Molecular breeding of oilseed rape (*Brassica napus*) with modified oil quality for nutritional and non-food purposes

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Introduction

The oil content of crop plants like oilseed rape (*Brassica napus*) is a major quality trait that predominantly determines the value and market-price of the commercial seed. The specific features of the seed oil and its use for food or non-food purposes are mainly determined by its respective fatty acid (FA) composition. In addition, seed oils contain valuable secondary compounds such as tocopherols (TOC, vitamin E). Therefore, the FA and TOC biosynthesis and the genetic modification of oil quality are important objectives of basic and applied research. Respective breeding activities aim at improving the competitiveness of rapeseed oil for different segments of the vegetable oil market. In addition to classical breeding, molecular approaches offer the possibility to modify plant storage lipids and valuable secondary compounds in order to meet specific demands, for nutritional, non-food or even therapeutic purposes (cf. Friedt et al. 2004). Here we report on recent molecular rapeseed breeding activities in our laboratory towards this purpose.

Modification of plant storage lipids

Today, double-low rapeseed (00, canola) is highly valued as a food raw material due to its favorable fatty acid composition, i.e. approximately 4% palmitic (C16:0), 3% stearic (C18:0), 65% oleic (C18:1), 18% linoleic (C18:2), 10% linolenic (C18:3) and 1% of other fatty acids. The high level of oleic acid combined with relatively high proportions of polyunsaturated fatty acids make rapeseed oil particularly suitable for nutritional purposes (e.g. margarine, salad oil). High oleic acid content also determines the usefulness of rapeseed oil for oleochemical (e.g. detergents, emulsifiers) and technical purposes (e.g. hydraulic and lubrication oil, biodiesel). Further improvement of nutritional quality could be achieved by a reduction of saturated fatty acid content (low sats) and an elevation of polyunsaturated fatty acid contents. Alternatively, increased sat contents would be helpful for special purposes such as the production of margarine or emulsifying agents, e.g. for cosmetic products. For technical uses, reduced linoleic and linolenic proportions would be desirable. Finally, the creation of novel rapeseed oil accumulating substantial amounts of medium-chain fatty acids would be welcomed in the oleochemical industry.

Aiming at an improvement of its non-food usefulness we have genetically engineered oilseed rape (*B. napus*) and developed progenies that accumulate medium-chain fatty acids (MCFA) in the seed oil. Relevant genes from the plant genus *Cuphea* encoding steps of MCFA synthesis were selected for *Agrobacterium tumefaciens*-mediated transformation. Gene constructs harbouring the β-ketoacyl-acyl carrier protein synthase III gene (wild- and mutant type) either from *C. lanceolata* (*Cl*KASIII) or *C. wrightii* (*Cw*KASIII) were used alone or in combination with different *Cuphea* thioesterase genes (*Ch*FatB2, *Cl*FatB3) involved in the biosynthesis of caprylic (C8:0) and capric acid (C10:0). Transgenic progeny could be regenerated with the following maximum contents recorded in T3 seeds: 2.6% C12+C14, 8.8% C10:0, and 2% C8:0. In a single genotype a total of more than 15% medium-chain fatty acids (C8-16) has been identified (Fig. 1). A high correlation between T2- and T3-fatty acid values indicates that the transgenic event is stable and heritable.

Another aspect of this work aimed at a significant increase of stearic acid (C18:0) in rapeseed oil. For this purpose, the activity of the endogenous stearoyl-ACP desaturase (SAD), catalysing the reaction step from stearic to oleic acid, was successfully down-regulated using an antisense approach. This regulation led to first prototypes of rapeseed (low- and high-erucic acid lines) with increased amounts of C18:0 in the oil. The result of SAD down-regulation was an approximately 10-fold increase of stearic content from 3.7 up to 32% in single seeds of transgenic low-erucic acid rapeseed (LEAR), while the high-erucic acid rapeseed (HEAR) showed a 4-fold increase from 1 up to 4%. In pooled T2 seed material of LEAR rapeseed we found that the stearic content was highly correlated with the down-regulation of SAD, as indicated by the stearate desaturation proportion (SDP). Furthermore, our results suggest that for elongation of C18 fatty acids an altered specificity of the β-ketoacyl-CoA synthase (KCS) enzyme in favour of stearate might have occurred. The importance of the promoter source was demonstrated by the fact that no change was achieved in the fatty acid composition of transgenic when the gene of interest was controlled by the FatB4 seed-specific promoter from *C. lanceolata.* Furthermore, the orientation of the gene of interest to the selectable marker seemed to influence the level of transcribed RNA.

