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Variation of carbohydrates and macronutrients during the flowering stage in canola (*Brasscia napus* L.) plants with contrasting seed oil content

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Abstract

Determination of dynamics of the physiological traits that affect the seed oil content in canola (*Brassica napus* L.) is essential for high seed oil content breeding programs. The main purpose of the present experiments was to explore the relationship between seed oil content and carbohydrates, N and P content of various canola aboveground tissues during flowering stage using two recombinant inbred lines RILS. Two field experiments were performed simultaneously in both 2010 and 2011. In experiment 1, seed yield and quality were compared between two RILs with contrasting seed oil content, that is, high oil content line (HOCL) and low oil content line (LOCL) at 50.4% and 41.4%, respectively. In experiment 2, our results showed the HOCL produced markedly more biomass in the reproductive organs by 9.9% and 30% at 35 days after anthesis (DAA) in 2010 and 2011, respectively. Furthermore, compared with LOCL, HOCL accumulated higher fructose, sucrose, and P content in the reproductive organs at the late flowering stage, with an average increment of 85.0%, 31.1%, and 12.4% at 35 DAA, respectively. However, the mean N content in the reproductive organs of HOCL was 17.27% lower than that in LOCL at 35DAA. These results reveal the importance of the higher reproductive organ C/N ratio in the regulation of the higher seed oil content of HOCL.Therefore, the C/N ratio of the reproductive organ may be a useful physiological indicator to screen canola lines for high seed oil content in future breeding program.

Keywords: Brassica napus; biomass; carbohydrate; oil content; nutrient.
Abbreviations: DAA-days after anthesis; HOCL-high oil content line; LOCL-low oil content line; RIL-recombinant inbred line.

Introduction

Canola is an important worldwide oil crop, and its seed oil has multiple functions with various applications in our daily life. Canola seed oil is also an ideal alternative for petroleum because it is renewable and environmentally friendly, which has been successfully adopted for the industrial use in several countries such as Germany and Canada (Nass et al., 2007; Karp and Richter 2011). Furthermore, several types of fatty acids from canola seed oil are particularly useful in industry. For example, erucic acid is harmful to humans as part of the vegetable oil but is widely used in plastic film and lubricant lines (Keller et al., 2001; Scarth and Tang, 2006). As a consequent, canola production had received increasing attention in many countries. Given the large demand for canola seed oil, breeders and researchers have been focusing on the research to increase canola seed oil content. Therefore, exploring the underlying physiological mechanisms of canola seed oil formation is one of the key components to assist breeding programs for high canola seed oil content. In addition, the ultimate aim of understanding seed oil biosynthesis is to apply it into breeding programs for high canola seed oil content through some effective methods such as genetic manipulation. Similar to many other traits of canola, seed oil content is also regulated by its genetic nature. Considerable investigations on the genetic control and quantitative trait locus (QTL) detection of seed oil content had been surveyed. This quality trait is very intricate with more than 10 QTLs have been identified (Zhao et al., 2005; Delourme et al., 2006; Qiu et al., 2006). Thus, isolation and cloning of major effect genes that control seed oil content is very difficult. On top of the genetic control, seed oil content is also influenced by many agronomic practices such as nitrogen (N) application as well as its direct and indirect developmental cues such as sink-to-source status (King et al., 1997; Jackson, 2000; Fortescue and Turner, 2007; Gan et al., 2008; Blackshaw et al., 2011). Flowering is a critical developmental stage of canola, where vegetative and reproductive growth demonstrates some overlaps. As a result, siliques and seeds develop rapidly, and most leaves in the lower stalk become senescent (Smith and Scarisbrick, 1990; Morrison et al., 1992). Normally, lots of nutrients will be transported from senescent leaves into young leaves, developing siliques, and seeds as a universal phenomenon in plants (Gombert et al., 2006; Vanacker et al., 2006; Wingler et al., 2006). Thus, young leaves in the middle and upper stalk of canola, stem, and siliques are composed of photosynthetic organs to produce carbohydrates, the basic material for synthesizing protein and oil (Triacylglycerols) in developing seeds (Focks and Benning, 1998; White et al.,

