Antisense suppression of *BnTT10* family genes causes retarded pigmentation and lignin reduction in seed coat of *Brassica napus*

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Abstract

Yellow-seed (*i.e.*, yellow seed coat) is one of the most important agronomic traits of *Brassica* plants and associated with seed oil and meal qualities. Previous studies on Brassicaceae plants, including *Arabidopsis* and *Brassica* species, proposed that the seed coat color trait was correlated to the flavonoid and lignin biosynthesis. A stable major QTL affecting seed coat color of *B. napus* in different populations and environments had been identified using SRAP technology. Based on microsynteny and comparative genomic analysis of the major QTL region with *Arabidopsis* genome sequences, the bi-functional gene *TT10* was the most important candidate gene. In this study, three *TT10* genes cloned from *B. napus* were progenitors of 2 and 1 *TT10* genes obtained from *B. rapa* and *B. oleracea*. Phylogenetic and bioinformatic analysis revealed that these *TT10* genes could be divided into two groups with obvious structural and functional differentiation. Spatial expression pattern assay showed that *Brassica TT10* genes were essentially expressed in developing seeds with differential expression patterns in yellow- and black-seeded near-isogenic lines. Furthermore, individual *BnTT10* gene probably inherited the expression pattern of its ortholog gene, for spatial and temporal characteristics, as well as association with yellow seed trait, indicating that different members of a gene family may play their own roles on yellow seed trait. However, overall *TT10* expression in developing seeds of yellow- and black-seeded NILs of *B. napus* showed no significant difference since the comprehensive roles of these gene members. For functional analysis, three black-seeded *B. napus* varieties were selected to develop *TT10* gene expression suppressed transgenic plants. Compared with control plants, identical phenotype of retarded pigmentation in seed coat at maturing and after-ripening stages were observed in transgenic plants of three varieties, implying the pigmentation and browning of black-seeded *B. napus* seed coat was affected by *BnTT10* genes. Increased soluble PAs (i.e. monomers or oligomers of proanthocyanidins) and decreased extractable lignin in the seed coats of transgenic plants detected by chemical composition analysis indicated that *BnTT10* genes possessed essential functions in proanthocyanidin polymerization and lignin biosynthesis. Furthermore, hypocotyl and root elongation of transgenic *B. napus* was significantly slower than that of control in seedling experiment for 5 days on MS medium. Interestingly, quantitative real-time RT-PCR showed a top-down temporal expression pattern in 5 days after seed germination, implied potential key roles of *BnTT10* in hypocotyl development and root elongation.

Keywords: *Brassica napus*; *Brassica rapa*; *Brassica oleracea*; *TRANSPARENT TESTA 10* (*TT10*); seed coat; proanthocyanidin; lignin

Introduction

B. napus is the second largest oilseed producing crop next to soybean and cultivated in many regions of the world. Yellow seed is one of the most important traits of *B. napus*. In previous study of our group, based on the sequence-related amplified polymorphism (SRAP) technology, a stable major

QTL was identified affecting seed color of *B. napus* in different recombinant inbred line (RIL) populations and different environments; based on microsynteny and comparative genomic analysis of the major QTL with *Arabidopsis* chromosome sequences, the target region should contain a bifunctional gene *TT10*, which was then considered as an important candidate gene for this QTL. In this study, *TT10* genes were simultaneously cloned from *B. napus* and its two parental species *B. rapa* and *B. oleracea*, and characterizated by expression pattern detection and functional identification by transgenic *B. napus*. Our findings provide evidence for understanding the role of *BnTT10* in PA metabolism, lignin synthesis and physiological properties in *B. napus* seed coat, and add some novel clues to reveal the molecular mechanisms of yellow seed traits formation of *Brassica* species.

Material and methods

Typical black-seeded *B. napus* line 5B, *B. rapa* line 06K130 and *B. oleracea* line 06K158 were used for gene cloning. *B. napus* near-isogenic lines (NILs) 09L588 (black-seed) and 09L587 (yellow-seed), *B. rapa* NILs 09L597 (black-seed) and 09L600 (yellow-seed), and *B. oleracea* NILs 09Bo-1 (black-seed) and 09Bo-4 (yellow-seed) were used for detection of expression patterns of *Brassica TT10* genes by RT-PCR. *B. napus* cv. Westar, Zhongyou821 and Zhongshuang10 were used for transgenic assays.

