Analysis of the lignin contents and related enzymes activities in seed coat between black-seeded and yellow-seeded rapes (*Brassica napus*)

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Abstract

A pair of near isogenic yellow/black seeded rape (*Brassica napus*) were used as experimental materials to study the changes of lignin contents and enzymes activities of 4-coumarate: CoA ligase (4CL), Cinnamyl alcohol dehydrogenase (CAD) and ferulate 5-hydroxylase (F5H) in seed coat during seed development. The results showed that the changes of lignin contents and enzyme activities of 4CL, CAD and F5H in seed coat had significant differences between black- and yellow-seeded rapes, and also between different development stages. Correlation analysis demonstrated that the lignin contents were positively related to the activities of all three enzymes investigated in the study, and the interactions between them in the seed coat of the two lines. For yellow-seeded rape, except the correlation coefficient of lignin content and 4CL was not significant, the other correlation coefficients were significant or even highly positively significant. For the black-seeded rape, only the lignin content was highly positively related to the activity of F5H, the other correlation coefficients were not significant. It is suggested that 4CL, CAD and F5H regulate the biosynthesis of lignin in the seed coat of the rapes, leading to the lignin contents in the seed coat of the yellow-seeded rape much lower than that of the black-seeded line, and affecting the thickness of seed coat in rapes.

Key words: *Brassica napus*, Lignin contents, 4CL, CAD, F5H

Introduction

The yellow-seeded rape has been one of the major foci in breeding research. Because yellow-seeded rape has lower seed coat ratio, higher oil content and more transparent oil than their black-seeded counterpart. However, it is hard to obtain genetically stable and true breeding lines of yellow-seeded types in *B. napus*. In recent years, great attentions have been paid to the mechanisms of its seed coat formation. Previous studies have shed light on the understanding of the biochemical mechanism of seed coat formation in yellow-seeded rapes. It has been found that polyphenols, anthocyanin, flavonoid and melanin contribute to the color and lustre in seed coat of black- and yellow-seeded rapes (Ye et al., 2002), and it is implied that anthocyanin and flavonoid were break down or transformed into the precursors of melanin biosynthesis during the late stage of the seed development (Ye et al., 2002).

It is well known that related substrates and enzymes are required for the pigments biosynthesis, and the enzymes of polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) in seed coat were found to be very important effect on the forming of the seed color of rapes (Wang et al., 1991; Ye et al., 2001, 2001); and it was implied that PAL, PPO and peroxidase (POD) affected the thickness of the developing seedcoats in rape by regulating the biosynthesis of lignin (Liang, et al., 2004).

Lignin is a complex polymer formed by the oxidative polymerization of hydroxycinnamyl alcohol derivatives termed monolignols, and 4CL, CAD and F5H were believed to be key enzymes involved in lignin biosynthetic (Boudet et al., 1995; Baucher et al., 1998). It was reported that lignin was one of the key factors that lead to the lower coat ratio in the seed coats of the yellow-seeded rape than that of the black-seeded rape (Liang et al., 2002). In order to explain the different roles of enzymes played in the leading of lower lignin contents in the seed coats of the yellow-seeded rape, and to better understand the mechanism of seed coat character's formation of yellow-seeded and black-seeded rapes with attempt to provide the theoretical assistance for the yellow-seeded rape breeding, the lignin contents and the three key enzymes activities were investigated, and the changes of lignin contents, related enzymes activities of 4CL, CAD and F5H were analyzed in seed-coats during seed development between black- and yellow-seeded rapes by near iso-genic lines.

Materials and Methods

A pair of near iso-genic line of rapes (*Brassica napus*), L1 (black-seed) and L2 (yellow-seed), were the descendants derived from the cross combination of *Brassica napus* and *Brassica juncea*. In 1995, an individual plant of black-seeded rapeseed was identified in the tenth progeny of the yellow inbred lines, which was identical to the yellow seeded one in both growth and appearance. The desirable plants were obtained in the third generation of the black inbred line by inbreeding the black-seeded individual plant, which was identical to the yellow seeded one except that the seed color was black. The stable inbred lines of black and yellow seeded rape seeds formed the near-isonegic pairs. In order to maintain genetic consistency of the near iso-genic lines, the offsprings were preserved by using the black-seeded individual plant as recurrent parent and adopting the BC_1 and F_1. The seed coats were sampled at 20, 25, 30, 35, 40, 45d till to the full maturation of seed at 50d after

flowering. All the analyses were performed in triplicate. The triplicate of L1 was marked L1-1, L1-2 and L1-3, and that of L2 was marked L2-1, L2-2 and L2-3.

Lignin contents were analyzed according to previously described protocols (Xing, 1981). Dried seed coats (0.5 g) were used for the analysis. After removing the concomitants, the lignin was oxidized by the $K_2Cr_2O_7$ with the H_2SO_4.