2000; Ekman et al., 2008). Furthermore, the silique is the most important part for photosynthesis among these organs (King et al., 1997). Consequently, developing siliques and seeds are actually replaced by the leaf as a new growth center. However, determining whether the genotype with high seed oil content of canola has a stronger sink and ampler source compared with that with low seed oil content is critical because this condition determines whether the former can produce enough carbohydrate for oil content. Unfortunately, related studies in canola are scare. In soybean, depodding treatment can increase sucrose concentration in the seeds, but shading can decrease its cotyledon indicating that the sink-to-source status modulates seed filling (Egli and Bruening, 2001). However, Fortescue and Turner (2007) reported that the removal of axillary branches can result in a larger seed size with reduced seed oil content in canola. Therefore, whether increased carbohydrate in the seed can correspondingly improve seed oil content is unknown. Although the genetic nature of seed oil content and lipid biosynthesis pathway is recently more lucid (Hajduch, 2006; Agrawal et al., 2008; Houston et al., 2009), little attention is given on the physiological and biochemical changes in the aboveground tissues at the flowering stage among canola genotype with high and low seed oil content. In the present study, we focused on the kinetics of carbohydrate and nutrient content in aboveground tissues at the flowering stage in low and high seed oil content genotypes. Comparison was performed to determine whether the variation in the aforementioned factors can partly uncover the physiological contribution to a higher oil accumulation in developing seeds.

Results

Seed yield and chemical composition of high oil content line (HOCL) and low oil content line (LOCL)

With amazingly high oil content at 50.4%, seeds of HOCL contained 21.4% more oil than that of LOCL (Table 1). On the contrary, the seed protein content in the HOCL was significantly lower (5.5%) than that in LOCL. Apart from seed oil content, HOCL exhibited higher seed yield, which was 18% more than that of LOCL. According to the fatty acid profile, the total unsaturated fatty acid content in both lines was nearly equal, which was 872 g kg $^{-1}$ in HOCL and 871 g kg^{-1} in LOCL, respectively. However, the seed oleic acid content in HOCL was 7.3% higher than that in LOCL, which has been reported to be beneficial in lowering blood pressure (Terés et al., 2008), indicating the high quality of the HOCL seed oil (Table1).

Dry matter accumulation of aboveground tissues in HOCL and LOCL

During the flowering stage, due to the overlap of vegetative and reproductive growth, the dry matter dynamics in the aboveground organs, such as the main stem and branches, leaves, and flowers and fruits (including the buds, developing siliques and seeds) reflects the developing condition of the canola plants. In both years, stem dry matter increased rapidly from 0 to 7 DAA and remained steady over the following four weeks, with averages at 15.98 and 18.43 g plant^{-1} for HOCL and LOCL, respectively (Fig 1. A). Similar trends were also found for the trials in 2011. Significant higher stem dry matter in HOCL was mostly observed at the first three weeks (0, 7, 14, and 21 DAA) of flowering stage in both years (Fig. 1. A and B). However, the stem dry weight was relatively stable compared with leaf and flower and fruit dry matter (Fig. 1). Over the five weeks, there was a dramatic decrease of leaf dry weight averaged at 85.7% and 94.6% for both lines in 2010 and 2011, respectively. Apart from higher leaf dry weight in LOCL at 7 DAA and in HOCL at 28 DDA, there was no obvious difference between leaf dry matter of HOCL and LOCL in 2010 and 2011 (Fig. 1). On the contrary, the accumulation of the dry matter of reproductive organs was very rapid over the 35 days, resulting in 14.6 and 13.2-fold increase for both lines at 2010 and 2011 (Fig. 1. A and B). The dry matter of the flower and fruit of the HOCL was significantly higher ($P < 0.05$) than that of LOCL except at 0 DAA in 2010. Similar results were only observed during the late flowering stage in 2011. For instance, HOCL gained 15.6% more dry weight of flower and fruit in comparison to LOCL (Fig. 1.B).

