Improved shatter resistance of canola by CRISPR/Cas9 and EMS mutagenesis

Janina Braatz
The natural seed dispersal mechanism of Brassica napus troubles farmers

- Dry siliques
- Fragile

- Weather
- Animals
- Machines

- Yield loss
- Volunteer plants
The dehiscence zone promotes seed shattering

Silique model

Silique cross-section

Underlying gene network (simplified)
CRISPR/Cas9 system can induce frameshift mutations at target locus

Model of CRISPR/Cas9-mediated gene knock-out

(Modified from Agrotis and Ketteler 2015)

BnALC

BnaA.ALC.a  ACGCCGCTTGTGCAGCCGCTGAAACT

BnaC.ALC.a  ACGCCGCTTGTGCAGTCGCTGAAACT

Cas9 target upstream of the bHLH domain

(Modified from Braatz et al. 2017)

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CRISPR/Cas9 construct was transformed into rapeseed hypocotyl explants

Transformation:

• Hypocotyl explants of spring cultivar ‘Haydn’

• Agrobacterium-mediated transformation

• 1 transgenic T<sub>1</sub> plant (transformation rate: 0.9%)

Regenerating plantlets
The T₁ plant contained four \textit{Bnalc} mutant alleles

<table>
<thead>
<tr>
<th>Allele</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A_1)</td>
<td>ACGCCG\textbf{CTT}--GTGCA\textcolor{red}{G}CCGCTGAAACT</td>
</tr>
<tr>
<td>(A_2), -2 bp</td>
<td>ACGCCG\textcolor{red}{C}---GTGCA\textbf{G}CCGCTGAAACT</td>
</tr>
<tr>
<td>(A_3), -7 bp</td>
<td>ACG\textcolor{red}{C}---\textbf{T}GCAGCCGCTGAAACT</td>
</tr>
<tr>
<td>(C_1)</td>
<td>ACGCCG\textbf{CTT}--GTGCA\textcolor{red}{G}CCGCTGAAACT</td>
</tr>
<tr>
<td>(C_2), +1 bp</td>
<td>ACGCCG\textbf{T}GTCAGCCGCTGAAACT</td>
</tr>
<tr>
<td>(C_3), -1 bp</td>
<td>ACGCCG\textbf{T}GTCAGCCGCTGAAACT</td>
</tr>
</tbody>
</table>

\textbf{BnaA.ALC.a}

\textbf{BnaC.ALC.a}

Sanger sequencing of cloned \textit{BnALC} PCR amplicons of the double heterozygous T₁ plant

(Braatz et al., 2017) © American Society of Plant Biologists
The T₂ progeny showed the expected Mendelian segregation

Inheritance of transgene and CRISPR/Cas9-induced BnALC mutations in 36 T₂ plants. 
O = observed, E = expected number of plants

<table>
<thead>
<tr>
<th>Transgene genotypes</th>
<th>alc genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A₂A₂ C₂C₂</td>
</tr>
<tr>
<td></td>
<td>A₂A₂ C₂C₃</td>
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<tr>
<td></td>
<td>A₂A₂ C₃C₃</td>
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<tr>
<td></td>
<td>A₃A₃ C₃C₃</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Transgenetic</th>
<th>Non-transgenic</th>
<th>Chi² testb</th>
<th>O</th>
<th>Ea</th>
<th>2.25</th>
<th>4.25</th>
<th>2.25</th>
<th>4.25</th>
<th>2.25</th>
<th>4.25</th>
<th>2.25</th>
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</thead>
<tbody>
<tr>
<td>Transgenic</td>
<td>Non-transgenic</td>
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<td>3</td>
<td>27</td>
<td>4</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>14</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Chi² testc</td>
<td></td>
<td></td>
<td>2.25</td>
<td>9</td>
<td>4.25</td>
<td>4.25</td>
<td>2.25</td>
<td>4.25</td>
<td>2.25</td>
<td>4.25</td>
<td>2.25</td>
</tr>
</tbody>
</table>

a: under the assumption that the T₁ parent CP1 was non-chimeric (A₂A₃/C₂C₃) 
b: 3:1 segregation, Chi²(0.999;2) = 13.82 

PCR test for T-DNA presence

(Braatz et al., 2017)
No off-target effects were detected in two homologous regions

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BnaA.ALC.a</td>
<td><code>CCGCTTGTGCAGGCGCTGAAACT</code></td>
</tr>
<tr>
<td>BnaC.ALC.a</td>
<td><code>CCGCTTGTGCAGTCGCTGAAACT</code></td>
</tr>
<tr>
<td>BnaC04g13390D</td>
<td><code>CCGCTTGTGCAGTCCTGGAAACT</code></td>
</tr>
<tr>
<td>Non-coding region on chr. C02</td>
<td><code>CCGCTTTTTCGCAGCCGGCAAGAAA--</code></td>
</tr>
</tbody>
</table>