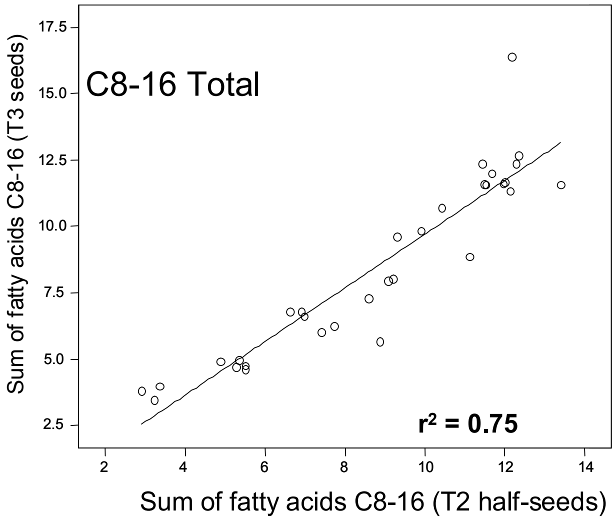

Fig. 1: Correlation between T2 half-seeds and T3 progeny seeds of rapeseed plants harbouring *Cl*KASIIIbmut/*Ch*FatB2 (sum of medium-chain fatty acids, 8-16 carbons).

(cf. Stoll et al. 2005)

A particular effect of the orientation of antisense cassettes was also observed. This can be explained by an impact of the inherent enhancer of p35S used in the selectable marker cassette on the napin promoter in pAS*Bn*DES3. An enhancer is assumed to act at a distance of about 2 kb. Therefore, the stronger antisense effect detected in plants transformed with the pAS*Bn*DES3 construct might be due to the fact that the distance between p35S and the napin promoter is shorter in pAS*Bn*DES3 than in pAS*Bn*DES2.

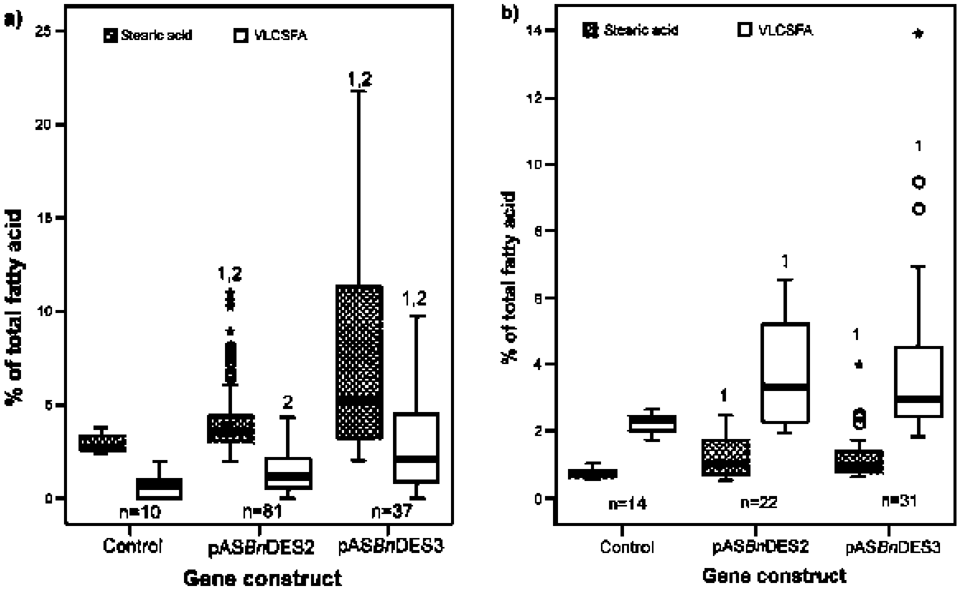

Fig. 2 a, b: Box and whisker plots of stearic and very long chain saturated fatty acid (VLCSFA) contents of T2 seeds in comparison to the controls in LEAR (a) and HEAR (b) lines. n = number of plants analysed. 1 = significantly different from the control ($p < 0.05$), 2 = significant difference between pAS*Bn*DES2 and pAS*Bn*DES3 at $p < 0.05$. o = outliers with a 1.5 distance from the nearest quartile, * = extreme values with more than 3 box lengths from the upper quartile (Zarhloul et al. 2006).