Carbohydrate dynamics of aboveground tissues in HOCL and LOCL

Fructose content

In order to find out whether the above difference of seed yield, seed oil content, and dry weight in HOCL and LOCL is related to the carbon partitioning in different tissues. We have conducted series of measurement on the dynamics of carbohydrate (Fig. 2). Similar dynamics of fructose was observed in the aboveground tissues in both HOCL and LOCL over two years (Fig 2. A and B). Stem and leaf fructose content decreased dramatically at the first week from anthesis and maintained at low level over the next four weeks. Furthermore, stem fructose content differed significantly between HOCL and LOCL only at 0 and 7 DAA in both years (Fig 2. A and B). However, the leaf fructose content in the HOCL was significantly higher ($P < 0.05$) than that in LOCL from 7 DAA throughout the flowering stage in 2010, however, little difference was observed in 2011. The flower and fruit fructose content accumulated quickly and peaked at 28 DAA in both lines over two years, but reduced by an average of 35.8% and 48.9% at 35 DAA in 2010 and 2011, respectively (Fig 2. A and B). In general, HOCL had significantly more fructose content in the reproductive organs ($P < 0.05$) than that of LOCL at the late flowering stage.

Sucrose content

In both years, stem and leaf sucrose content was very high when the two canola lines started flowering. However, it declined dramatically to 72.3% (mean value of the two lines and years) after one week and gradually recovered to 63.3% of that of 0 DAA after five weeks (Fig 2. C and D). Overall, it was illustrated that no significant difference in the stems and leaves sucrose content in the two lines. In contrast, sucrose content in the flower and fruit increased constantly over the flowering stage. In addition, significant difference ($P < 0.05$) between the two lines was detected especially at the late flowering stage in both years. This gap between the two lines was particularly evident in 2011, and the sucrose content in HOCL was about 15 mg g^{-1} higher than that in LOCL at 35 DAA (Fig 2. C and D).

Starch content

Starch content in the canola aboveground tissues of the HOCL and LOCL were much more variable between two years compared with fructose and sucrose content (Fig 2. E

and F). For example, the stem starch content in the LOCL was slightly higher than that in HOCL at 35 DAA in 2010, whereas the reverse result was observed in 2011. The starch content in leaves, and flowers and fruits of both cultivars peaked at the middle flowering stage in two years, and the starch content in the reproductive organs was above 30 mg g^{-1} at 14 DAA.

Nitrogen (N) and phosphorus (P) dynamics of aboveground tissues in HOCL and LOCL

In addition to the carbohydrates, N and P partitioning and conversion in different tissues also affect canola seed yield, seed oil content, and dry weight. Therefore, we conducted series of measurements on the dynamics of N and P in HOCL and LOCL (Fig. 3).

N content

N is one of the most important macronutrient elements and is involved in many physiological and biochemical metabolisms. Stem N content was, on average, about 30 mg g-1, however, it decreased to less than half of the value after 7 DAA and kept at this low level throughout the flowering stage. There was no difference in stem N content between the two lines in both years (Fig 3. A and B). Also, stem N content was much lower compared with that in the leaf, and flower and fruit. Further, leaf N content was more variable over the five weeks of flowering in both years, which was markedly higher ($P < 0.05$) in the LOCL than that in HOCL at 0 and 7 DAA in 2010, whereas no significant difference was observed between the two lines in 2011. However, the leaf N content in the HOCL was significantly higher ($P < 0.05$) than that in LOCL from 14 to 35 DAA in 2011 (Fig 3. A and B). Conversely, the dynamics of N content was observed in the reproductive organs with the LOCL showing a higher N content in most cases in comparison to the HOCL (Fig 3. A and B). In short, the results indicate a significant difference of N content in the leaf, and flower and fruit of the two lines.

