



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

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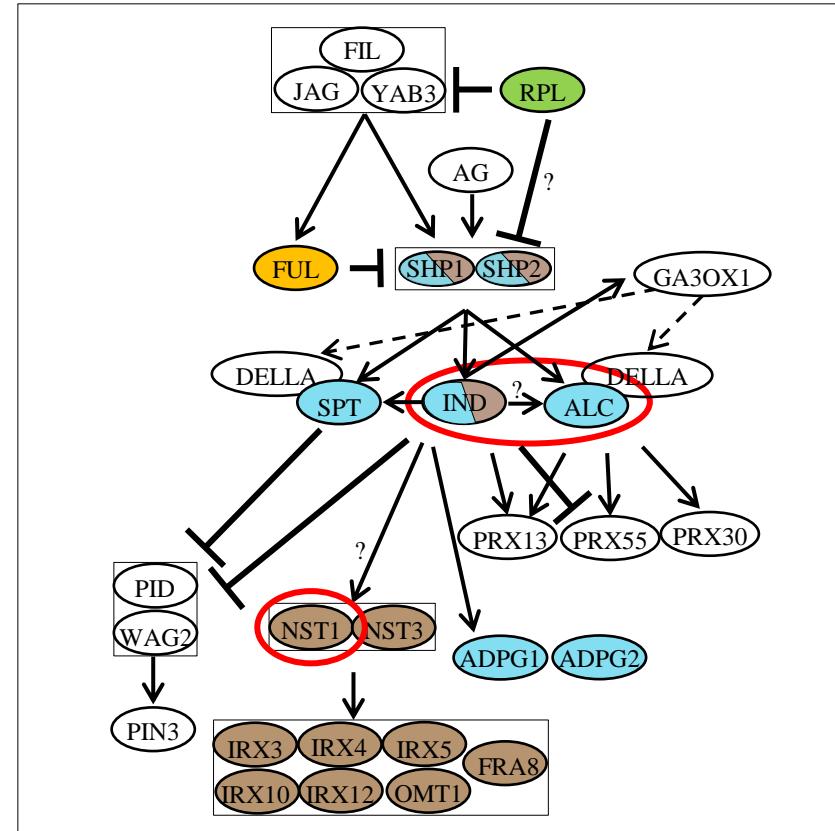
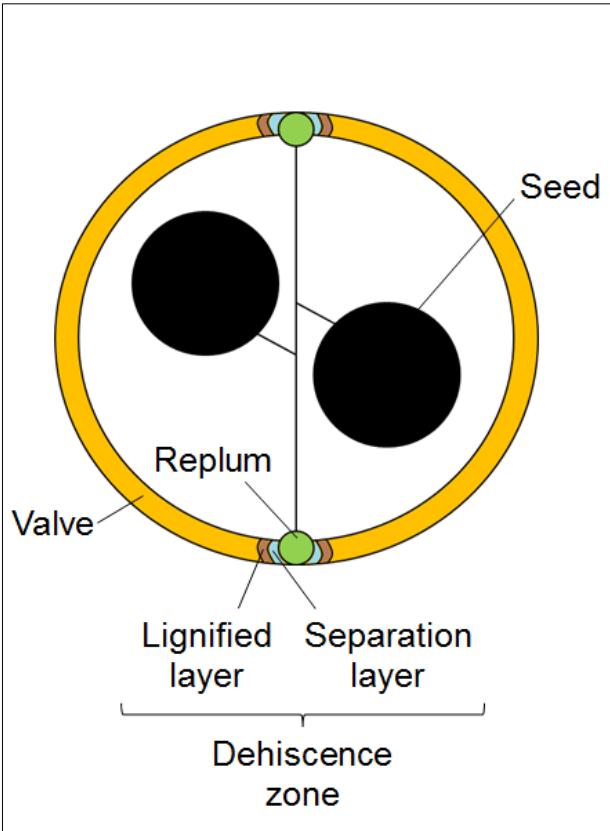
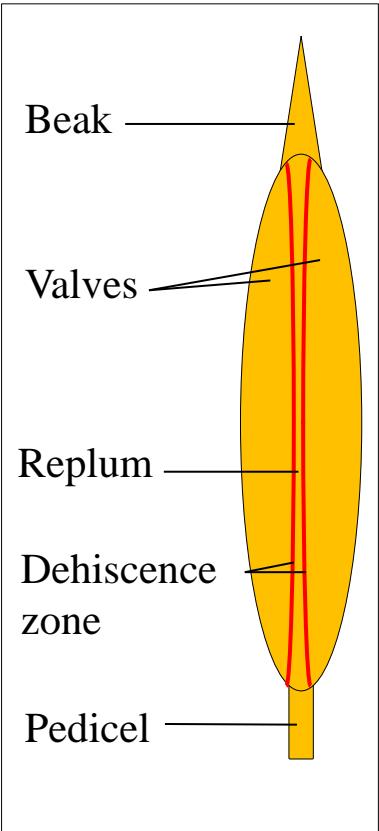
# The natural seed dispersal mechanism of *Brassica napus* troubles farmers

