Biochemical and molecular analyses of flavonoid metabolism in *Brassica napus* seed: identification of key factors for seed coat pigmentation

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Introduction

Oilseed rape is a worldwide major oil crop that also supplies oil-free meal with high protein content (38-40%) and well-balanced amino acid composition. Currently, the quality of oilseed rape meal is still altered by secondary metabolites such as procyanidins. These compounds are flavonoid end-products that accumulate specifically in the seed coat during seed embryogenesis and maturation as colorless polymers that turn to brown-to-dark upon oxidations during seed desiccation (Nesi *et al.*, 2009). Despite their biological effects in seed sheltering, they are considered as anti-nutritionals since they impact negatively on animal production (especially for non-ruminant livestock) by altering feed intake and/or by lowering live weight gains.

Yellow seediness, which is thought to be associated with a reduction of procyanidin content, represents a major agronomic trait for Brassica crop improvement and has concentrated important research efforts over the last two decades. Indeed, the yellow-seed trait is linked to increased seed oil content and lower dietary fibre content associated with a thinner seed coat (Simbaya *et al.*, 1995; Slominski *et al.*, 1999). Therefore, the resulting meal has an improved energy value. Such yellow-seeded lines naturally exist for several *Brassica* spp., but not for *B. napus*. Breeding of *B. napus* yellow-seeded lines through interspecific crosses often results in unstable lines with poor agronomic properties. Therefore, development of yellow-seeded lines requires better knowledge of the seed flavonoid pathway in black- and yellow-seeded *B. napus* lines. The present work aimed to identify the biochemical determinants linked to the yellow color of *B. napus* seed coat.

Materials and Methods

Plant material

A breeding program is ongoing since 20 years at the INRA of Rennes to select yellow-seeded *B. napus* lines with superior agronomic properties and producing seeds with low erucic acid and glucosinolate contents. The yellow-seed trait originates from the interspecific cross between a canadian *B. rapa* line and a spring-type and black-seeded *B. napus* genotype. An inbred line, 'YS', was used in our breeding schemes and was crossed with competitive winter-type black-seeded genotypes. Following self-fertilization, yellow-seeded inbred lines displaying low-erucic acid and low-glucosinolate contents as well as stable seed colour phenotype and yield were obtained.

Amongst these lines, we focussed on the F_6 offspring from the PR3984*YS*Aviso cross and on the respective parental lines PR3984, Aviso (black-seeded) and YS. Details for oilseed rape growth conditions in greenhouse were reported previously (Nesi *et al.*, 2009). To determine procyanidin and flavonol composition of the seed coats, tagged siliques were collected from individual plants 30, 37

and 45 days after pollination (dap). Four siliques were collected from one plant and seeds were carefully removed. Seed coats were manually separated from the embryos and immediately frozen into liquid nitrogen. The sampling was done in triplicate for each developmental stage, considering three independent plants grown together.

Extraction, characterization and quantification of seed coat flavonoids (Auger et al*., 2010)*

Samples were lyophilized and ground. Methanol/acetone/water/TFA mixture (40:32:28:0.05, v/v/v/v) was added to the samples that were sonicated. After centrifugation, the pellet was extracted further with the same mixture. After a second centrifugation, the two supernatants were pooled and separated in two equal parts. Extracts were concentrated under nitrogen flow. The first part of extracts was dissolved in 1% acetic acid in methanol to analyze flavonol content. The second part was submitted to de-polymerization for quantification of soluble procyanidins (Kennedy and Jones, 2001). Briefly, extracts were dissolved in 0.1 N HCl in methanol containing phloroglucinol and ascorbic acid. After incubation at 50°C, the reaction was stopped by adding one volume of 0.2 M aqueous sodium acetate. The molar amount of soluble procyanidins was quantified as the sum of phloroglucinol-derivatized extension units and free terminal units. All the samples were filtered through Teflon membrane before LC–ESI–MS^n analysis. HPLC was performed with a water/acetonitrile gradient applied through a XDB–C18 Eclipse column and mass analyses were carried out with a LCQ Deca mass spectrometer equipped with an ESI source used in the negative ion mode. Data collection and processing were done with Xcalibur software v. 1.2. Retention times and MS^n mass spectra of compounds were recorded and compared with commercial standards or published data when available, in order to characterize and quantify flavonoids.

