

Research Article

Different Levels of N Supply Impacts on Seed Yield by Modulating C and N Metabolism in *Brassica Napus*

Bok-Rye Lee^{1,2,†}, Hyo Lee^{1,†} and Tae-Hwan Kim^{1,*}

¹Department of Animal Science, Institute of Agricultural Science and Technology, College of Agriculture & Life Science, Chonnam National University, Gwangju 61186, Korea.

²Asian Pear Research Institute, Chonnam National University, Gwangju 61186, Korea.

ABSTRACT

Oilseed rape is known to crop having low nitrogen use efficiency (NUE) but requires high levels of N fertilizer. NUE is associated with N remobilization from source to sink organ, consequently affects seed yield. Remobilization of leaf N is also related to transport of C/N metabolites in phloem. However, interaction between seed yield and phloem transport was not fully documented. In response to seed yield, N and C metabolites and their transport into seed from bolting to pod filling stage investigated in two contrasting genotypes (Capitol and Pollen) cultivated under ample (HN) or limiting nitrate (LN) supply. Seed yield was significantly reduced in N limitation and its reduction rate was much lower in Capitol than in Pollen compared to HN treated plants. Amino acid and protein content was higher in Capitol than in Pollen at bolting stage. They gradually decreased during plant development but not significant between two cultivars and/or two treatments. Glucose, fructose and sucrose content were 1.8-, 1.6- or 1.25-fold higher in LN condition than in HN condition, respectively. Amino acid and sucrose content in phloem were largely higher in Capitol than in Pollen under LN condition. These results indicate that the higher seed yield might be related to greater transport ability of amino acid and sucrose in phloem under LN condition.

(Key words): Oilseed rape, Seed yield, Amino acid and sucrose transport, N limitation)

I. INTRODUCTION

Oilseed rape (*Brassica napus* L) is a major crop for vegetative oil production in the world and grown for biodiesel and animal feed. This crop requires high capacity of mineral nitrogen (N) absorption (160-250 kg N ha⁻¹ year⁻¹) but only 50% of N from fertilizer is recovered in the seeds at harvest (Schjoerring et al., 1995). Besides, high level of residual N (up to 100 kg N ha⁻¹ year⁻¹) was observed in fallen leaves of oilseed rape (Dejoux et al., 2000). This low N use efficiency (NUE) is related to low N remobilization (NRE) during natural senescence as transition from vegetative to reproductive stage of oilseed rape development, as reported in several studies (Tilsner et al., 2005; Gombert et al., 2006). Indeed, this low NRE strongly impacts on agronomic potential and final yield (Noquet et al., 2004). Avice and Etienne (2014) reported that seed yield was increased by improving N remobilization from leaves or reducing the residual N in falling leaves, as suggested by Malagolic et al. (2005b). N remobilization

therefore is considered as crucial determinant for oilseed rape yield.

Several studies reported that N remobilization from leaves to seed is closely associated with protease activity (Gombert et al., 2006; Girondé et al., 2015; Poret et al., 2019) which is related to degradation of soluble proteins in oilseed rape. Poret et al. (2016) reported that activity of several proteases such as PLCPs, VPEs, CPs increased by leaf senescence of rape seed, remained to late stages of leaf senescence and their activity was more increased under N deficit condition. Indeed, N remobilization in two cultivars of rape seed in response to N limitation depended on protease activities (Girondé et al., 2015). In addition, N remobilization is affected by phloem transport. Amino acid produced by protein degradation in leaves is transported into the phloem to develop sink organs such as young leaves, pods and seeds. Lee et al. (2016) reported that amino acid transporter genes, AAT1, AAT2, AAT4 and AAT6, were largely induced in leaves during reproductive stage. Overexpression of amino acid permease1 (AAP1) in pea enhanced N uptake and utilization

[†]These authors have contributed equally to this work.

*Corresponding author: Tae-Hwan Kim, Department of Animal Science, Institute of Agricultural Science and Technology, College of Agriculture & Life Science, Chonnam National University, Gwangju 61186, Korea.
Tel: +82-62-530-2126, Fax: +82-62-530-2129, E-mail: grassl@chonnam.ac.kr

efficiency, consequently improved production of seeds (Perchlik and Tegeder, 2017). Therefore, N remobilization is associated with protease activity and amino acid transport in phloem which are potentially important factors influencing NUE (Xu et al., 2012; Mu et al., 2015), but their effects on N restricted condition at different stage of plant growth was not yet fully understood.

