Generating an oilseed rape mutant with non-abscising floral organs using CRISPR/Cas9 technology

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Dear Editor,

Oilseed rape (OSR, Brassica napus) is an important edible oil crop consumed worldwide. As an ornamental, its fascinating seasonal flowers generate up to $1.57 million in flower tourism revenue per day in China (Xiao et al., 2021). Sclerotinia stem rot (SSR), caused by Sclerotinia sclerotiorum, is a detrimental fungal disease for OSR. SSR is initiated by air-borne ascospores that germinate to form hyphae with the help of nutrients and moisture from abscised petals adhered to leaves and petioles (Supplemental Figure S1). These infected petals serve as secondary inocula invading other plant tissues (Hegedus and Rimmer, 2005). Petal infection is thus crucial to the prevalence of SSR in OSR. OSR varieties with abscission-defective floral organs were predicted to be less susceptible to Sclerotinia infection and to have a longer flowering period to enhance tourism income. To date, such germplasm is unavailable in OSR. Here, we generated OSR lacking abscission of floral organs.

In Arabidopsis (Arabidopsis thaliana), Inflorescence Deficient in Abscission (IDA) encodes a small secreted protein with an N-terminal signal peptide, and IDA knockouts retain floral organs indefinitely (Butenko et al., 2003; Cho et al., 2008). Five OSR homologs to AtlIDA, gleaned from the released OSR genome (Chalhoub et al., 2014), were cloned from the pure OSR line J9712 (Figure 1A). BnC06.IDA and BnA07.IDA share a highly conserved amino-acid sequence and exhibit 76.9% sequence similarity to AtlIDA (Figure 1A). Transcriptomic analysis revealed that BnC06.IDA and BnA07.IDA were preferentially expressed in the floral abscission zone (AZ), and in other floral organs, including petals, sepals, and filaments. In contrast, the other three copies of BnIDA were barely expressed in floral organs (Figure 1B). Our results suggest a role for BnC06.IDA and BnA07.IDA in determining the fate of floral organ abscission.

To investigate the function of IDA on floral organ abscission, BnC06.IDA and BnA07.IDA were knocked out using CRISPR/Cas9 genome editing. To that end, two guide RNAs (gRNAs), gRNA1 and gRNA2, were designed to specifically target two regions of the coding sequence (Tgt1 and Tgt2) of BnC06.IDA and BnA07.IDA (Figure 1C). The CRISPR/Cas9 construct contained two gRNAs driven by Arabidopsis U3b and U3d promoters, and a Cas9 gene driven by the 35S promoter (Figure 1C).

The construct was transformed into J9712 through Agrobacterium-mediated transformation, and a total of 30 independent positive transformants were obtained. Seven of the 30 (23.3%) T₀ plants exhibited editing events at the designed target sites (Figure 1D). All mutant plants were single heterozygous mutants with single nucleotide deletions or insertions that led to translational frame shifts (Figure 1D). No editing events occurred at BnC04.IDA, BnA02.IDA, and BnC02.IDA.

Mutant plants were self-pollinated and the obtained T₁ progeny contained single homozygous mutations in bna07.ida and bnc06.ida. However, floral abscission in bnc06.ida and bna07.ida single mutants was similar to wild-type plants.

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Figure 1 Generation of bnida mutants in B. napus by CRISPR/Cas9-mediated gene editing. A, Phylogenetic analysis of IDA homologs in B. napus and Arabidopsis. A neighbor joining tree is presented based on the deduced amino acid sequences. Bootstrap values (1,000 replicates) are shown at each branch as a percentage. A branch length scale bar indicates the number of amino acid residue substitutions per site. B, Expression patterns of BnIDAs in floral organs of B. napus. The expression of BnIDAs in the AZ of the flower at position 3 was determined by transcriptome sequencing of line J9712. The expression patterns in petals, sepals, and filaments were acquired from the BnTIR transcriptomic database (Liu et al., 2021). TPM, transcripts per million mapped reads. C, Schematics of the target sites (Tgs) of BnA07.IDA and BnC06.IDA and the Cas9/gRNA vector. NLS, nuclear location signal; HPT, hygromycin phosphotransferase. D, Sequences at the sgRNA target sites in the T0 generation according to TA cloning and Sanger sequencing. Dashes represent deletions; red letters represent insertions. The protospacer-adjacent motif (PAM) is underlined. E, Allele-specific markers were developed to discriminate the wild-type, heterozygous, and homozygous mutants for BnA07.IDA (AA, Aa, and aa, respectively) and BnC06.IDA (CC, Cc, and cc, respectively).

(J9712) (Figure 2A), suggesting functional redundancy between the BnIDA homologs. To obtain bnc06.ida bna07.ida double mutants, we crossed single mutants of bna07.ida (line #10) and bnc06.ida (line #3). To discriminate wild-type, heterozygous, and homozygous mutants, allele-specific PCR markers for BnA07.IDA and BnC06.IDA were developed (Figure 1E and Supplemental Table S1). Marker-assisted selection and a comprehensive speed breeding system (Song et al., 2022) allowed us to successfully obtain F2 progeny with bnc06.ida bna07.ida double mutants within 6 months.