- Dry siliques
- Fragile

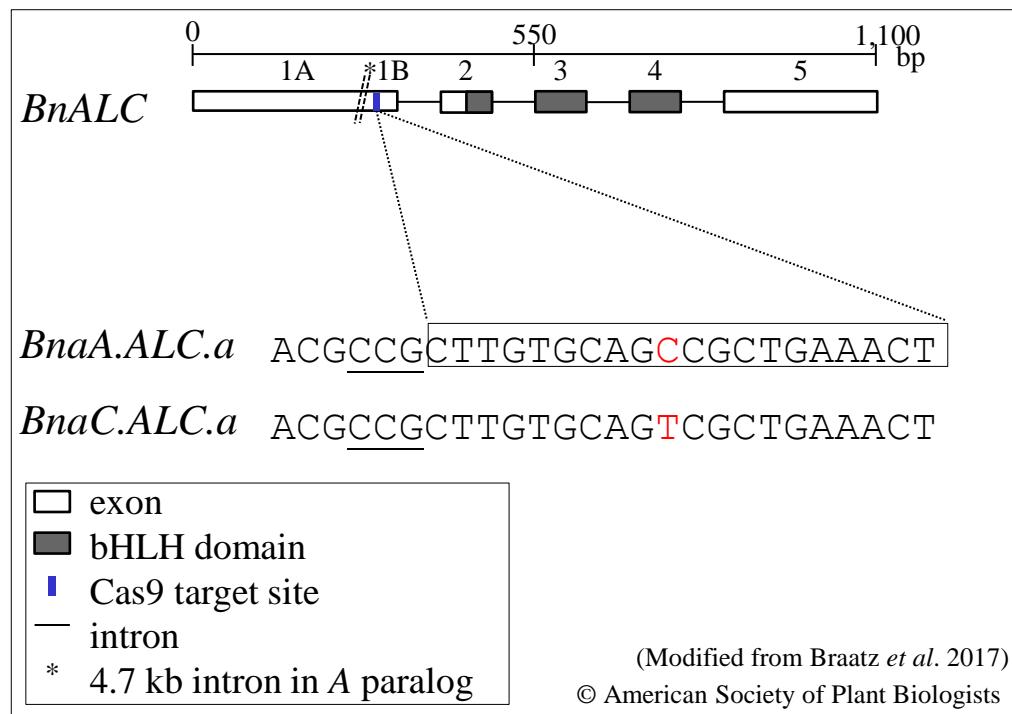
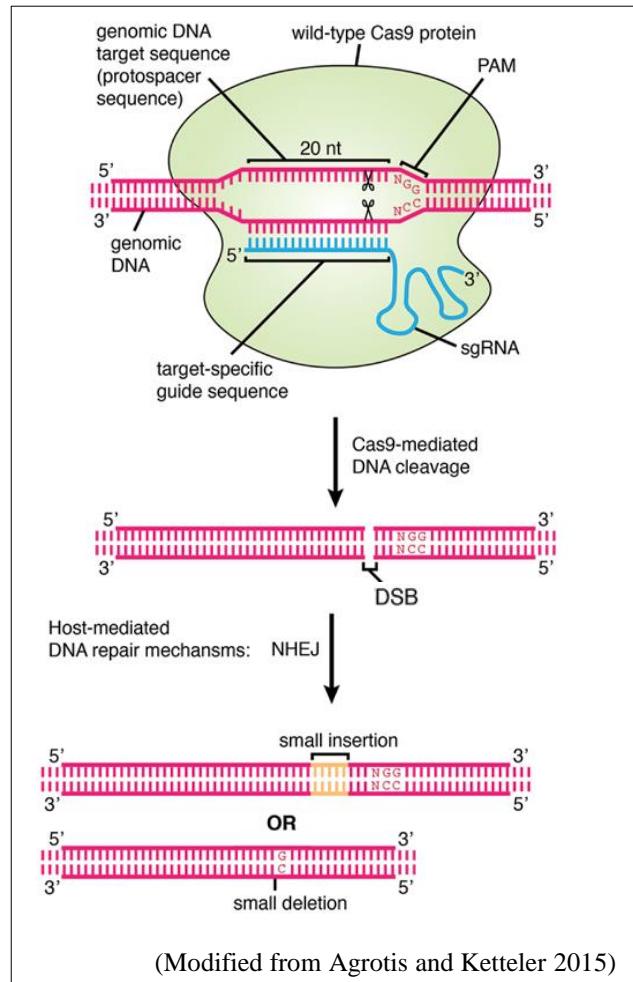