The cDNAs of *Brassica TT10* genes were cloned by RACE method. Expression patterns of *TT10* genes in various organs of indicated *Brassica* species were detected by semi-quantitative and real-time quantitative RT-PCR. A 951-bp conserved fragment of *Brassica TT10* genes was cloned in antisense orientation into the binary vector pCAMBIA2301G and this antisense *TT10* expression construct was transferred into *B. napus* using *Agrobacterium tumefaciens* strain LBA4404. To observe seed coat color during seed development, seed pods were regularly sampled at 40, 45, 50, 55 and 60 DAF and the whole stripped pods were observed under a low-power stereoscope. To observe seed coat color during seed after-ripening, the pods sampled at 40, 50, and 55 DAF were also stripped and observed after 5 and 15 days of *in vitro* storage at ambient temperature. The PA content was determined using the butanol-HCl method and LC-MS. The extractable lignin content was determined using the acetyl bromide method. To examine the effect of the inhibited expression of *TT10* on hypocotyl and root elongation, seeds were grown in a square Petri dish ($10 \times 10 \times 1.5$ cm^3) containing 35 ml of sterile solid medium consisting of 1 x MS salt, 2% sucrose, and 0.3% phytagel at pH 5.8. Freshly harvested transgenic or wild-type seeds (10 each) were firstly surface sterilized and arranged on the surface of the solid medium. The plates were placed vertically on a rack so that the hypocotyls and roots grew upward and downward on the surface of the growth medium respectively. Hypocotyl and root growth was tracked for 5 days by length measurement at the same time each day.

Results and discussion

1. Major members of *TT10* gene families from *B. napus* and its parental species *B. rapa* and *B. oleracea* could be divided into two groups

Using homology-based Rapid Amplification of cDNA Ends (RACE) technology, three *TT10* genes were isolated from *B. napus*, three and two *TT10* genes, representing 3, 2 and 1 unique alleles according to sequence similarities and origination relationships, were isolated from its parental species *B. rapa*, and *B. oleracea*, respectively. The highly homologous sequences of full-length mRNA, identical deduced protein sequences and similar expression patterns indicated that *BnTT10-1* and *BnTT10-3* are originated from *B. rapa BrTT10-1A* and *BrTT10-2*, and *BnTT10-2* is originated from *B. oleracea BoTT10-1*, respectively. Based on sequence alignments and similarity of expression patterns, eight *Brassica TT10* genes could be divided into two groups. *BnTT10-1*, *BrTT10-1A*, *BrTT10-1B*, *BnTT10-2* and *BoTT10-1* show high identities with each other and form Group II, *BnTT10-3* and *BrTT10-2* share higher homology to *Arabidopsis TT10* gene (*AtTT10*) than Group II members at full-length mRNA level and form Group I.

2. *BnTT10* gene family participates in the polymerization of proanthocyanidin monomers and lignin synthesis in seed coat of *B. napus*

A fragment conserved in *BnTT10*, *BrTT10* and *BoTT10* gene families was reversely inserted into pCAMBIA2301G to generate the antisense vector. By *Agrobacterium*-mediated transformation using hypocotyl explants, it was successfully transformed into three cultivars of black-seeded *B. napus*, standard laboratory stock Westar, typical commercial cultivar Zhongyou821 DH line, and double-low commercial cultivar Zhongshuang10. Quantitative RT-PCR detection revealed a 0-83% down-regulation of *BnTT10* overall expression in these transgenic lines, and similar phenotype modifications were observed from the transgenic lines of the three cultivars.

We determined the acetone-soluble PA in seed coat of transgenic plants with the butanol-HCl method and LC-MS. Our results showed that the inhibition of *TT10* expression resulted in increase of soluble PA content in seed coat, and the degree of increase of soluble PA content was also well in agree with the degree of inhibition of *TT10* expression, indicating that *Brassica TT10* possibly has the similar function as *AtTT10*, i.e. being involved in the polymerization of proanthocyanidin monomers. Our study also showed that the unknown PAs content increased in seed coats of valid transgenic seed coats, though the difference was not significant. This finding implies that *BnTT10* perhaps further oxidize PA polymers into larger polymers, but it needs further study.

