids (VLCFAs) in the seed oil for industrial niche market needs. More than 1000 patents have been issued for the utilization of erucic acid, behenic acid and their derivatives as feedstocks in the manufacture of surfactants, plasticizers, nylon 1313, surface coatings, high temperature lubricants, photographic materials, industrial plastics, emulsifiers, etc. (Leonard, 1994; Sonntag, 1991, 1995). The current market for high erucate oils exceeds $100 million US/annum. Worldwide erucic acid demand is predicted to increase from about 40 million pounds (Mpds) in 1990 to about 80 Mpds by the year 2010. Similarly, demand for the derivative, behenic acid, is predicted to triple to about 102 Mpds by 2010 (Sonntag, 1995). In recent years, production has lagged behind market needs and high erucic acreage in western Canada is currently at a record high (D. Males, Saskatchewan Wheat Pool, personal communication). A *Brassica* cultivar containing erucic acid levels approaching 80% would significantly reduce the cost of producing erucic acid and its derivatives and could meet the forecast demand for erucic and behenic acids as renewable,

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environmentally friendly industrial feedstocks (Leonard, 1994; Taylor et al., 2002).

In the current study, our objective was to determine whether the supply of oleate (18:1) moieties available for elongation to erucic acid is rate-limiting in *Brassica carinata*. The oleate desaturase *FAD2* (EC 1.3.1.35; 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine desaturase) is one of the most important enzymes for the production of polyunsaturated fatty acids in plants (Okuley et al., 1994). By altering the level of expression of the microsomal oleate desaturase encoded by the *FAD2* gene in a seed-specific manner, it is theoretically possible to increase the pool of 18:1 which is available for chain elongation by the fatty acid elongase complex to produce more erucic acid. Interestingly, the seed oils of the *FAD2-1* and *FAD2-5* EMS-mutants of *A. thaliana* reported several years ago exhibited a 24–30% decrease in linoleic acid, resulting in 22–38% increases in oleic acid and 6–9% increases in the proportion of 20:1, the major VLCFA in *Arabidop*sis seed oil (Okuley et al., 1994).

In order to investigate whether enriching the fatty acid pool with increased proportions of 18:1 could result in increased biosynthesis and accumulation of erucic and other VLCFAs in higher Brassicaceae, we have studied the effect of co-suppression of the endogenous *FAD2* gene (AF124360) in HEA *B. carinata* (Abyssinian mustard, $n = 17$). In parallel, using the homologous *B. napus FAD2* (AF243045) gene, we have examined the effect of an antisense approach to down-regulate the expression of the *B. carinata FAD2*.

2. Results and discussion

2.1. Silencing of endogenous FAD2 gene in B. carinata by co-suppression

When using *B. carinata* for genetic transformation, the co-suppression approach is attractive, because the high transformation rate in this species affords the opportunity to generate the high numbers of transgenic lines necessary to observe a co-suppression event (Babic et al., 1998). Co-suppression of *FAD2* has been used successfully to increase the proportions of 18:1 in transgenic soybean, canola-*B. napus* and *B. juncea* lines (Kinney et al., 2002; Stoutjesdijk et al., 2000). By silencing the *FAD2* gene in somatic soybean embryos, transgenic plants were produced that contained about 85% oleic acid with trace proportions of 18:2 and about 5% 18:3 (Kinney and Knowlton, 1998). In canola *B. napus*, it was necessary to silence both the *FAD2-1* and *FAD2-2* genes in order to obtain transgenic lines with the highest proportions (88%) of oleic acid (DeBonte and Hitz, 2000). Co-suppression of *FAD2* in canola-quality *B. juncea* raised the oleate content of the seed oil from 45% to 73% (wt/wt) (Stoutjesdijk et al., 2000). None of these studies utilized HEA rapeseed cultivars.

In this study, we have determined the effects of down-regulating the desaturation of oleic acid to enhance substrate availability for the biosynthesis of VLCFAs and erucic acid in particular, via elongation. The diagram in Fig. 1 illustrates the anabolic pathway of the synthesis of polyunsaturated C_{18} fatty acids via desaturation and VLCFAs via elongation reactions. Using a co-suppression approach, we have transformed 160 cotyledons to produce a total of $33 T_0$ plants that were both resistant to kanamycin and PCR positive for the *FAD2* gene (transformation rate of 21%). Transgene copy numbers were determined by Southern analyses (Southern, 1975) and transformants with only 1 copy were retained for further studies. For each generation, individual plants were selected based on their seed oil composition (i.e., low levels of 18:2 and 18:3, high levels of 18:1, 20:1 and 22:1, and a high proportion of monounsaturated over polyunsaturated) as analyzed by GC. For each selected line, 10 individual plants were seeded and the progeny analyzed up to the T_3 generation for their oil content and composition in triplicates. Finally, segregation analyses showed the 3:1 ratio expected for a single insertion event in each of the selected transgenic lines (data not shown).

As expected, GC analysis of the seed lipids identified some lines displaying the *FAD2* sense over-expression fatty acid phenotype (decreased proportions of 18:1,

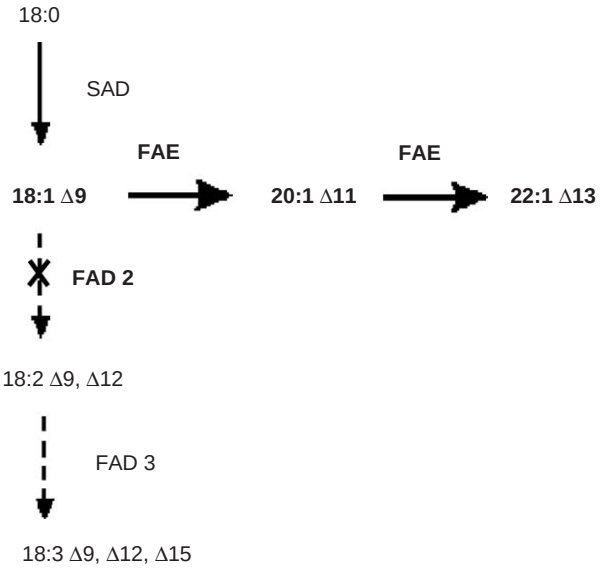

Fig. 1. Schematic representation of the metabolic pathways for the production of 18:2 and 18:3 via desaturation and 20:1 and 22:1 via elongation. The hypothesis of re-directing oleate moieties toward the synthesis of VLCFAs by down-regulating the expression of oleate desaturase is represented by the relative intensity of the reaction pathway arrows. FAE: fatty acid elongase; *FAD2*: Δ12 oleoyl desaturase; *FAD3*: Δ15 linoleoyl desaturase; SAD: stearoyl-ACP desaturase.