Anthocyanin extraction from plantlets

Glycosylated cyanidin derivatives were looked for in 10-days old plantlets grown on MS medium supplemented with 1%, 5% or 10% sucrose to create an osmotic stress that induced anthocyanin production. To extract anthocyanins, three plantlets were grounded in 0.3 M HCl/ethanol 75:25. Chloroform was subsequently added to eliminate chlorophylls. After centrifugation, two phases were observed with anthocyanins in the upper phase and chlorophylls in the lower phase.

Results

*Pigmentation of YS and PR3984*YS*Aviso seed coat was not uniform*

The seed coat of the two “yellow-seeded” lines was not entirely yellow since the micropylar-chalazal area remained black and the yellow seed body was spotted with brown to black spots of uneven frequency (Fig. 1).

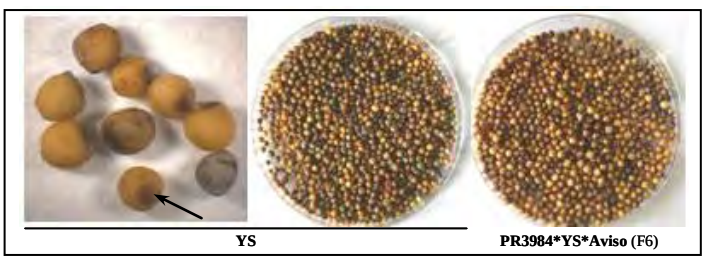

Figure 1. Phenotype of YS and PR3984*YS*Aviso mature seeds. Arrow highlighted the black micropylar-chalazal region.

Seed coats of yellow-seeded lines were almost deprived of soluble procyanidins

We chose to quantify soluble procyanidins in seed coats during the seed maturation stage, when their content was maximal in black-seeded lines. Indeed, it was previously shown that soluble procyanidins accumulate from mid-embryogenesis onwards and that their content increased throughout seed development to reach a maximum level during the early seed maturation stage (30 dap) that corresponded to 10% of the seed coat dry weight (DW). Then, from 40 dap onwards soluble procyanidin amount decreased concomitantly with the onset of seed browning (Auger *et al.*, 2010). Therefore, seed coats were harvested at 30, 37 and 45 dap. According to previous results, procyanidin content reached 103.5 *µ*g/mg seed coat DW for PR3984 and 73.2 *µ*g/mg seed coat DW for Aviso at 45 dap. However seed coats of yellow-seeded lines accumulated few or no procyanidins (Fig. 2A).

Flavonol content was not altered in yellow-seeded lines

In an earlier work, seven main flavonols were detected by LC–ESI–MS in flavonoid extracts from *B. napus* seed coats and were isorhamnetin, quercetin and kaempferol derivatives (Auger *et al.*, 2010). These seven flavonols were retrieved in 30, 37 and 45 dap-seed coat extracts of yellow-seeded lines, as well as a new isorhamnetin derivative with $[M-H]^-$ at *m/z* 719 that was tentatively identified as isorhamnetin-dihexoside-sulfate after MS^n analysis. Flavonol accumulation during seed maturation followed the same kinetic in black- and yellow-seeded lines. Most of flavonol contents varied depending on the accession: ANOVA analysis demonstrated that there were as much significant differences between PR3984 and Aviso than between black and yellow seeded lines ($P<0.05$). But interestingly, at the three developmental steps, isorhamnetin-sinapoyl-trihexoside content was significantly lower in YS/PR3984*YS*Aviso than in PR3984/Aviso ($P<0.05$). On the contrary, isorhamnetin-dihexoside-sulfate amount was higher in yellow-seeded lines, especially during late-maturation (Fig. 2B).

