Genetic and chemical investigations of antinutritive phenolic compounds (tannins) in rapeseed (*Brassica napus* L.)

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Abstract

Rapeseed meal is an important product for animal nutrition and is also a potential source of vegetable protein for human nutrition. The meal possesses around 40% protein with a favorable composition of amino acids and high contents of the essential amino acids methionin and cystein. However, due to the high proportion of crude fibre and antinutritive components the utilization of rapeseed meal/protein in animal and human nutrition is limited. During this study genetic and chemical analyses of antinutritive phenolic compounds in rapeseed (principally condensed tannins) were performed. Using this analysis, the relationship between seed color and phenolic compounds in *B. napus* was investigated with a view to improving the rapeseed meal quality. The investigations of proanthocyanidin (PAs) content were carried out by quantitative and qualitative methods (Vanillin- and HPLC assay). The obtained values were used to identify *quantitative trait loci* (QTL) for the respective traits. The candidate loci for the target traits were genetically mapped and compared with the positions of the corresponding QTL.

Introduction

Oilseed rape / canola (*Brassica napus* ssp. *napus*, 2n=38, genome AACC) represents a potentially valuable source of vegetable protein due to its favorable composition of essential amino acids. However, the use of rapeseed protein for human nutrition is presently not possible due to the presence of major anti-nutritive compounds, which also reduce the value of rapeseed meal as a source of animal feed. Especially relevant in this regard are dietary fibre, dark-coloured tannins and bitter-tasting sinapate esters.

Chemically three groups of tannins are distinguishable: phlorotannins, hydrolysable and condensed tannins (*syn.* proanthocyanidins). Condensed tannins are dimers, oligomers and polymers of flavan-3-ols which, upon acidic hydrolysis, produce anthocyanidins and are therefore also known as proanthocyanidins.

In rapeseed condensed tannins are largely responsible for the dark colour of the seed coat, where they accumulate predominantly in the endothelium cell layer between the outer integument and the aleurone layer. Shahidi and Naczk (1988, 1989) found that canola varieties contained 0.68-0.77% condensed tannins. Naczk et al. (1994) indicated that canola hulls contained up to 2000 mg of soluble condensed tannins per 100 g of oil-free hulls as determined by the vanillin assay. The proportion of condensed tannins in dark-seeded *B. napus* extracted by proanthocyanidin assay can comprise up to 6% of the seed coat, with a total content of up to 5231 mg/100g oil-free hulls (Naczk, 2000). The variability of tannin content in the reported results is due by the differences existed in the environmental growing condition, as well as the differences in the solvent extraction systems employed for the recovery of tannins and methods used for their quantification.

The mutated gene loci that affect the synthesis and/or accumulation of proanthocyanidins in the seed coat of *Arabidopsis thaliana* represent interesting candidate genes for the analogous character in oilseed rape. The seed-specific genes *tt1* (Sagasser et al. 2002) and *tt16* (Nesi et al. 2002) are involved in development of the seed endothelium, in which proanthocyanidins accumulate, and in flavonoid biosynthesis.

By localising quantitative trait loci (QTL) for condensed tannin content in *B. napus* seeds and comparing these to the positions of promising candidate *tt*-genes, we hope to develop closely-linked molecular markers for selection regarding important genes involved in the accumulation of antinutritive tannins in rapeseed meal.

Methods and Materials

Using the software JoinMap 3.0 a dense genetic map was generated from the DH mapping population YE2 with 166 doubled-haploid (DH) lines derived from a cross between an inbred line of the black-seeded German winter oilseed rape cultivar 'Express' and the true-breeding, yellow-seeded line '1012-98', both with 00-seed quality. Significant QTL involved in seed colour, total and individual flavonoid compounds were mapped using the software PLAB-QTL based on analyses of seeds grown in field trials from Rauischholzhausen, Einbeck and Gross Gerau (Germany). Seed colour was measured quantitatively based on digital reflectance values. Total condensed tannins were estimated by spectrometry based on the vanillin assay, while individual and total flavonoid compounds were quantified via HPLC using standards to identify peaks.

Results and discussion

A major part of this work concentrated on the phenotyping of the plant materials regarding seed color and phenolic compounds in the seed meal. The seed color values for the genotypes of the DH population ranged from 2.9 (yellow seeds) to 8.1 (black seeds) and the condensed tannin content (via Vanillin assay) from 240.7 to 511.6 mg/100 g oil-free rapeseed meal (Figure 1). The seed color was significantly correlated with the total PA content (r = 0.33) but not correlated with the total flavonoids content (r = 0.09). The week correlation between seed color and total PA content leads to the conclusion that the yellow seed coat color alone cannot be used as a selection marker for a reduction of total PA or flavonoid content.

