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Short communication

Pod removal responsive change in phytohormones and its impact on protein degradation and amino acid transport in source leaves of *Brassica napus*

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ABSTRACT

To characterize the hormonal regulation of nitrogen remobilization from source to pod filling in *Brassica napus*, the hormonal level, proteolytic process, and amino acid transport were assessed in mature leaves of pod-removed or control at the early pod-filling stage. Pod (sink) removal decreased salicylic acid (SA), and significantly increased jasmonic acid (JA). The SA/JA ratio decreased with pod removal, accompanied by low degradation of foliar proteins and Rubisco content. A significant decrease in protease activity was observed in pod-removed leaves, confirmed by in-gel staining of protease. Pod removal reduced the expression of four amino acid transporter genes (*BnAAP1*, *BnAAP2*, *BnAAP4*, and *BnAAP6*) in mature leaves and reduced amino acid loading into phloem. These results indicated that a decrease in SA resulting from pod removal down-regulated nitrogen remobilization accompanied by a decrease in proteolytic activity and amino acid transport in mature leaves at the pod-filling stage.

1. Introduction

In plants, the source–sink relationship refers to the integration of photo-assimilates and amino acid production in photosynthetic tissue or organ (source) with the export of assimilates into the site of assimilate utilization (sink) in growth, storage, maintenance, and production. Source and sink metabolism is closely coordinated to maintain the balance between supply and demand. For example, the source activity drives the sink metabolism and vice versa, which in turn are related to C and N metabolism (Egli and Bruening, 2001; Iqbal et al., 2011; Zhang et al., 2015). The alteration of source activity during the regenerative stage usually results in a corresponding change in pod and seed number, indicating a source limitation. Source limitations during seed filling seem to be relatively common based on changes in seed size, but seed growth rate is not as responsive to changes in source activity and can be sink limited (Egli, 1999; Egli and Bruening, 2001). However, the response of sources and sinks during pod filling does not clearly define the grain yield and quality limiting processes.

The production of the reproductive organ (sink) is closely associated with the remobilization of assimilates from source leaves (Feller, 2004). The partitioning of photo-assimilates and starch hydrolysis in response to source–sink alteration have been widely reported (Egli, 1999; Egli and Bruening, 2001; Lee et al., 2008), whereas relatively less information on N remobilization is available. Besides, oilseed rape (*Brassica napus* L.) is characterized by low nitrogen use efficiency (NUE), which is mainly due to a lack of nitrogen remobilization capacity with less than 50% of nitrogen stored in source leaves used for seed filling (Rossato et al., 2001; Desclos et al., 2009). Some studies have suggested that the weak N remobilization in *B. napus* is primarily associated with poor hydrolysis of foliar proteins (Tilsner et al., 2005; Girondé et al., 2015) and a weak transport of amino acids (Tilsner et al., 2005; Zhang et al., 2015). Although N remobilization efficiency at the early regenerative stage is a crucial determinant for ultimate seed productivity and quality, regulatory mechanisms linked to the proteolytic process and amino acid transport is still not clearly established.

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Phytohormones are active signal compounds involved in grain development (Wang et al., 2006), with their regulation on sink potential via increasing cell differentiation such as plastid biogenesis and DNA amplification, and/or by controlling the import of assimilates and accumulation of dry matter in developing reproductive organs (Iqbal et al., 2011; Jibran et al., 2013; Albacete et al., 2014). Improvement of the harvest index in response to phytohormones has often been observed (Kuiper, 1993; Wang et al., 2006; Iqbal et al., 2011). Hormonal changes resulting from abiotic stresses, have a significant influence on crop productivity by mediating source–sink relations (Rivero et al., 2007; Ghanem et al., 2011). Our recent work provided evidence of salicylic acid-mediated protein degradation and amino acid transport in mature leaves of *B. napus* (Zhang et al., 2015).

In the present study, we hypothesized that 1) sink (pod) removal modifies endogenous hormonal status, and 2) this modification has a significant influence on proteolytic processes and amino acid transport at the early pod filling stage. To test this hypothesis, the N assimilate pool, protein profile, protease activity, amino acid transporter gene expression, and phloem loading of amino acids in relation to the hormonal changes resulting from pod removal were assessed.

2. Materials and methods

2.1. Plant culture and experimental procedure

The surface-sterilized seeds of *Brassica* napus L. cv. Naehan was sown into bed soil in trays. When seedlings were grown to the 4-leaf stage, they were transferred to field of the Agro-Bio Industry Technical Support Center, Chonnam National University. Oilseed rape plants at the early pod filling stage (about 3 weeks after flowering) were selected by morphological similarity and divided into two groups. As indicated in Fig. 1, in one group the pod was completely removed from the branch of plants and in the other group (control) the pod was not removed during the same period. After 5 days of treatments, leaves were separated by the order of ontogenic appearance, which was designated as leaf rank number (i.e., leaf No. 1 was the oldest leaf). In the present study, green mature leaves numbered 5–8 were considered. After sampling, leaf tissues were frozen immediately in liquid nitrogen and stored in a deep freezer until further analysis.