It has been well reported that carbon and nitrogen metabolism is closely linked. For example, nitrogen deficiency induced accumulation of carbohydrate in leaves and increased translocation of sucrose to sink organ improve N availability (Hermans et al., 2006). Sucrose is the main source of carbon and energy in the sink tissues of plants. During *Brassica napus* seed development, sucrose synthesized in the mature leaves is loaded into phloem to sink tissues. Therefore, sucrose transport is an important to developing seed growth. In rice, inhibition of sucrose efflux into phloem lead to reduction of seed production (Eom et al., 2011). We hypothesized that sucrose transport in source organs contribute to plant NUE and that their activities affect plant growth and development in N restricted condition.

Recently, we found that NUE in 8 genotypes of oilseed rape defined three genotypic profiles with different behaviors during vegetative stage senescence that were associated with drought (Lee et al., 2016). We selected two genotypes, Capitol and Pollen, which are having contrast NUE. Capitol is able to maintain high NUE under drought stress-induced N deficient condition. The aim of this work is to investigate effects of nitrogen limitation on seed production in relation to amino acid and sucrose transport in different two *Brassica napus* cultivars during plant development.

II. MATERIALS AND METHODS

1. Plant growth and treatment

Two cultivars of *Brassica napus* ‘Capitol’ and ‘Pollen’ were grown in greenhouse. After bolting stage, plants were divided with 2 groups and supplied different levels of nitrogen, 0.2 mM (LN, low nitrogen) and 2 mM (HN, high nitrogen). Plants were harvested at different growing stage; bolting stage, flowering stage, pod filling stage, and mature seed stage. Plants were separated into roots, leaves, stem, siliques, seeds and pod walls. After harvest, the tissue of leaf was frozen immediately in liquid nitrogen and stored in deep freezer (-80°C) for further analysis.

2. Determination of amino acids and protein

The amino acid concentration was measured by the ninhydrin colorimetric method (Sun et al., 2006). About 200 mg of fresh ground samples were extracted with 1 mL 20 mM Tris-HCl, pH 7.5 and centrifuged. The supernatant was mixed with 100 mL ninhydrin solution and 25 mL 4.0 M lithium acetate buffer and boiled for 10 min. After cooling, 5 mL of 50% alcohol was added into the mixture. The absorbance of the reaction mixture was measured at 570nm and calculated with glycine as a standard. Soluble proteins were extracted by suspending freeze-dried sample with 100mM sodium phosphate buffer (pH 7.0). The protein concentration was determined using Bradford reagent (Sigma), with BSA as the standard protein.

3. Soluble sugars and starch analysis

For sugar extraction, leaves (0.2 g) were homogenized in 1 ml of 80% ethanol and centrifuged, then supernatants were collected for analysis. Sucrose content was determined according to the method by Handel (1968). The reaction mixture containing 100 μ l of the supernatant and 100ul of 30% KOH, and 3ml of 0.15% anthrone reagent was incubated at 40 °C for 15 min. After reading 620 nm, absorbance was calculated using sucrose as the standard. Glucose content was measured by following the method of Koehler (1952). The supernatant was mixed with 0.2% athrhone reagent and boiled at 100 °C for 8 min. Then, absorbance was measured at 625 nm and calculated using glucose as the standard. Fructose was assayed by methods described previously from Davis and Gander (1967). For this, the supernatant was mixed with 12N HCl and 0.05% resorcinol reagent and then incubated at 77 °C for 8 min. After incubation, the reaction mixture was recorded at 420nm and calculated using fructose as the standard. Starch analysis was carried on by followed the method of Baxter et al. (2003). The ethanol-extracted residue was dried at 80 °C to remove ethanol. Deionized water was added and boiled for 10 min to gelatinize the starch. After cooling, 400 ml of 0.2 N Na-acetate buffer (pH 5.1) was added. Starch was digested by adding 0.2 U of amyloglucosidase (Sigma product A3514) and 40 U of α -amylase (Sigma product A0273) in the acetate buffer to each sample. Tubes were incubated at 50 °C for 24 h with occasional shaking and centrifuged. Glucose in the supernatant was determined as mentioned above. Starch concentrations were calculated multiplying a factor of 0.9 to glucose concentration.