Enzyme extraction and the enzymes activities assays: the enzyme extraction was using 0.1 mol L-1 Tris-HCl buffer (pH 7.5; 20 mmol L^{-1} mercaptoethanol; and 5% [w/v] polyvinylpolypyrrolidone), protein concentrations were determined using the Bio-Rad reagent (Bradford, 1976). 4CL activity was measured at room temperature by spectrophotometric assay to detect the formation of CoA esters of *p*-coumaric acid (Knobloch et al., 1977). CAD activity assays were carried out following Abbott's protocol (Abbott et al., 2002) with some modification. For F5H activity was determined by a NADPH regenerating system (Humphreys et al., 1999).

The data was analyzed with the DPS statistical software.

Results

As showed in Fig.1, the lignin contents reached to its maximum level when the seeds had grown for 40 days in the 2 lines of rapes. The maximal lignin content of the black-seeded rape was 13.56% while that of the yellow-seeded rape was only 4.92%. When the seeds nearly got ripen at 50 days, the lignin content of the yellow-seeded rape (3.15%) was a little higher than that (2.41%) in the early seed development stage, but that of the black-seeded one (13.32%) was much higher than that (5.94%) in the early stage.

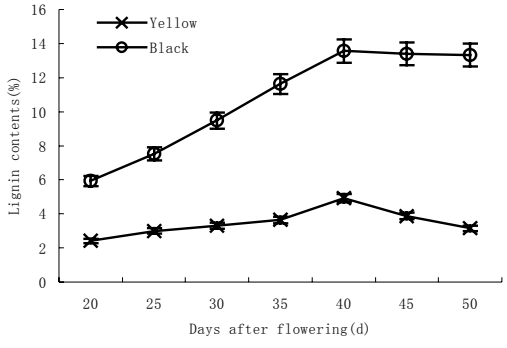

Fig.1 Changes of lignin content in seed coat

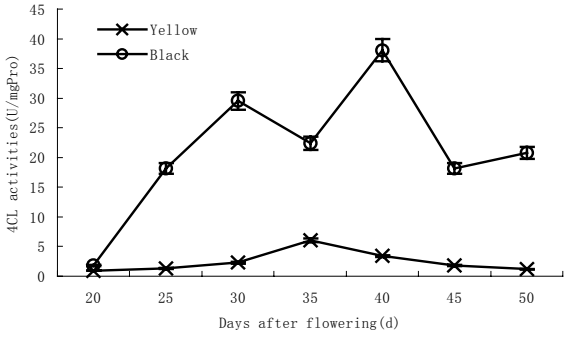

Fig.2 Changes of 4CL activities in seed coat

The changes of 4CL activities in seedcoats of the yellow-and black-seeded rapes were shown in Fig.2. In general, the 4CL activities of the two species increased at first, then decreased and increase again. The change of black-seeded rape is sharper than that of the yellow-seeded one. Enzyme activity of black-seeded reached its maximum level (38.09 U mg^{-1}Pro) at 40 days, and that of yellow-seeded one reached the maximum (6.10 U mg^{-1}Pro) at 35 day. The black one reached the second peak (29.55 Umg^{-1}Pro) at 30 days.

As showed in Fig.3, CAD activities of the two species firstly rose, and reached maximum at 40 days (Yellow-seeded rape: 0.2290 U mg-1Pro, Black-seeded rape: 1.4083 U mg-1Pro), then fell. But the enzyme activities of the black-seeded one increase significantly at 30 days and then dropped suddenly after reached its maximum; however the enzyme activity of the yellow-seeded rapes had two peaks including the maximum peak value, and begun to increase 5 days latter than the black one (Fig.3).

Fig. 4 demonstrated the changes of F5H activities in seedcoats of yellow-seeded and black-seeded rapes. The patterns of the F5H activity of the two NILs were different. The activity of F5H in the black-seeded rape reached its peak (1.8119 U $^{-1}$mg Pro) at 45 d while it reaches its peak at 40 days in the yellow one (0.2285 U mg-1 Pro).

As showed in table 1, in the seedcoats of the yellow-seeded rape, besides the correlation coefficient (0.4964) of lignin content and 4CL activity was not noticeable, the correlation coefficient (0.7262) of lignin content and the interaction between 4CL and F5H was noticeable, and the lignin contents were marked positive correlative to the activities of CAD and F5H, the interaction between 4CL and CAD, and the interaction between CAD and F5H in seedcoats of the yellow-seeded rape, the correlation coefficients of them were 0.9213, 0.9150, 0.8907, and 0.9214 respectively.

As showed above in table 2, in the seedcoats of the black seeded rape, only the lignin content was noticeable correlative to the activity of F5H, and the correlation coefficient was 0.772949, the other correlation coefficients of lignin contents to 4CL, CAD activities, the interactions between the three enzymes were 0.672363, 0. 541259, 0.503105, 0.703452, and 0.531204 respectively, they were not noticeable, but they all were above 0.5000.