We observed a weak correlation between transgene copy number and stearic acid content, which however may be due to the small number of genotypes tested. For LEAR and HEAR plants not just the amount of transcribed antisense RNA is important, but also the time frame of gene expression in order to obtain an efficient inhibition.

As in other studies the particular importance of the promoter for an appropriate expression of the transgene was demonstrated. Although in the present work the same antisense gene was used in all three constructs, we assume that the FatB4 promoter driving the antisense SAD was too weak to achieve the desired phenotype. To ensure the sufficient expression of a gene of interest and subsequently achieve the desired altered phenotype, a strong tissue-specific promoter is needed.

Modification of rapeseed tocopherol content and composition

Although modern rapeseed oil is considered a high-value dietary vegetable fat, further enhancements of its nutritional value and health effects would be welcome. Lipid soluble tocochromanols, only synthesised in photosynthetic organisms, are industrially interesting compounds because of their antioxidative properties and their essential function in nutrition. In particular, the tocopherols (TOC) as part of this class of compounds are important phytonutrients in edible oils possessing bioactivity as vitamin E and reducing the autoxidation of unsaturated fatty acids, the production of off-flavours and rancidity. Normally, TOC content of rapeseed oil varies from 500 to 700 mg kg^{-1} oil. In the course of a metabolic engineering approach, our strategy has been to elevate TOC levels by increasing the flux through the pathway by heterologous overexpression of enzymes that catalyze relevant steps in TOC biosynthesis, such as 4-hydroxyphenylpyruvate dioxygenase (HPPD), homogentisate phytyltransferase (HPT) and tocopherol cyclase (TC) genes.

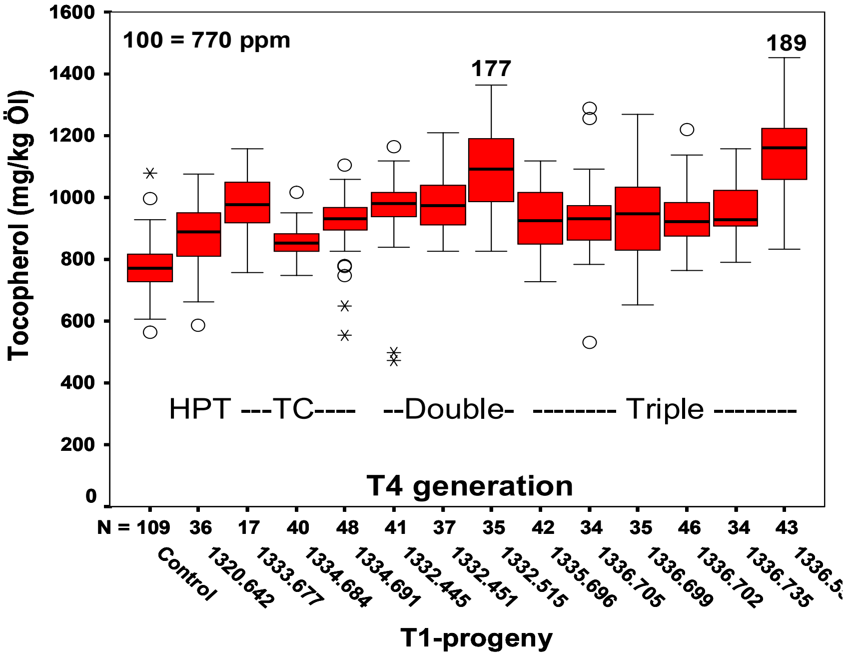

Fig. 3: Variation of Tocopherol content in genetically modified seedoil (T4 generation, pooled T3-seeds); N= Number of plants in T2; All mean values differ significantly from the control ($P < 0,001$, Tamhane); HPT= Nap_HPT; TC = Nap_TC; Double = DC3_HPPD2, Nap_HPT; Triple = DC3_HPPD2, Nap_HPT, Nap_TC (cf. Kumar et al. 2005, Raclaru et al. 2006)