Wild-type and mutant plants were subsequently grown in a climate chamber. The sepals, petals, and stamens abscised at flower positions 10–11 in wild-type and bna07.ida bnc06.ida plants (Figure 2, A and B). Strikingly, the double mutants displayed a floral abscission-defective phenotype in which floral organs remained attached to developing siliques, and dry and colorless senesced floral parts remained attached to mature siliques (Figure 2, A and B). In contrast, the detached petals and stamens in wild-type and single mutants of bna07.ida and bnc06.ida adhered to leaves or petioles (Figure 2A).
Figure 2 Phenotype of bna07.ida (aa), bnc06.ida (cc), and bna07.ida bnc06.ida (aacc) compared with the wild-type (AACC). A, The whole inflorescences of wild-type and BnIDA mutants. Red arrows indicate petals and stamens adhering to leaves or petioles after floral organ abscission. Flowers from bna07.ida bnc06.ida plants displayed an abscission-defective phenotype. Scale bar = 5 cm. The upper right photograph shows that flowers remained attached to mature siliques. B, Representative siliques from wild-type and BnIDA mutants at flower positions 11, 15, and 20. Scale bar = 1 cm. Position 1 refers to the youngest flower with visible yellow petals at the top of the inflorescence. C, Petal breakstrength of flowers at different positions of the wild-type and BnIDA mutants. Error bars indicate standard deviations (n = 12) for each position. D and E, Morphology of floral AZ at different flower positions. D, Secretion of the white substance at petal (Pt), sepal (Se), and stamen (St) AZ after
To quantify the extent of floral organ abscission, the mechanical force required to remove petals from flowers was measured with a high-precision tension meter (Figure 2C). The breakstrength of wild-type petals dramatically decreased at positions 8 and 10, and were zero after position 10 since the petals were already detached (Figure 2C). By contrast, mechanical force was required to remove the petals at all tested positions in the \textit{bnc06.ida bna07.ida} plants (Figure 2C).

We further compared the morphological differences at the floral AZ between the wild-type and \textit{bna07.ida bnc06.ida} plants. At position 4, floral organs were forcibly removed, forming a fracture plane with broken cells at the floral AZ in both genotypes (Figure 2, D and E). After abscission, beginning at position 11 of wild-type plants, the floral AZ was covered with a white substance rich in arabinogalactan (as described by Stenvik \textit{et al.}, 2006; Figure 2D), and the AZ cells were enlarged and rounded to form an abscission scar (Figure 2E). Instead of rounded cells and white substance, broken cells were found in the floral AZ of double mutant plants at position 11 (Figure 2, D and E), indicating incomplete dissolution of the middle lamella in the AZ cells. Apart from floral abscission, the majority of agronomic traits and fertility between \textit{bna07.ida bnc06.ida} and wild-type plants were not substantially different (Supplemental Table S2 and Supplemental Figure S2).

To test sensitivity of the mutants to SSR, petals were inoculated with a mycelial suspension of \textit{S. sclerotiorum}. In wild-type plants, inoculated petals were removed and adhered to leaves, while inoculated petals remained attached in the mutants. The majority of infected petals caused necrosis to the leaves of wild-type plants, whereas disease lesions were undetectable in the petals and leaves of the double mutants (Figure 2F).

In summary, our results demonstrate that \textit{BnA07.IDA} and \textit{BnC06.IDA} are functionally redundant and are involved in the regulation of floral abscission in OSR. We successfully generated OSR plants with abscission-defective floral organs by simultaneously inactivating two \textit{BnIDA} genes using CRISPR/Cas9 (Figure 2G). The double mutant line, with its allele-specific markers, can be applied for breeding OSR cultivars with non-abscising floral organs that are anticipated to be or not to be a pathogen. FEMS Microbiol Lett \textit{251}: 177–184.

Song \textit{et al.} (2022) Comprehensive speed breeding: a high-throughput and rapid generation system for long-day crops. Plant Biotechnol J \textit{20}: 13–15.

The following materials are available in the online version of this article.

\textbf{Supplemental Figure S1}. Petals adhered to leaves and petioles after abscission, spreading SSR.

\textbf{Supplemental Figure S2}. Fertility performances of \textit{bna07.ida bnc06.ida} and wild-type plants.

\textbf{Supplemental Table S1}. Sequences of the primers used in this study.

\textbf{Supplemental Table S2}. Agronomic traits of wild-type and \textit{bna07.ida bnc06.ida} plants grown in the climate chamber.

\textbf{Supplemental Materials and Methods}.

\textbf{Acknowledgments}.

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\textbf{Conflict of interest statement}.

The authors declare no conflicts of interest.

\textbf{References}.

Butenko \textit{et al.} (2003) \textit{Inflorescence deficient in abscission} controls floral organ abscission in Arabidopsis and identifies a novel family of putative ligands in plants. Plant Cell \textit{15}: 2296–2307.

Chalhoub \textit{et al.} (2014) Early allopolyploid evolution in the post-Neolithic \textit{Brassica napus} oilseed genome. Science \textit{345}: 950–953.

Choi \textit{et al.} (2008) Regulation of floral organ abscission in \textit{Arabidopsis thaliana}. Proc Natl Acad Sci USA \textit{105}: 15629–15634.

Chiquet \textit{et al.} (2006) Overexpression of \textit{INFLORESCENCE DEFICIENT IN ABSCSSION} activates cell separation in vestigial abscission zones in \textit{Arabidopsis}. Plant Cell \textit{18}: 1467–1476.

Song \textit{et al.} (2022) Comprehensive speed breeding: a high-throughput and rapid generation system for long-day crops. Plant Biotechnol J \textit{20}: 13–15.

Xiao \textit{et al.} (2021) Rapeseed as an ornamental. Horticulturae \textit{8}: 27.

\textbf{Figure 2 (Continued)}

abscession in wild-type plants. Scale bar = 1 mm. E, Scanning electron microscopy of the petal AZ cells at positions 4 and 11. Scale bar = 30 µm. F, Assessment of the sensitivity of \textit{bna07.ida bnc06.ida} mutant and wild-type plants to \textit{S. sclerotiorum}. The enlarged images show magnified views of the red boxes above. G, Schematic diagram showing the generation of oilseed rape with non-abscising floral organs by CRISPR/Cas9.