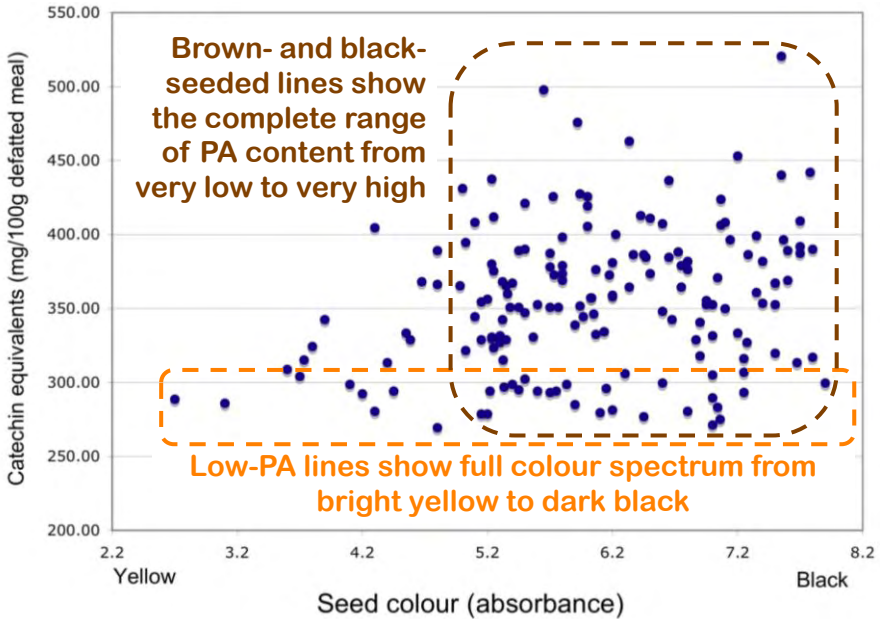

Figure 1 - Correlation between seed colour and total seed flavonoid content in YE2 DH-Population

A total of 176 polymorphic markers (126 AFLP and 50 SSR- markers) covering 1171 cM were localized in the genetic map from the YE2-DH population. The linkage groups were designated based on known marker positions using the standard N1 to N19 nomenclature for *B. napus,* with the exception of one unidentified group that was designated KG14.

Using quantitative data obtained by HPLC analysis it was possible to detect QTL for total and individual flavonoid compounds (Figure 2). In addition to some loci that co-localized with significant QTL for seed color in this population, a number of seed colour-independent QTL for different phenolic substances were detected. These loci presumably represent genes involved in biosynthesis of colorless flavonoids, and therefore exert a nutritionally effect on the flavonoid composition independently of the seed colour.