4. Collection of phloem exudates

The phloem exudates were collected in EDTA using the facilitated diffusion method, in accordance with the methods described by Lee et al. (2009). The forth fully extended leaf was cut and immediately immersed in 20mM EDTA solution (pH 7.0) for 5 min, and then transferred to a new tube containing 5mM EDTA solution, and kept for 6 h in a growth chamber with 95% relative humidity under dark condition. Amino acid and sucrose in phloem exudates were measured as mentioned above

5. Statistical analysis

The experiment was performed in a completely randomized design with three replicates for four treatments and sampling date. Duncan's multiple range test was used to compare the means of separate replicates. All statistical measures were performed using SAS 9.1 (SAS Institute Inc. 2002-2003). Differences at $P \leq 0.05$ were considered significant.

III. RESULTS AND DISCUSSION

1. Impact of N limitation on seed yield

We expected that Capitol having high NUE at vegetative stage might produce higher seed yield regardless of N application level. When plants were well-supplied with nitrate (HN), however, the seed yield was higher for Pollen (23.7g) compared with Capitol (18.4g) at mature seed stage (Fig. 1).

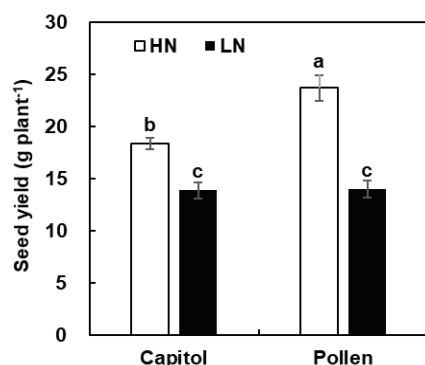


Fig. 1. Seed yield of 'Capitol' and 'Pollen' under ample (HN, 2mM) or low N supply (LN, 0.2mM). Data are means \pm s.e. of three replicates. Different letters at each plant stage are significantly different ($P < 0.05$) according to Duncan's multiple range test.

In low nitrate (LN) condition, no difference in seed yield was observed between genotypes. As expected, seed yield was reduced in LN condition. Reduction rate of seed yield was higher in Pollen (-40.9% compared to HN) than in Capitol (-24.5% compared to HN). Our results showed the greater ability of Capitol plants to adapt to N limitation, indicating NUE levels at vegetative stage mostly impacts N limitation condition during reproductive stage but not in well-supplied condition. It might be difference of main source organ for N remobilization between early- and late reproductive stage, as suggested by Girondé et al. (2015).

2. Genotype difference in N assimilation and amino acid transport in response to N limitation

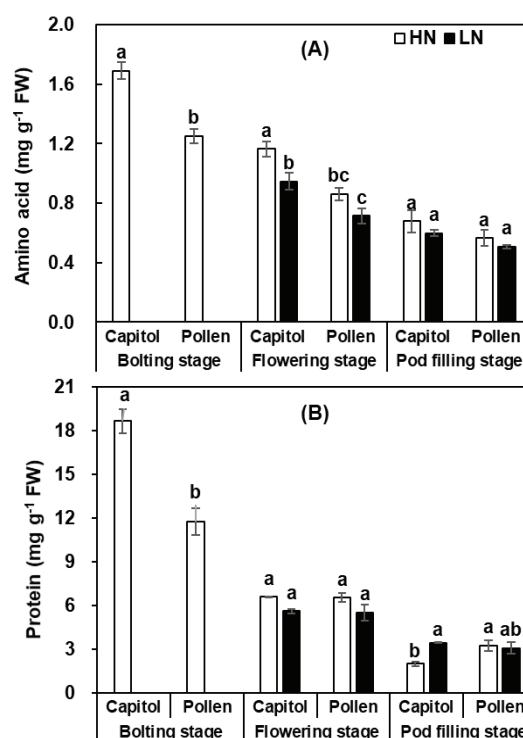


Fig. 2. Kinetics of Amino acid (A) and protein content (B) in mature leaves of 'Capitol' and 'Pollen' under ample (HN, 2mM) or low N supply (LN, 0.2mM). Data are means \pm s.e. of three replicates. Different letters at each plant stage are significantly different ($P < 0.05$) according to Duncan's multiple range test.