P content

Similar dynamics of P content in the stem, leaf, and flower and fruit was observed in the HOCL and LOCL in two years (Fig 3. C and D). Overall, the flower and fruit were higher in P content than the stem and leaf. P content in the stem of the two canola lines exhibited no significant difference throughout the entire flowering stage (Fig 3. C and D). Although the leaf P content in the HOCL was slightly higher than that in LOCL, the difference among most of the developmental stages was not significant. P content in the flower and fruit in the HOCL was significantly higher than that in LOCL, except at 0 DAA in 2010. However, in 2011, no significant difference was found until 21.2% and 10.7% ($P < 0.05$) higher P content was identified for HOCL at 28 and 35 DAA in the two lines. These results revealed that the P content differed between the two lines mostly in the reproductive organs.

Discussion

Selection and breeding of high oil content canola cultivars has received particular attention because of its commercial value for both agriculture and industry. For instance, the HOCL with high percentage of oleic acid is a good quality trait of canola due to its health benefits (Bondia-Pons et al., 2007; Terés et al., 2008). Therefore, based on the current and previous research work in our lab, we proposed that the HCOL in this study is a desirable canola line for extensive cultivation in the future. In HOCL and LOCL, most of the leaves in the lower and middle of stalk were senescent at flowering stage (Fig. 1), leading to the relocation of N and P nutrients from these leaves into young ones and siliques (Fig. 3 C, D, Gombert et al., 2006; Vanacker et al., 2006; Wingler et al., 2006). Consequently, the export of N from old leaves to new ones and the developing siliques must have a strong competition. Given that the leaf sample was a mixture of old and new one, its total N content decreased because N content of the old leaves decreased rapidly at the early flowering stage. Thereby, a peak of leaf N content in the two lines was appeared at 21 DAA in both years. Interestingly, the leaf N content in the HOCL was significantly higher than that in LOCL whereas the reverse trend was observed in the reproductive organs. Seeds contain moderately low N content has been considered advantageous to increase seed oil content. In some reports, excessive N application into the soil can be absorbed by canola plants and will result in excessive synthesis of seed protein, resulting in the reduction of seed oil content (Cheema et al., 2001, 2010). Recently, Zhang et al. (2010) found that disrupting the *Amino Acid Permease 2* (*AAP2*) gene in *Arabidopsis* led to the depression of the leaf amino acid transportation (as N form) into the seed without significant effects on seed carbon content. As a consequence the increased ratio of C/N resulted in high seed oil content. Another similar result was also documented in corn, in which the C/N ratio in the mature endosperm is directly associated with the final kernel composition of starch and protein (Seebauer et al., 2010). These results in *Arabidopsis* that highly homologous to canola and cereal crops suggest the importance of C/N ratio in the regulation of the main reserves during seed development. In our present study, carbohydrate, particularly fructose and sucrose, content in the reproductive organ of the HOCL, was significantly higher than that in LOCL, indicating a higher C/N ratio in the seeds of HOCL. Therefore, according to the previous reports (Seebauer et al., 2010; Zhang et al. 2010), a higher seed oil content may be obtained in the HOCL. However, whether the decrease of seed N in the HOCL is also related with the expression levels of homologs of the *Arabidopsis APP2* gene in the reproductive organ of the HOCL should be further investigated. Besides N, P is also necessary for fatty acid synthesis. In the present study, we found that the P content of flower and fruit was generally higher than the leaf and stem. At this stage, stem, young leaves, and developing siliques were composed of important photosynthetic organs, however, flowers and siliques were the main growth center. Thus P nutrient would be prioritized to meet flower and silique development. P is a part of the nuclear acid component and is involved in lipid and protein biosynthesis regulation. Furthermore, during canola seed development, Agrawal and Thelen (2006) reported that lots of proteins including metabolic enzymes need to be reversibly phosphorylated such as sucrose synthase and sucrose phosphate synthase. In our present survey, silique contained significantly higher P content in the HOCL than that in LOCL at the late flowering stage. Whether this is beneficial for seed lipid accumulation also requires strong evidences. It is well known that carbohydrate plays an important role in the regulation of protein, lipid, and other secondary metabolites such as cellulose and flavonoid biosynthesis because numerous genes and proteins that were identified involve carbohydrate metabolism such the tricarboxylic acid cycle (Ruuska et al., 2002; Hajduch et al., 2006; Hua et al., 2007; Noguchi et al., 2009, Li et al., 2011). In the present investigation, a marked

Table 1. Comparison of yield, oil and protein content, and fatty acid profile between high oil content line (HOCL) and low oil content line (LOCL) in 2010 and 2011.