Alignment of the CRISPR-Cas9 target sequence with two potential off-target sites identified by a BLAST search

Sanger sequencing of the T<sub>1</sub> plant and five T<sub>2</sub> progeny showed only wild type sequences in the two potential off-target sites.
Whole genome shotgun sequence of T₁ plant was produced

- gDNA of T₁ plant
- (www.illumina.com)
  HiSeq 2500, 1 lane
  Paired-end sequencing
- 412 mio. raw data reads
- Quality trimming and mapping against transformation vector and Darmor-bzh reference
  (BWA mem, SAMtools, Novosort, R)
- Average 20x genome coverage
- Information about inserted sequences

(Sandra Driesslein, Nils Stein, Axel Himmelbach and Martin Mascher, IPK, Gatersleben, Germany)
The T₁ plant carried vector backbone insertions

Transgenic T₁

Position in vector sequence (kb)

Read depth

Mapping of genome sequences against the transformation vector sequence

Average genome coverage, half & double of the coverage

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The general plant growth of $T_1$ and $T_2$ resembled the wild type

$T_1$  
$T_2$  
Haydn

$alc$ mutants

(Modified from Braatz et al., 2017)
Bench-top phenotyping of single siliques assesses shatter resistance

- Measure: Peak tensile force separating valves and replum

Tensile force measurement
Cas9-induced *Bnalc* shatter resistance was masked by transformed genotype

**Conditions:**
Greenhouse, 16 h light, 22 °C, *Bnalc* mutations in ‘Haydn’ background, 30 siliques/5 plants/genotype

**Statistics:**
Regression at SL 5.5 cm, standard error, ANCOVA, same letters no significant difference ($p \geq 0.05$)
‘Haydn’ shows high shatter resistance

Conditions: Greenhouse, 16 h light, 22 °C, 30 siliques/ 5 plants/ genotype
Statistics: Regression at SL 5.5 cm, standard error, ANCOVA, same letters no significant difference (p ≥ 0.05) (Braatz et al., under review)

Tensile force measurements

**Winter varieties**

- 'Express'
- 'Apex'
- 'Artoga'
- 'Avatar'

**Spring varieties**

- 'Express'
- 'Drakkar'
- 'Haydn'
- 'Mozart'
- 'Westar'

(Braatz et al., under review)
EMS mutants confirmed the Cas9-induced Bnalc shatter resistance

Tensile force measurements

- **Conditions:** Greenhouse, 16 h light, 22 °C, Bnalc mutations in ‘Express’ background, 30 siliques/5 plants/genotype
- **Statistics:** Regression at SL 5.5 cm, standard error, ANCOVA, same letters no significant difference \((p \geq 0.05)\)

(Braatz et al., under review)
Preliminary field data support \textit{Bnind} shatter resistance

Overview of field trial in full bloom

Seed collection tray

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fieldTrialOverview}
\caption{Overview of field trial in full bloom.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{seedCollectionTray}
\caption{Seed collection tray.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{harvestData}
\caption{Average harvest per plot (kg).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{lossData}
\caption{Pre-harvest loss (%).}
\end{figure}

\textbf{Conditions:} 2016/17, Rheinbach (Bonn), randomized block design, three repetitions, 3 x 3 m plots, 11 cm row distance, 55 seeds/m², backcrossed F\textsubscript{3} \textit{Bnind} mutations in ‘Express’ background, two 1 x 0.1 m seed collection trays per plot (28.06.17), seed loss until 31.07.17, harvest 02.08.17, direct cutting of 4.86 m² central plot.

\textbf{Statistics:} n = 3 (harvest), n = 6 (loss), bars show std. dev./ median, t-tests, same letters no significant difference (p \geq 0.05)
Four *BnNST1* homoeologs were targeted by CRISPR/Cas9 in resynthesized rapeseed RS306

![Diagram showing CRISPR/Cas9 target site and exon/intron structure]

- Indel mutations in all four gene copies of primary transformant
- Multiple alleles per gene → chimeric mosaic T₁ plant
- Inheritance to T₂ currently under investigation
- Phenotyping pending

(Braatz, unpublished)
This study provided

- Insights into the efficiency of CRISPR/Cas9-mediated mutagenesis of polyploid rapeseed
- Novel mutations for breeding shatter resistant rapeseed
- Information on the effect of \textit{Bnalc} and \textit{Bnind} mutations on shatter resistance
Future efforts will involve

- Phenotypic assessment of *Bnnst1* mutants
- Marker-assisted backcross of mutant alleles into elite material
- Establishment of DNA-free transformation protocol to produce non-GMO mutants for the European market
I would like to acknowledge

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IKMB, University of Kiel
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