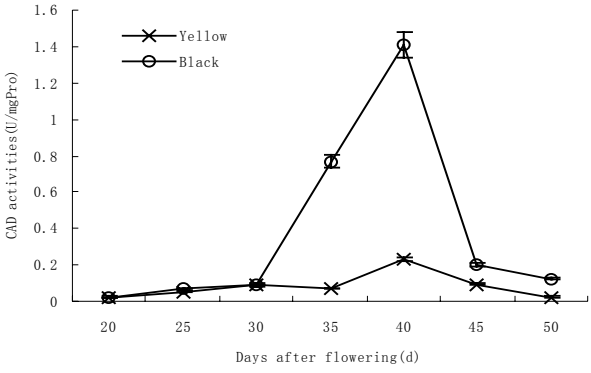

Fig.3 Changes of CAD activities in seed coat

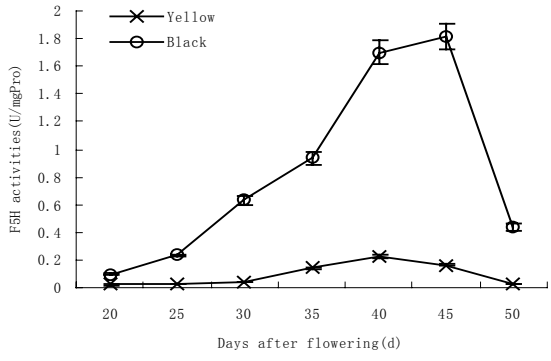

Fig.4 Changes of F5H activities in seed coat

Table.1 The correlation coefficients of lignin contents, 4CL, CAD and F5H activities in seed coat of yellow- seeded rape

Correlation coefficients	4CL	CAD	F5H	4CL × CAD	4CL × F5H	CAD × F5H	Lignin contents	Significant levels
4CL	1.0000	0.3725	0.5808	0.6869	0.8997	0.3648	0.4964	0.2571
CAD	0.3725	1.0000	0.8327	0.9124	0.6565	0.9601	0.9213	0.0032
F5H	0.5808	0.8327	1.0000	0.8576	0.8522	0.8834	0.9250	0.0028
4CL × CAD	0.6869	0.9124	0.8576	1.0000	0.8833	0.9106	0.8907	0.0071
4CL × F5H	0.8997	0.6565	0.8522	0.8833	1.0000	0.7069	0.7620	0.0465
CAD × F5H	0.3648	0.9601	0.8834	0.9106	0.7069	1.0000	0.9214	0.0032
Lignin contents	0.4964	0.9213	0.9250	0.8907	0.7620	0.9214	1.0000	1E-08

Table.2 The correlation coefficients of lignin contents, 4CL, CAD and F5H activities in seed coat of black- seeded rape

Correlation coefficients	4CL	CAD	F5H	4CL × CAD	4CL × F5H	CAD × F5H	Lignin contents	Significant levels
4CL	1.0000	0.6782	0.6278	0.6998	0.7770	0.6880	0.6724	0.0980
CAD	0.6782	1.0000	0.6424	0.9774	0.8359	0.9719	0.5413	0.2096
F5H	0.6278	0.6424	1.0000	0.6259	0.9133	0.6889	0.7729	0.0416
4CL × CAD	0.6998	0.9774	0.6259	1.0000	0.8645	0.9951	0.5031	0.2498
4CL × F5H	0.7770	0.8359	0.9133	0.8645	1.0000	0.8999	0.7035	0.0778
CAD × F5H	0.6880	0.9719	0.6889	0.9951	0.8999	1.0000	0.5312	0.2199
Lignin contents	0.6724	0.5413	0.7729	0.5031	0.7035	0.5312	1.0000	1E-08

Discussion

The seed coat color development are well documented (Ye et al., 2002, 2002), some enzymes involved in pigment biosynthesis have also been reported (Wang et al., 1991; Ye et al., 2001, 2001); it is reported that the lignin was one of the key factors that leaded to the lower coat ratio in seed coats of the yellow-seeded rape than that of the black seeded one (Liang et al., 2002), and it was implied that PAL, PPO and POD affected the thickness of the developing seedcoats in rape by regulating the biosynthesis of lignin (Liang, et al., 2004). 4CL, CAD and F5H are considered as key enzymes participating in lignin biosynthesis (Liang, et al., 2004). Therefor, it was anticipated that the lignin components might be different between yellow- and black-seeded rapes, and the S/G of the yellow-seeded rape were lower that of the black one, the activity of 4CL caused great difference in lignin contents, it was CAD and F5H leaded the quantitative difference of G- and S- monolignol between yellow- and black-seeded rapes. So, it was feasible to change the seedcoat ratio by over expressing or suppressing the activities of theses three enzymes or one of them. Whether the difference of the enzymes in the two species was due to DNA or RNA or even protein changes is still unknown, it needs to be further investigated by utilizing molecular biology or biotechnology techniques.

Hence, it is necessary to investigate the lignin biosynthesis, this is useful not only for elucidating the characters of the seed coat of rapes, but also the lignin biosynthetic mechanism in various cell types, tissues and organs of different genetic rapes. It is feasible to cultivate new kinds of rapeseed, which can not only provide more qualified oil but also become more adaptive to kinds of stresses by bioengineering methods in combination with conventional breeding techniques.

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