Lines	Yield (kg ha^{-1})	1000-weight (g)	Oil content (%)	Protein content (g kg^{-1})	Fatty acid profile (g kg^{-1})							
					Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Arachidic acid	Docosanoic acid	Erucic acid
HOCL	3572.5a	4.3a	50.4a	257b	93a	25a	633a	181b	56a	3a	1a	2a
LOCL	2920.0b	4.2a	41.5b	272a	92a	24a	587b	218a	64a	4a	1a	2a
F value	52.12**	7.06	596.45**	11.93*	1.50	2.53	304.85**	153.52**	8.61*	3.69	0.00	0.33

Data for the oil and protein content, and fatty acid profile over two years were combined after *F* test because of no significant difference of these traits between two years. Different lowercase letters in the same column show a significant difference at $P < 0.05$. The * and ** indicate statistical significance at the $P < 0.05$ and $P < 0.01$, respectively.

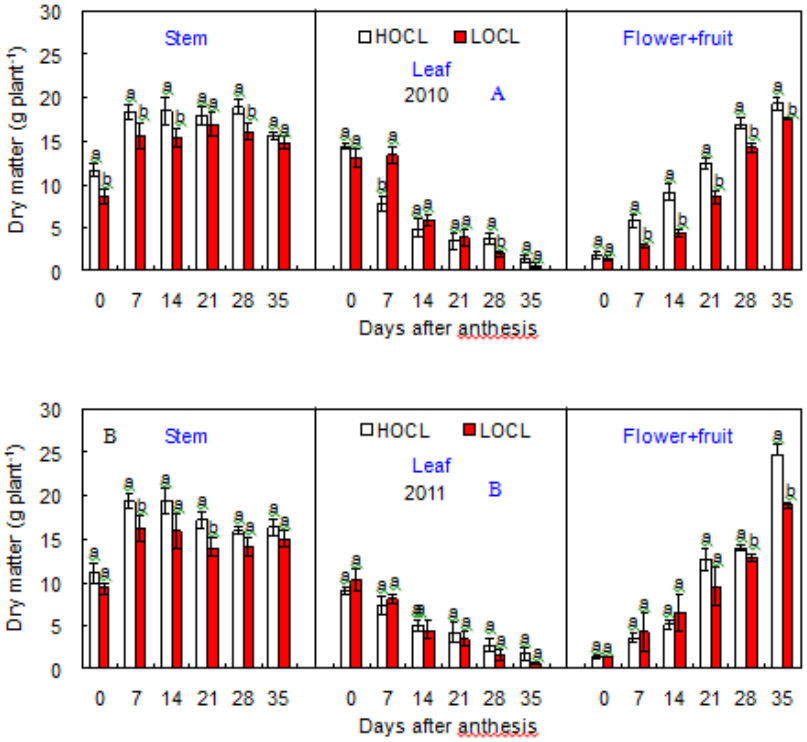

Fig 1. Dry matter of aboveground tissues in the high oil content line (HOCL) and low oil content line (LOCL) from 0, 7, 14, 28, and 35 d after anthesis during 2010 (A) and 2011 (B). Each value is the mean of 15 canola plants, five from each of three replicate plots. Error bars indicate standard errors of mean. Different lowercase letters at each development stage implicate a significant difference at $P < 0.05$ between two lines in both years.

