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Identification of miRNAs that regulate silique development in *Brassica napus*

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ABSTRACT

MicroRNAs (miRNAs) are a class of non-coding small RNAs (sRNAs) that play crucial regulatory roles in various developmental processes. Silique length indirectly influences seed yield in rapeseed (*Brassica napus*); however, the molecular roles of miRNAs in silique length are largely unknown. Here, backcross progenies of rapeseed with long siliques (LS) and short siliques (SS) were used to elucidate these roles. Four small RNA libraries from siliques in an early stage of development were sequenced, and a total of 814 non-redundant miRNA precursors were identified, representing 65 known and 394 novel miRNAs. Expression analyses revealed that 17 miRNAs were differentially expressed in LS and SS lines. Furthermore, through degradome sequencing, we identified 522 cleavage events. Correlation analysis of the differentially expressed miRNAs and their targets suggested that miR159 and miR319 represses cell proliferation and miR160 regulates auxin signal transduction to control silique length. Additionally, the upregulation of miR2111, miR399, miR827, and miR408 reflected restricted silique development due to inorganic phosphate/copper deficiency. More significantly, high expression of miR160 in rapeseed may repress auxin response factors and result in increased silique length, illustrating that silique length might be regulated via an auxin-response pathway.

1. Introduction

Brassica napus L. is an allopolyploid (AACC) brassicaceous species derived from two ancestors, *Brassica rapa* and *Brassica oleracea* [1]. Rapeseed, which has low erucic acid and glucosinolate contents, is the third leading source of edible oil worldwide. Brassicaceae produce non-fleshy fruits known as siliques, which develop from the gynoecium following ovule fertilization. The silique is a highly specialized plant organ that allows for the successful maturation and the eventual dispersal of the seeds, while protecting seeds from biotic and abiotic stresses [2]. It has been proven that the silique is not only an important source organ for photosynthesis for the developing seeds contained therein, but is also an important sink organ that stores carbohydrates synthesized in the vegetative plant parts [3]. Several silique-related traits have been shown to be related to yield (i.e., siliques per plant [SP], seeds per silique [SPS], and seed weight [SW]) and thus, are significant for rapeseed breeding [4]. In general, silique length (SL) has a significant positive relationship with SPS and SW, where longer siliques produce more seeds and seeds of greater weight than short siliques [3,5]. Therefore, long silique is a desirable trait in rapeseed breeding for high seed yield.

A full understanding of the genetic mechanisms that govern SL could have considerable implications for the improvement of seed yield. Many studies have demonstrated that cell proliferation and cell expansion are two major factors that affect organ size [6]. Some transcription factors, such as GROWTH-REGULATING FACTORs (GRFs), are actively engaged in cell proliferation, while TEOSINTE BRANCHED1/CYCLOIDEA/PCFs (TCPs) repress cell proliferation [7,8]. In addition, basic helix-loop-helix (bHLH) transcription factors play important roles in the control of cell elongation to regulate cell size, which could be important for the determination of organ size and shape [9]. The MADS-box transcription factor gene FRUITFULL (*FUL*) was found to promote valve cells to elongate and differentiate in *Arabidopsis* [10]. Ubiquitin-activated peptidase *DA1* promotes the cleavage and destabilization of its activating E3 ligases and TCP transcription factors to maintain normal cell numbers and regulate final organ size [11]. Various phytohormones, such as auxin, gibberellin, cytokinin, brassinosteroids, and jasmonic acid also mediate fruit development [12,13]. The *Arabidopsis* AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE (ARGOS) and its homolog ARGOS-LIKE (ARL) promote organ growth via cell expansion downstream of auxin and brassinosteroid signaling [14,15]. In addition, *ARGOS* can upregulate the downstream

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transcription factor AINTEGUMENTA (*ANT*) to further extend the period of cell division [16]. Analyses of the vast degree of natural variation in organ size using quantitative trait locus (QTL) mapping indicated that AUXIN RESPONSE FACTOR18 (*ARF18*) regulates cell growth in the silique wall by acting via an auxin-response pathway that controls SL and SW in rapeseed [17].

Endogenous non-coding small RNAs (sRNAs) are known to be important regulators of gene expression at the transcriptional and post-transcriptional levels in eukaryotes [18]. microRNAs (miRNAs) are a class of abundant sRNAs of 21–24 nt in length and have been identified as important regulators of gene expression in both animals and plants [19]. miRNAs bind to Argonaute (AGO) to form the RNA-induced silencing complex that directs mRNA-targeted degradation or inhibits the translation of target mRNAs through partially or fully complementary sequence homology with their targets [20]. To date, hundreds of miRNAs have been isolated by sRNA and degradome sequencing, allowing the large-scale discovery of miRNA targets in many plant species [21,22].

miRNAs have been shown to play key roles in various biological processes, including cell proliferation, hormone responses, and stress responses [23,24]. For example, miR396 represses GRFs, which promote leaf growth in *Arabidopsis* [25]. Strong overexpression of miR319 resulted in crinkly leaf owing to excessive cell proliferation at the leaf periphery [26]. In addition, the transcripts of several auxin response factors (ARFs) are directly regulated by miRNAs and trans-acting siRNA (ta-siRNA). For example, *ARF6* and *ARF8* transcripts targeted by miR167, *ARF10/16/17* transcripts targeted by miR160, and *ARF2/3/4* transcripts targeted by miR390 produce ta-siRNAs [27–30]. Of these, the transcriptional repressor *ARF2* restrains cell proliferation, and thus organ size, by repressing the activity of *ANT* [31]. miR393 represses auxin perception by down-regulating the expression of the *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*)*/AUXIN SIGNALLING F-BOX* (*AFB*) auxin receptors in *Arabidopsis* [32]. Overexpression of the miR171-targeted GRAS transcription factor SlGRAS24 in tomato causes smaller fruits via the regulation of gibberellin and auxin homeostasis [33].