To investigate effect of genotype difference on production of seed yield, we compared N assimilates such as amino acid and proteins, and amino acid in phloem during plant development. At

bolting stage, amino acid and protein content were much higher in Capitol than in Pollen (Fig. 2A, B). In HN plants, amino acid was 27.9% higher in Capitol than in Pollen at flowering stage (Fig. 2A), while it was decreased by about 56.7% at pod filling stage. In response to LN supply, amino acid reduction for both genotypes was highlighted at flowering stage, where the amino acid of Capitol was 18% higher than Pollen. However, no genotype/treatment interaction was not observed in amino acid content at pod filling stage. Protein content was gradually decreased from bolting stage to pod filling stage in both cultivars regardless of N supply levels (Fig. 2B). At flowering stage, protein content was 6.5 mg g⁻¹ FW in HN-treated plants and 5.6mg g⁻¹ FW in LN-treated plants but no difference in both genotypes. At pod filling stage, in response to HN condition, protein content was 62.5% higher in Pollen than in Capitol. However, it was slightly higher in Capitol than in Pollen under LN condition but not significant. It has been well reported that reduction of protein content is associated with reduction of N assimilation and enhancement of protein degradation during natural- or N deficit stress-induced senescence (Girondé et al., 2015; Poret et al., 2019). Protein degradation leads to increase

of amino acid content in leaves and consequently increase amino acid transport into phloem (Lee et al., 2016). In the present study, however, amino acid content in phloem was remarkably decreased to over 78% at flowering stage and 90% at pod filling stage in both treatments (Fig. 4A). Absolute amino acid content in phloem was higher in Capitol than in Pollen at bolting- and flowering stage. Compared to Akela, amino acid content in phloem of Capitol was 2.6-fold higher in HN treatment and 5.7-fold higher in LN treatment at flowering stage, while it was 40.3% lower in HN treatment but 1.7-fold higher in LN treatment. It has been demonstrated that seed yield is highly related to amino acid transport (Xu et al., 2012; Mu et al., 2015; Perchlik and Tegeder, 2017). Despite of high amino acid transport in Capitol at bolting- and flowering, seed yield was much lower than Pollen in HN condition. Contrary, it was higher in Pollen than in Capitol at pod filling stage. These results suggest that seed yield is more affected by amino acid transport at pod filling stage under HN condition, while amino acid transport from source organ to sink organ is mostly important to seed yield under LN limitation during plant development.

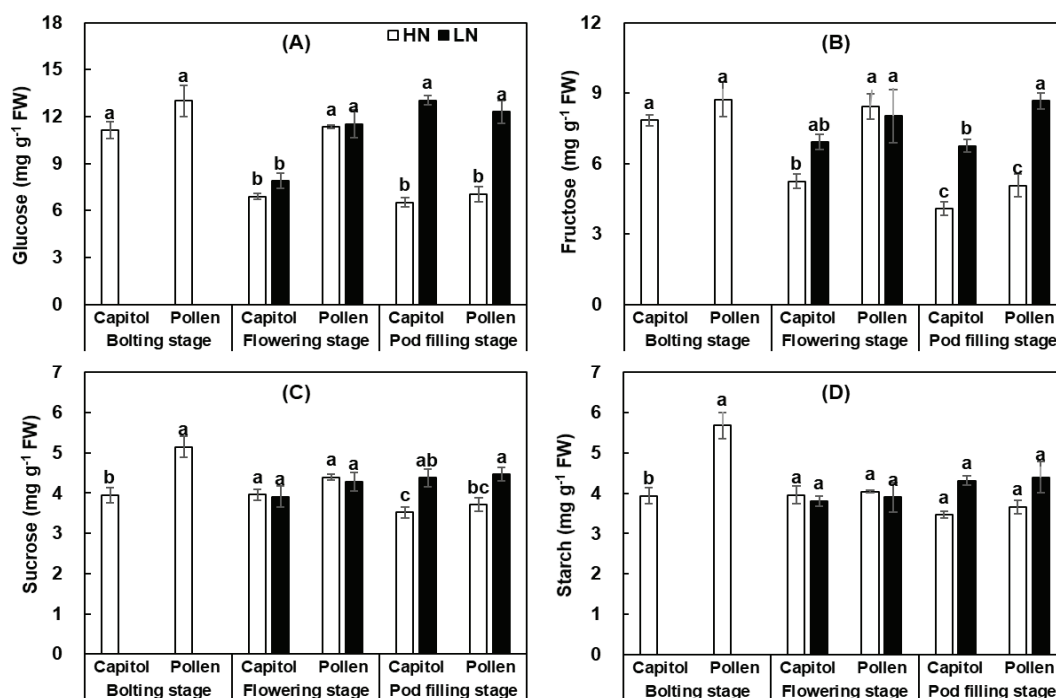


Fig. 3. Kinetics of glucose (A), fructose (B), sucrose (C) and starch content (D) in mature leaves of 'Capitol' and 'Pollen' under ample (HN, 2mM) or low N supply (LN, 0.2mM). Data are means \pm s.e. of three replicates. Different letters at each plant stage are significantly different ($P < 0.05$) according to Duncan's multiple range test.