Antisense suppression of *TT10* resulted in reduction of lignin content in transgenic seed coats when measured with the acetyl bromide method, revealing that *BnTT10* genes, like *AtTT10*, are involved in lignin synthesis in *B. napus* seed coats.

3. Presumable functional divergence among *Brassica TT10* genes

Our study indicated presumable functional divergence between the two groups of *Brassica TT10* genes in PAs oxidative polymerization. Group II members, excluding *BoTT10-1* and *BoTT10-1pse*, all expressed highest in middle-stage (25-30 DAF) seeds, with decreasing trends in seeds during gradual maturing process. In addition, the predicted subcellular localization of Group II proteins is the endoplasmic reticulum, in vesicles derived from which PAs are synthesized as colorless polymers (Stafford, 1988; Pourcel et al., 2005), indicating that Group II genes probably mainly act at the early step of PA synthesis, though the product may be not colorless (Pourcel et al., 2005). On the contrary, Group I members *BnTT10-3* and *BrTT10-2* exhibited strong expression during the whole process of seed development, with slightly increasing trends in expression during maturing process and showed highest expression in the late-stage seeds after pigmentation. At the same time, like *AtTT10*, the Group I genes are predicted to be secreted in the apoplast, where epicatechin and PAs would interact with *TT10* and become oxidized and polymerized during the seed desiccation period (Pourcel et al., 2005). It is possible that Group I genes mainly participate in the step of further oxidization PAs.

4. The *BnTT10* family influences early seedling growth of *B. napus*

In the process of seed germination and seedling growth, hypocotyls and root elongation of T_3 progenies of transgenic lines with inhibited *BnTT10* expression were significantly reduced compared to control lines, suggesting that *BnTT10* could influence the physiological properties of the seed. In 5 days after seed germination, qPCR results showed a gradually strong to weak overall *BnTT10* expression in hypocotyls, consequently with the weak to strong temporal expression pattern in roots, implied potential key roles of *BnTT10* in hypocotyls development and root elongation.

5. *BnTT10* family participates in the pigmentation of seed coat in *B. napus*

In this study, the transgenic plants with inhibited *BnTT10* expression showed delay in pigmentation of seed coats, implying the pigmentation of black-seeded *B.napus* seed coat is affected by *BnTT10* genes. The inhibition of *TT10* expression may be influence the oxidization polymerization of PA and furthermore affects the accumulation of pigments in seed coats. This may results in the

effect that seeds of transgenic plants were delayed to turn red or brown. However, the seeds of transgenic plants also can pigmentation, may be caused by the remained *TT10* activity or by non-enzymatic reaction. This finding is also verified by in vitro observation of seed pigmentation. When compared to seed pigmentation of control plants, seeds harvested at 40 DAF from transgenic plants also showed pigmentation delay after 5 days of after-ripening, and the color could not turn black after a month of after-ripening, indicated the role of *BnTT10* in pigment precursor accumulation of seed coat could not be compensated by autoxidation.

6. *BnTT10* genes are regulated by upstream yellow seed major genes of *B. napus*

Expression patterns of *TT10* genes in various organs of black- and yellow-seeded lines of *B. napus* and its two parental species were detected by RT-PCR. These members were essentially expressed in developing especially maturing-stage seeds and exhibited differential expressions in black-seeded and yellow-seeded lines of *B. napus*, *B. rapa* and *B. oleracea*. Besides, different members showed distinct relevance to yellow seed trait.

Our transgenic seeds with inhibited *BnTT10* expression, either those of T_2 or T_3, show reduction of lignin content and partial perturbation of PA polymerization, but the transgenic seeds still showed black color like the control. Previous studies of our group have found that many other loci, such as the *TT12* gene family, are also down-regulated in yellow-seeded *B. napus*. Thus we deduce that down-regulation of *BnTT10* gene family is one of determinative factors contributing to the yellow seed trait, however it is not the upstream yellow-seed gene but are downstream genes regulated by this major gene. *BnTT10* gene family has its usefulness in molecular breeding of transgenic yellow-seed trait of rapeseed, but simultaneous manipulation of multiple loci including *BnTT10* is necessary.

References Omitted

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