2.2. Collection of phloem exudates and determination of amino acids and protein

The phloem exudates were collected in EDTA using the facilitated diffusion method, in accordance with the methods described by Lee et al. (2009). The amino acid concentration was measured by the ninhydrin colorimetric method (Sun et al., 2006). The protein concentration was determined using Bradford reagent (Sigma), with BSA as the standard protein.

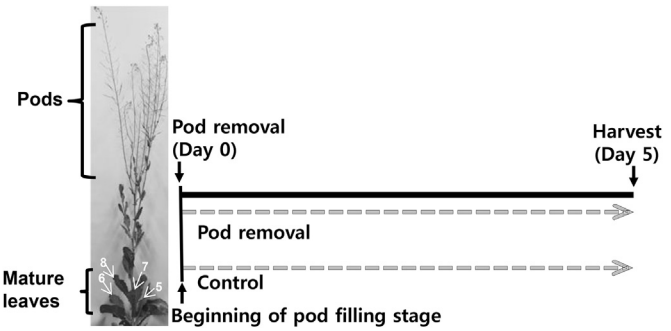

Fig. 1. Schematic diagram of the experimental design. The pod was removed from branches at the beginning of the pod filling stage. Mature leaves (leaf ranks 5–8) of *Brassica napus* were harvested 5 days after treatments.

2.3. Analysis of protein profiles by SDS-PAGE

To investigate the protein profile, SDS-PAGE was performed in a mini vertical electrophoresis system (Bio-Rad, Mini-PROTEAN). An equal quantity of protein (3 μg) from each sample was loaded into 12.5% gel and Precision Plus Protein Dual Color Standard (Bio-Rad) was incorporated into the gel to determine the molecular weight of the bands. The resulting gel was stained with the silver staining procedure described by Blum et al. (1987). The absolute integrated optical density (IOD) of each band was analyzed by Gel-Pro Analyzer software 4.0 (Media Cybernetics Inc., Bethesda, MD, USA).

2.4. Total protease activity and in-gel staining of protease

Total proteolytic activity of leaf extraction was determined according to the method of Beyene et al. (2006). One unit of enzyme activity was equal to the conversion of 1 μmol substrate per min. Visualization of proteases after electrophoretic separation in 12.5% SDS-PAGE containing gelatine was performed according to Beyene et al. (2006).

2.5. Hormone analysis

Quantitative analysis of salicylic acid (SA) and jasmonic acid (JA) in leaf tissue was performed according to Pan et al. (2010). Hormone extract from 50 mg of well-ground leaves was injected into a reverse phase C18 Gemini high-performance liquid chromatography (HPLC) column for HPLC electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS) analysis. An Agilent 1100 HPLC (Agilent Technologies), Waters C18 column (150 × 2.1 mm, 5 μm), and API3000 MSMRM (Applied Biosystems) were used for the analysis.

2.6. RNA extraction and quantitative RT-PCR

Total RNA was isolated from 100 mg leaf tissue using the SV Total RNA Isolation System (Promega). The first-strand cDNAs were synthesized using the GoScript Reverse Transcription System (Promega). Gene expression level was quantified on a light cycler real-time PCR detection system (Bio-Rad) with SYBR®*Premix Ex Taq*™ (TaKaRa, DALIAN). The qRT-PCR amplifications were performed using F-box (Bra006317; forward: 5′-GAGA-TAAGTCGCTTCCTACCG-3′ and reverse: 5′-TGTTCCCATTGCCCTGTG-3′). The primers of amino acid permease (*AAP*) were the following: *AAP1* (AY188953.1; forward: 5′-TGCTTACGCCACGGTTCTCA-3′ and reverse: 5′-GCTGCGCGAACACCTGATAG-3′), *AAP2* (AY188954.1; forward: 5′-CGGTACTGTTTGGACCGCAA-3′ and reverse: 5′-GACACGGGTCTTTTCCTCCG-3′), *AAP4* (AY188955.1; forward: 5′-CGCTGGACCTGCAGTGATGT-3′ and reverse: 5′-CCGCTCTCGTGAAAGCAGTTT-3′), and *AAP6* (AJ565848.1; forward: 5′-GCGGCCGTAATGTCCTTTTC-3′ and reverse: 5′-ACCCACA-CACCCGCATAACA-3′). The qRT-PCR was performed with three biological replicates using a triplicated PCR reaction for determining the cycle threshold (Ct). The relative expression level of target genes was calculated from Ct by using *F-box* as an internal control.

2.7. Statistical analysis

A Student's *t*-test was employed to compare the means of each