Although many studies have investigated the molecular mechanisms underlying organ size, the roles of miRNAs in the control of SL in *B. napus* are largely unknown. The silique begins to elongate after ovule fertilization, and elongation is completed after about three weeks in rapeseed. The early stage of silique development may be an important factor determining SL, as numerous genes are expressed at higher levels in this stage. The aim of the present study was to systematically identify miRNAs that may be involved in early silique development. To this end, we constructed sRNA libraries from siliques of *B. napus* backcross progenies with long and short siliques at the early stage of silique development. Expression profiling analysis of miRNAs and their targets will provide novel information on the regulatory network of miRNAs during silique development.

2. Materials and Methods

2.1. Plant materials and RNA isolation

Populations of backcross progenies with long siliques and short siliques (LS and SS), derived from the cross of 8008 × 4942, were grown in an experimental field at the Huazhong Agricultural University (Wuhan, Hubei Province, China). '8008′ is an inbred *B. napus* line with long siliques in comparison to '4942′. '4942′ is a doubled haploid line obtained by a microspore culture of a short-silique line. Pistils/siliques from LS and SS lines were harvested at 0–7 days after pollination (DAP). When the flower was opened, pistils were harvested (0 DAP). Then, inflorescences were labeled to harvest siliques of defined stages. For each stage, siliques were harvested from ~12 plants, directly frozen in liquid nitrogen, and stored at −80 °C. Total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The tissue samples used in RNA extraction were collected from two replicated experiments.

2.2. Construction of sRNA and degradome libraries

sRNA library construction and deep sequencing were carried out as previously described [34]. Total RNA quantity and purity were assayed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) to ensure quality. Then, sRNAs of 18–30 nt were purified from 10 μg of total RNA by 15% polyacrylamide gel electrophoresis. Subsequently, 5′ and 3′ RNA adapters were ligated on both ends using T4 RNA ligase. The adapter-ligated sRNAs were transcribed to cDNA using Super-Script II Reverse Transcriptase and PCR-amplified. Finally, the PCR products were purified and sequenced on an Illumina Genome Analyzer at the Beijing Genomics Institute (Shenzhen, China).

Degradome libraries were constructed as previously described [22]. Approximately 200 μg of total RNA was used for the purification of polyadenylated RNA molecules using the Oligotex mRNA kit (Qiagen, Valencia, CA, USA). Then, using T4 RNA ligase (Ambion, USA), an adaptor was ligated to the 5′-monophosphate of cleaved products. The ligated products were purified and reverse-transcribed in five PCR cycles, then digested with *MmeI*, and ligated to a 3′ double DNA adaptor. Finally, the products were amplified by another 20 cycles of PCR, and gel-purified PCR products were sequenced on an Illumina Genome Analyser.

2.3. Bioinformatics analysis of sequencing data

The 49–50 nt raw sequences were pre-processed using the Fastx-toolkit pipeline (http://hannonlab.cshl.edu/fastx_toolkit/) to remove trim adaptor sequences, low quality reads, reads smaller than 18 nt and contamination formed by adaptor-adaptor ligation. Length distribution of the clean reads was summarized and common and unique reads between samples were identified.

For the sRNA sequencing data, the high-quality reads were aligned to the genome of *B. napus* (version 5.0) with the SOAP software (http://soap.genomics.org.cn/). After removing unexpected sRNAs, such as ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and degraded mRNA by aligning clean reads to GenBank and Rfam (10.1), the remaining perfectly genome-matching sRNA sequences were used for identification of miRNAs. Mireap (http://sourceforge.net/projects/mireap/) was used to predict miRNAs with the following parameters: the hairpin structure with free energy lower than $-18\ \text{kcal mol}^{-1}$; space between 5p and 3p miRNA sequences is less than 300 nt; more than 16 matched nucleotides and fewer than four nucleotide bulges between 5p and 3p miRNAs; the miRNA-5p/miRNA-3p duplex containing a 2 nt overhang at the 3′ end. Hairpin structures of the filtered pre-miRNA sequences were determined using MFOLD. By comparison with miRBase 21.0 (http://www.mirbase.org, release 21), a miRNA was considered as a known miRNA if its mature sequence had two or fewer nucleotide mismatches to the known miRNA and as a novel miRNA when there were more than two nucleotide mismatches.

For the degradome sequencing data, the clean reads that matched with sequences in the GenBank and Rfam databases were removed. Degraded sequences with single base over 70% in the sequence were defined as polyN and were removed. The filtered reads were mapped to *B. napus* cDNAs to identify potential miRNA target sites using the TargetFinder v1.6 script in the CleaveLand pipeline v3.0.1. The degradome peak at each position was categorized as follows. Categories $0-3$ have more than 1 raw read at the position. Category 0: only one single maximum on the transcript; Category 1: more than one maximum per transcript and equal to the maximum; Category 2: peak is lower than the maximum but higher than the median of the transcript; Category 3: peak is equal to or lower than the median of the transcript; Category 4: only one raw read at the position.