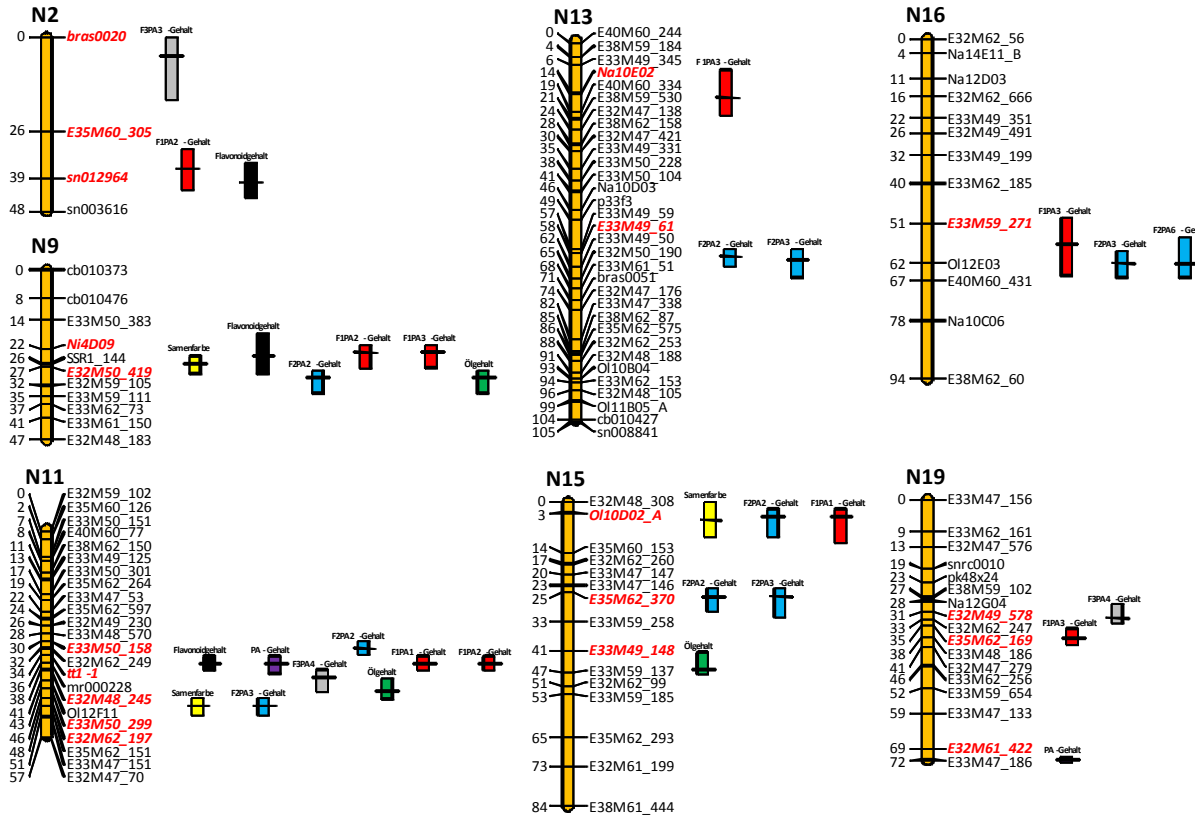

Figure 2 – QTL results for YE2 DH population

HPLC analysis of crude phenolic extracts showed two main peaks for phenolic acids. It is believed that these represent sinapine (F1PA1) and sinapoylglucose (F1PA2), respectively, the two most abundant phenolic acids in rapeseed meal. The main QTL for F1PA1 content was located at the same position on chromosome N11 with QTL for F1PA2, total flavonoid and proanthocyanidin content, and in the immediate area of a QTL for seed color, oligomeric and polymeric PAs (F2PA3, respectively F3PA4). A minor QTL for F1PA1 content (presumably sinapine) is located on linkage group N15 in the same interval (2-8 cM) with QTL for seed color and oligomeric PAs (F2PA2). On chromosome N9 two loci for F1PA2 (presumably sinapoylglucose) and F1PA3 co-localize with the main QTL for seed color (part. R^2 = 40.9%) and additionally with QTL for total flavonoid compounds, ADF, NDF and ADL content. In terms of breeding for rapeseed meal quality improvement this result is significant because it demonstrates an association between the seed coat thickness, the concentrations of phenolic acids and other antinutritive compounds. Using marker assisted selection (MAS) with respect to this locus it could be possible to obtain a significant reduction of phenolic acids and fiber components in rapeseed meal independently of the seed color.

The HPLC studies of mono- and oligomeric PAs showed that the crude extract from rapeseed meal contains no monomeric proanthocyanidins (PAs) in detectable quantities. Interestingly, some DH lines with dark seed coats possessed low oligomeric PA contents comparable with the levels found in yellow seeds. These observations confirm the existence of genetic variation for seed cell wall thickness and associated traits influencing seed color and rapeseed meal quality in dark seeded *B. napus*. In dark seeded DH lines from the YE2-DH population a large variation of phenolic compounds was observed, therefore a selection regarding meal quality can be made independently of the seed color.

Because of its role in the regulation of seed coat development and flavonoid biosynthesis the *tt*1 gene is an important candidate gene for seed color and associated quality traits in canola meal. The preliminary QTL and segregation data for phenolic compounds suggest that the inheritance of these traits in *B. napus* is different, but a single, dominant gene has an important role relating the reduction of these antinutritive compounds. Highly significant QTL for F1PA1, F1PA2, total flavonoid and total PAs content were co-localized at the same position as the SSR marker tt1_1 on chromosome N11, and QTL for seed color, oil-, F2PA2-, F2PA3- and F3PA4 content were also found nearby. This indicates that this SSR marker, and possibly a physically linked *B. napus* copy of the *tt*1 gene, could be responsible for variation in these traits in the YE2-DH population. From the mapping position of the SSR marker only a weak correlation with seed color in *B. napus* can be explained, because there was

no direct connection with the seed color QTL on chromosome N11. The co-localization of the tt1_1 marker with the above named QTL suggests a possible direct effect of the *tt*1 gene on the formation of endothelial cells and an indirect impact of the gene product on enrichment with proanthocyanidins.

A marker-assisted breeding strategy based on the results of this study could accelerate the generation of pure-breeding, light-colored winter rapeseed varieties with improved meal quality – both in respect to a utilization of seed meal as animal feed or as a basis for the extraction of highly nutritious protein for animal diet and human nutrition.

Closely linked molecular markers that were identified during this study to the seed color and phenolic compounds can be valuable tools for breeding of new yellow or dark seeded genotypes with reduced levels of antinutritive compounds. Potential candidate genes, e.g. *Bntt*1, could play an important role in this direction.

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