- Weather
- Animals
- Machines
- Yield loss
- Volunteer plants

# The dehiscence zone promotes seed shattering



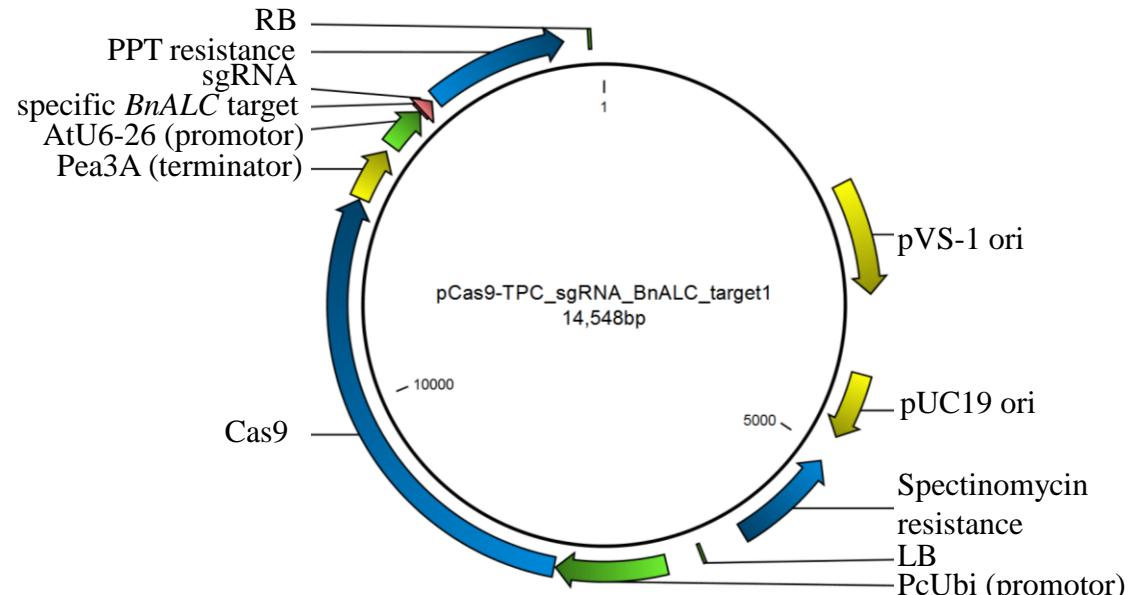
# CRISPR/Cas9 system can induce frameshift mutations at target locus