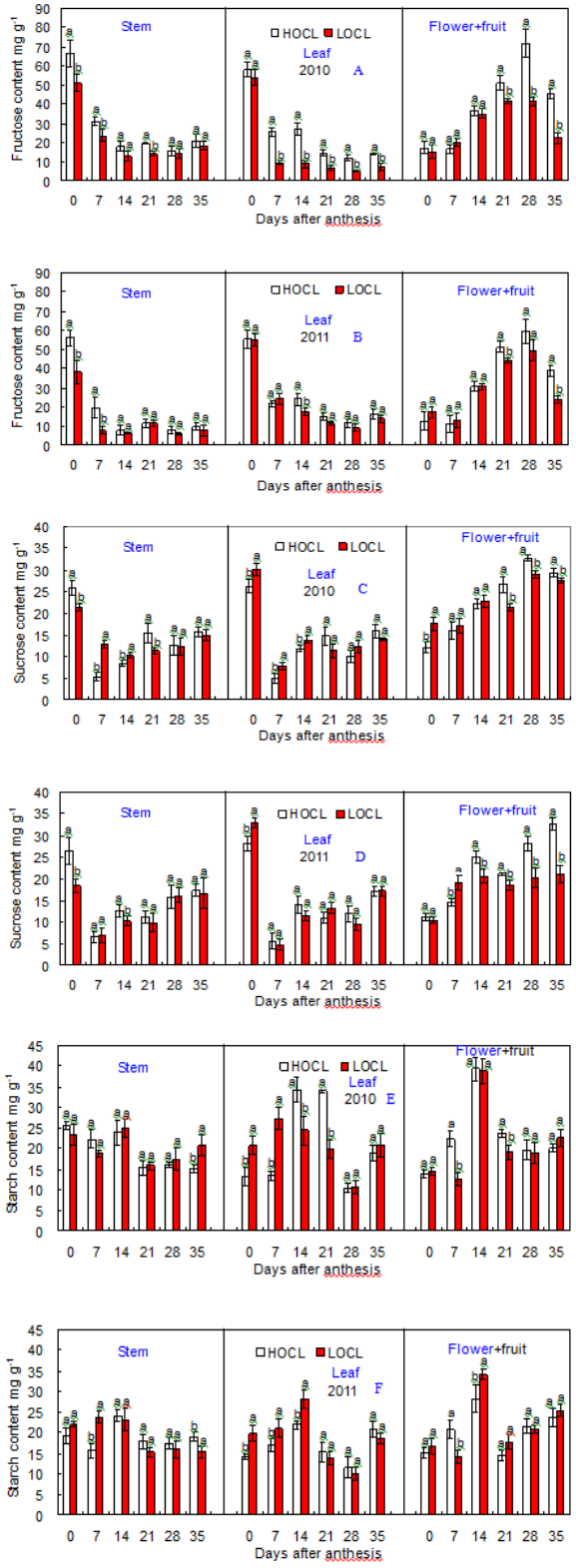

Fig 2. Fructose (A and B), sucrose (C and D), and Starch (E and F) content of aboveground tissues in the high oil content line (HOCL) and low oil content line (LOCL) from 0, 7, 14, 28, and 35 d after anthesis during 2010 and 2011. Each value is the mean of 15 canola plants, five from each of three replicates. Error bars indicate standard errors of mean. Different lowercase letters at each development stage implicate a significant difference at $P < 0.05$ between two lines in both years.

difference in the carbohydrate content was generally found in reproductive organs but not in stem or leaf indicating that the growth of the reproductive organs was much vigorous than the leaf. Furthermore, the fructose and sucrose content in the HOCL in the flower and fruit was significantly higher than that in LOCL. Consequently, ample material for lipid biosynthesis combined with low N content in the developing silique and seed is a key factor to guarantee higher seed oil content in the HOCL compared with LOCL. More recently, Tan et al. (2011) manipulated *Brassica napus LEAFY COTYLEDON1* (*BnLEC1*) and *LEC1-LIKE* (*Bnl1L*) genes in the developing seed, which resulted in an increase oil content by a maximum of 20%, which was from 35.57% to 42.72% in the third generation of transgenic canola seeds. They also found that expression these two genes upregulated *SUCROSE SYNTHASE* gene, which can cleave into fructose and glucose, in the silique walls and developing seeds. Furthermore, the developing seeds of transgenic lines of the sucrose and fructose content were significantly higher than Westar (control). Therefore, these results strongly support that enough carbohydrate content is beneficial for more lipid accumulation and seed oil formation.