In order to increase the tocochromanol content in the seed oil of transgenic plants, we aimed at engineering the flux of substrates and intermediates through the pathway. To this end, we overexpressed genes encoding hydroxyphenylpyruvate dioxygenases, alone or in combination with chimeric homogentisate phytyltransferase and tocopherol cyclase genes, in seeds of transgenic *B. napus* plants and analysed total tocochromanol content and composition. Overexpression of chimeric HPPD genes, both in the cytosol or in the plastids of developing seeds, yielded a slight although significant increase in total tocochromanol level. Coexpression of a HPPD gene with both a HPT and a TC gene elevated this effect with maximum increases of up to approx. 2-fold in individual lines (Fig. 3), and this phenotype was found to be stably inherited through T2 and subsequent generations. These data showed that the three enzymes are critical in determining the total tocochromanol content in the seed oil of rapeseed plants, while the TC, unlike HPPD and HPT, had an additional effect on the relative abundance of individual tocochromanol species and resulted in an increase of δ-tocopherol and plastochromanol-8 in the seeds (cf. Kumar et al. 2005, Raclaru et al. 2006).

In summary, coexpression of two or three genes essential for tocopherol synthesis in developing seeds of transgenic rapeseed plants resulted in an increase of the tocopherol content in the seed oil. To achieve a further improvement of tocopherol content, amongst others the plastidial phytylPP pool and the properties of the HPT would need to be improved. This assumption is in line with recent data showing that a boost in the plastidial homogentisate pool in transgenic plants resulted in the activation of an endogenous homogentisate geranylgeranyltransferase (HGGT) activity and, thus, in the accumulation of tocotrienol, whereas the tocopherol content was hardly effected (Kumar et al. 2005, Raclaru et al. 2006). A concomitant increase in the plastidial phytylPP pool in such transgenic plants will show whether the tocopherol content is primarily controlled by the substrate pools available to the HPT or the properties of enzymes. Altogether, the data obtained by the analysis of the tocochromanol biosynthesis indicate that a major increase of the tocochromanol content in the seeds of transgenic plants requires an up-regulation of the pathway by overexpressing multiple rate-limiting enzymes as well as an increase in the supply of the aromatic and isoprenoid precursors. The analyses of the present transgenic plants emphasised the complex mechanisms that regulate the metabolic flux of substrates and intermediates in the tocochromanol pathway and provide a useful basis for future studies of the underlying regulatory mechanisms.

Conclusions

The absolute changes of fatty acid contents achieved in this work were small but consistent and heritable; KAS-Fat double-gene constructs proved to be superior to single gene-constructs regarding the formation of MCFA. This is thought to be due to limitations in the storing or channelling process, e.g. the necessity of a specific acyltransferase (PDAT). Modification of further enzymes, e.g. PDAT, is expected to lead to higher MCFA contents. Regarding tocopherol content and composition, the best individual transgenic plants showed nearly twice the tocopherol content of the controls. The combination of an optimum ratio of C18:2 to C18:3 (approx. 2:1) with a high tocopherol content characterizes a superior rapeseed oil quality for nutritional usages. Overall it can be concluded that genetic engineering is not only a powerful tool to elucidate the genetics of biosynthetic pathways, but also useful for creating new phenotypes. A next step will be the incorporation of these prototypes into oilseed rape breeding programmes in order to create environmentally stable, high-quality genotypes combined with high agronomic value and market potential.

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References

Friedt W, Leckband G, Frauen M, 2004. NAPUS 2000 - Research for an overall improvement in rapeseed. INFORM 15: 295-297

Kumar R, Raclaru M, Schüßeler T, Gruber J, Sadre R, Lühs W, Zarhloul KM, Friedt W, Enders D, Frentzen M, Weier D, 2005. Characterisation of plant tocopherol cyclases and their overexpression in transgenic *Brassica napus* seeds. FEBS Lett. 579: 1357-1364

Raclaru M, Gruber J, Kumar R, Sadre R, Lühs W, Zarhloul KM, Friedt W, Frentzen M, Weier D, 2006. Increase of the tocochromanol content in transgenic *Brassica napus* seeds by overexpression of key enzymes involved in prenylquinone biosynthesis. Molecular Breeding 18: 93-107

Stoll C, Lühs W, Zarhloul MK, Friedt W, 2005. Genetic modification of saturated fatty acids in oilseed rape (*Brassica napus*). Eur. J. Lipid Sci. Technol. 107: 244-248

Zarhloul MK, Stoll C, Lühs W, Friedt W, 2006. Breeding high-stearic oilseed rape (*Brassica napus*) with high- and low-erucic background using optimised promoter-gene constructs. Molecular Breeding (in press)

Note: Compare the references in the cited papers above for further information and publications on the subject.