Anthocyanin biosynthesis was induced in plantlets grown under osmotic stress

Besides flavonoid accumulation in seed integuments, the possible impact of the mutation(s) leading to the yellow seed trait on anthocyanin biosynthesis in the vegetative parts was studied by growing plantlets under osmotic stress conditions. Ten days after germination, plantlets of YS and PR3984*YS*Aviso were able to accumulate anthocyanins, as the parental lines PR3984 and Aviso (Fig. 2C).

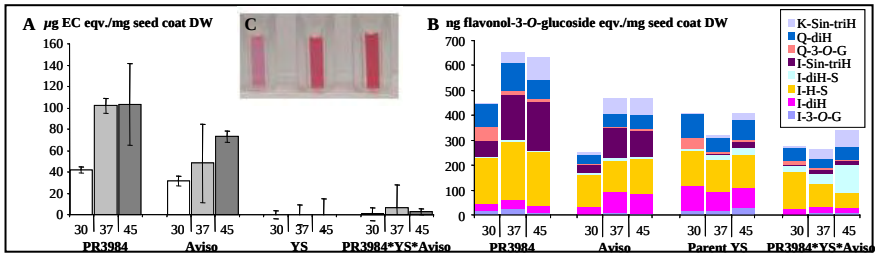

Figure 2. Flavonoid content in *B. napus* black- and yellow-seeded lines (A), Accumulation of soluble procyanidins after acidic cleavage in the presence of excess phloroglucinol and (B), Time-course accumulation of flavonols depending on their aglycone. Quantifications were made in the seed coat during seed maturation using LC–ESI–MS. (C), Anthocyanin production in 10 days old PR3984*YS*Aviso plantlets grown in MS medium supplemented with 1%, 5% and 10% sucrose (from left to right).

Q-3-*O*-G, quercetin-3-*O*-glucoside; Q-diH, quercetin-dihexoside; I-3-*O*-G, isorhamnetin-3-*O*-glucoside; I-diH, isorhamnetin-dihexoside; I-H-S, isorhamnetin-hexoside-sulfate; I-diH-S, isorhamnetin-dihexoside-sulfate; I-Sin-triH, isorhamnetin-sinapoyl-trihexoside; K-Sin-triH, kaempferol-sinapoyl-trihexoside.

Discussion

Procyanidin content differed between black- and yellow-seeded lines

Seed coat of PR3984*YS*Aviso did not contain procyanidins but displayed an unaltered flavonol content. Further studies would therefore be interesting to specify the role of procyanidins in *B. napus* seed coat. The only significant difference in flavonol content brought by YS was that isorhamnetin-dihexoside-sulfate might be preferentially accumulated instead of isorhamnetin-sinapoyl-trihexoside during seed maturation. The presence of such original hexoside and acylated derivatives of isorhamnetin, quercetin and kaempferol in *B. napus* seed coat justified the interest to breed lines specifically altered in procyanidin metabolism. Indeed, these flavonols may certainly have an important physiological role and their content must not be modified by breeding. Moreover, the anthocyanin pathway was unaffected in YS and PR3984*YS*Aviso plantlets. Then amongst flavonoids, the mutation harbored by the YS line seemed to essentially affect the procyanidin pathway. Maybe that some yellow seeded lineages displaying poor agronomic properties and described in the literature were affected before the flavonol/procyanidin or anthocyanin/procyanidin split and then in the whole plant and not specifically in the seed coat.

Which brown pigments did accumulate in the seed coat of the yellow-seeded lines?

Seed coats of yellow-seeded lines accumulated few or no procyanidins but still exhibit brown to black spots, notably at the micropylar-chalazal level. Maybe that a small amount of oxidized procyanidins was enough to display a local brown pigmentation. In fact, during seed maturation, weak epicatechin amount was quantified in some seed coats. However, this residual pigmentation could also be linked to the accumulation of polyphenols like lignans or other phenylpropanoids, as detected in mature seed coats of *B. carinata* (Li *et al.*, 2010). In the future, unraveling the nature of these brown pigments will be overriding since no other anti-nutritional compound (sinapic acid esters, lignins…) should accumulate in the seed coats of yellow-seeded lines instead of procyanidins.

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