Materials and methods

Plant materials

Field experiment was carried out in 2009-2010 and 2010-2011, two growth seasons at the experimental station of the Zhejiang Academy of Agricultural Sciences in Hangzhou, China. Two recombinant inbred lines, that is high oil content line (50.4%) and low oil content line (41.4%), derived from two parental lines (Huyou 15 as male parent and Zheshuang 6 as female parent) crossed in 2000. The seeds of the two lines were sown in a seedling bed on the 3rd and 5th of October, in 2009 and 2010, respectively. Necessary field practices, such as weed, disease, and pest control were conducted to obtain high yield and good quality. Seedlings with uniform growth were selected and transplanted after one month. The soil type in the experimental station was loamy clay (loamy, mixed, and thermic Aeric Endoaquepts). Before transplanting, 150 kg ha^{-1} of urea was evenly applied in the soil. Additional 75 kg ha^{-1} urea was applied at the end of January in 2010 and 2011, respectively. The field was not irrigated during canola growth season because rainfall is sufficient during this period

Experimental design

Two experiments were conducted simultaneously. In experiment 1, it was a randomized complete block design with three replications using HOCL and LOCL as two treatments. The seeds were harvested for seed yield and quality determination. Experiment 2 was performed with a randomized complete block design in a split-plot with three replications. HOCL and LOCL were used as the main plots and different sampling intervals as sub-plots. The sampling intervals were composed of 0, 7, 14, 21, 28, and 35 DAA six treatments. Each plot consisted of eight rows, 0.35 m apart between rows and 20 m length with 0.2 m apart between each plants.

Harvesting treatment and sampling procedure

In experiment 2, as for sub-plot treatments, samples were

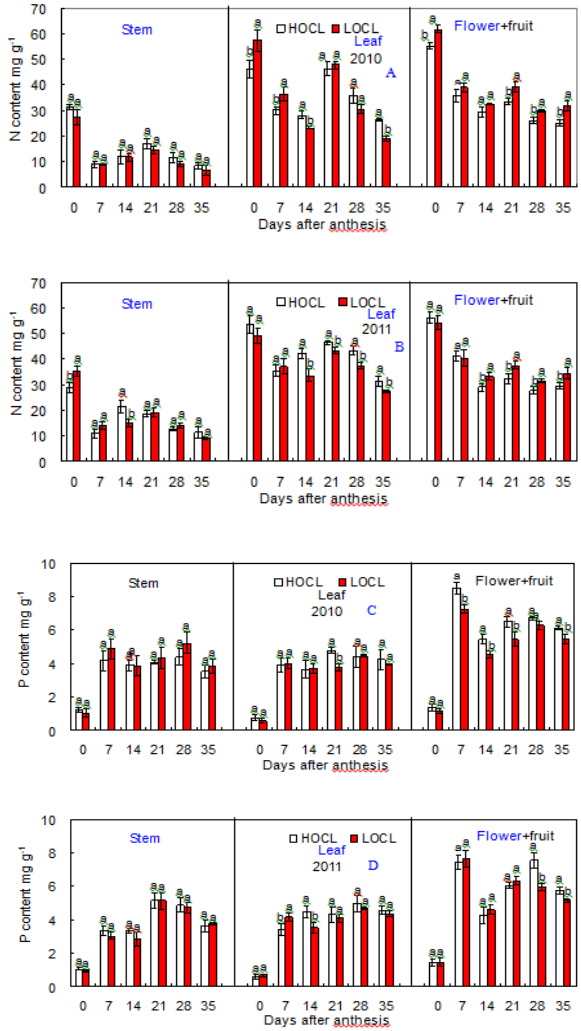

Fig 3. N (A and B) and P (C and D) content of aboveground tissues in the high oil content line (HOCL) and low oil content line (LOCL) from 0, 7, 14, 28, and 35 d after anthesis during 2010 and 2011. Each value is the mean of 15 canola plants, five from each of three replicate plots. Error bars indicate standard errors of mean. Different lowercase letters at each development stage implicate a significant difference at $P < 0.05$ between two lines in both years.