3. Genotype difference in C assimilation and sucrose transport in response to N limitation

It has been well reported that N and C metabolism are highly interacted. The number of researches have reported that N deficiency alternate photosynthesis, sugar metabolism and carbohydrate partitioning between source and sink tissues (Morvan-Bertrand et al., 1999; Boussadia et al., 2010). For instance, N deficiency increase carbohydrates such as sugars and starch in leaves because of low demand of carbon in sink organ (Remans et al., 2006). Therefore, the high susceptibility of carbon allocation to N limitation may be associated with sucrose transport from source leaves to sink organ. Under N deficiency, sucrose content was found to accumulate in source leaves, which was explained as a result of either inhibited starch synthesis or inhibited phloem export of sucrose. In the present study, glucose and fructose content were significantly decreased in HN condition, while it was largely increased in LN condition in both genotypes (Fig. 3A, B). In both treatment, glucose content was 1.6-fold higher in Pollen than Capitol at flowering stage but not significantly different at pod filling stage. At pod filling stage, however, glucose content was 2-fold higher in HN condition than in LN condition. Similar tendency was observed in fructose content (Fig. 3B). At pod filling stage, fructose content was higher 25% in Pollen than Capitol in both treatments. Sucrose content was slightly decreased in HN condition during plant development, while it remained largely constant in N limitation (Fig. 3C). Starch content was 44% higher in Pollen than in Capitol at bolting stage but not significantly different at flowering- and pod filling stage (Fig. 3D). Our results are consistent with previously reported results by Remans et al. (2006) and Boussadia et al. (2010). Sucrose content in phloem in both cultivars was the highest at bolting stage and it showed 5.5-fold higher in Capitol than in Pollen (Fig. 4B). After bolting stage, sucrose content in phloem of Capitol was largely decreased to pod filling stage, whereas it was largely constant in Pollen under HN condition. In response to LN condition, sucrose content in phloem was 4.8-fold or 1.4-fold higher in Capitol than in Pollen at flowering stage or at pod filling stage, respectively. These results indicate that greater ability of sucrose transport is related to tolerance to N limitation in Capitol than in Pollen as a results of higher seed yield.

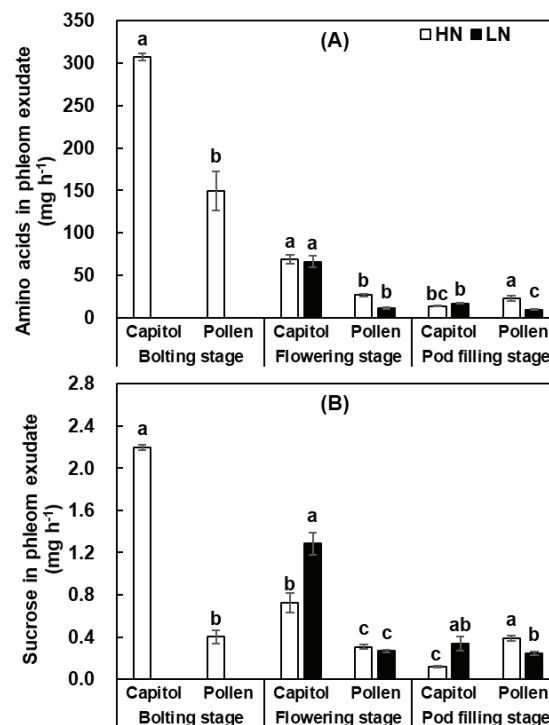


Fig. 4. Kinetics of amino acid (A) and sucrose (B) content in phloem of 'Capitol' and 'Pollen' under ample (HN, 2mM) or low N supply (LN, 0.2mM). Data are means \pm s.e. of three replicates. Different letters at each plant stage are significantly different ($P < 0.05$) according to Duncan's multiple range test.

IV. CONCLUSION

N limitation was conducted reduction of seed yield in both genotypes. However, Capitol having high NUE at vegetative stage showed lower seed yield compared to Pollen under well-supplied N condition. Reduction of seed yield was much lower in Capitol than in Pollen in LN condition. It is well known to have a close correlation between seed yield and amino acid or sucrose transport. In the present study, amino acid and sucrose content in phloem were significantly higher in Capitol than in Pollen in both flowering- and pod filling stage under N limitation. Therefore, our results suggest that the greater transport ability of amino acid and sucrose into the seed might impact on seed yield when plant exposed to N limitation.

V. ACKNOWLEDGEMENTS

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