collected from the first flower anthesis, which was designated as 0 days after anthesis (DAA) and applied as harvesting treatment 1. And then 7, 14, 21, 28, and 35 DAA were applied as other five harvesting treatments. Only the aboveground tissues were harvested for carbohydrate and nutrient determination. Five plants in the middle of each plot, which was the central four rows (10.5 m^2) of each sampling plot (21 m^2), were randomly collected and aboveground tissues were cut by a sharp knife at the base of stem. The sampling of plants in the middle of each plot was a destructive process, therefore those plants were not used for any experimental analysis.

Yield and quality analysis

Plants (exception of border ones to avoid from boarder effect) in half of each 20-m long plot were used for yield determination whereas the other half was used for quality analyses. The method for fatty acid extraction was according to Hajduch et al. (2006). The assay of fatty acids was performed using a Shimazu gas chromatograph (GC-2014, Japan) with a flame ionization detector and a 30 m (length) × 0.25 mm (inner diameter) × 0.25 μm (liquid membrane thickness) column (Supelco wax-10, Supelco). Conditions of gas chromatography were followed on as described by Hajduch et al., (2006). Seed oil and protein content was determined by a Near Infrared instrument (Bruker, Vector 22/N, Germany).

boveground dry matter Determination

Plant samples were harvested at 0, 7, 14, 21, 28, and 35 DAA as six harvesting treatments, which was a mixture of 5 plants from every plot. The plants were immediately taken back to the laboratory and separated into stalk, leaf, flower and fruit (including buds, flowers, and siliques). Fresh samples were cut into small pieces, dried at 105 °C for 30 min to stop the cell metabolism and then kept at 70 °C until constant weight.

Carbohydrate content measurement

The dried tissue samples were ground into fine powder by a pulverizer. The powder of each sample was boiled in 30 mL of 80% (V/V) ethanol for 30 min twice and centrifuged at 10000 × *g* after the sample extract was cooled into room temperature. Two hundred milligrams of active charcoal was added to remove chlorophyll the supernatant of each sample and was then used for fructose and sucrose determination according to the method described by Hendrix (1993). The pellet was retained for starch analysis. The starch in the pellets was digested with amyloglucosidase for 100 min at 55 °C following the protocol demonstrated by Hendrix (1993).

N and Phosphorus (P) assay

Dried samples were ground, passed through a 0.5-mm screen, and wet digested. N content in tissues was measured by the standard Kjeldahl method, whereas and P was determined by the vanadomolybdate yellow color using a spectrophotometer (SHIMADU UV-2550, SHIMADU Co., Kyoto, Japan) (Olsen and Sommers, 1982).

Statistical analysis

The statistic analysis was performed using SPSS software for Windows (version 11.0). For experiment 1, the seed yield, oil content, protein content, and fatty acid profile over two years were combined using average data based on the Bartlett's Chi square test, indicating no significant difference between the two years. In experiment 2, *F* value was calculated for the split-plot experimental design. The dry weight of stalks, leaves, and flowers, the carbohydrate, N, and P contents of two lines were compared using Duncan's test at a probability of 0.05 within each developmental stage (0, 7, 14, 21, 35 DAA) in both years.

Conclusion

In the present study, significant difference of aboveground biomass, carbohydrate content including fructose, sucrose, and starch, and N and P content was observed in HOCL and LOCL at flowering stage. The higher biomass that produced by HOCL in aboveground tissues especially in the reproductive organ resulted in the higher seed yield, implicating its higher photosynthetic capacity. More

carbohydrate content and less N content were observed in reproductive organs of HOCL benefiting the synthesis seed oil, while lower carbohydrate content and higher N content in LOCL promoting protein synthesis in seeds. Therefore, this C/N ratio can be used as a physiological indicator to screen genotypes for high seed oil content in canola breeding program.

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