Cas9 target upstream of the bHLH domain

Model of CRISPR/Cas9-mediated  
gene knock-out

# 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%)

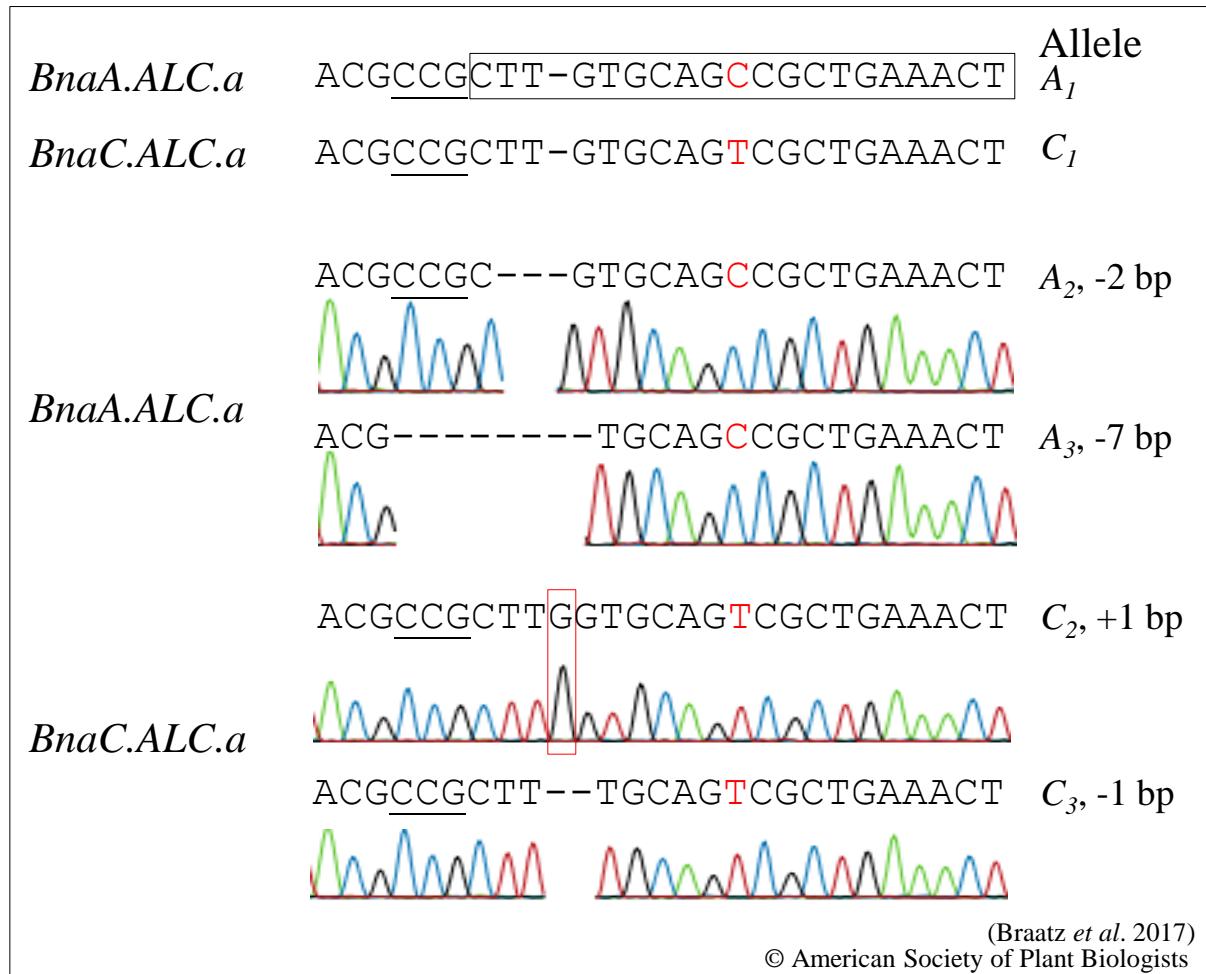


Regenerating plantlets

# The T<sub>1</sub> plant contained four *Bnalc* mutant alleles



T<sub>1</sub> plant



Sanger sequencing of cloned *BnALC* PCR amplicons  
of the double heterozygous T<sub>1</sub> plant

# The T<sub>2</sub> progeny showed the expected Mendelian segregation

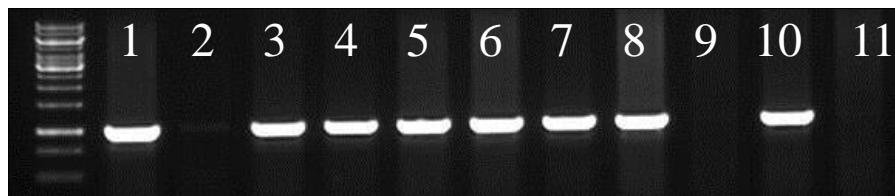
Inheritance of transgene and CRISPR/Cas9-induced *BnALC* mutations in 36 T<sub>2</sub> plants.  
 O = observed, E = expected number of plants

	Transgene genotypes			alc genotypes									
Transgenic	Non-transgenic	Chi <sup>2</sup> test <sup>b</sup>	A <sub>2</sub> A <sub>2</sub>	A <sub>2</sub> A <sub>2</sub>	A <sub>2</sub> A <sub>2</sub>	A <sub>2</sub> A <sub>3</sub>	A <sub>2</sub> A <sub>3</sub>	A <sub>2</sub> A <sub>3</sub>	A <sub>3</sub> A <sub>3</sub>	Chi <sup>2</sup> test <sup>c</sup>			
O	27	9	0	3	4	0	3	14	2	1	6	3	8.52
E <sup>a</sup>	27	9		2.25	4.25	2.25	4.25	9	4.25	2.25	4.25	2.25	

<sup>a</sup>: under the assumption that the T<sub>1</sub> parent CP1 was non-chimeric (A<sub>2</sub>A<sub>3</sub>/C<sub>2</sub>C<sub>3</sub>)

<sup>b</sup>: 3:1 segregation, Chi<sup>2</sup><sub>(0.999;2)</sub> = 13.82

<sup>c</sup>: 1:2:1:2:4:2:1:2:1 segregation, Chi<sup>2</sup><sub>(0.999; 8)</sub> = 26.12



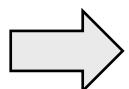
PCR test for T-DNA presence

# No off-target effects were detected in two homologous regions

<i>BnaA.ALC.a</i>	<u>CCGCTTGTGCAGCCGCTGAAACT</u>
<i>BnaC.ALC.a</i>	<u>CCGCTTGTGCAG</u> <b>T</b> CGCTGAAACT
BnaC04g13390D	<u>CCGCTTGTGCAG</u> <b>T</b> <b>C</b> TCTGAAACT
Non-coding region on chr. C02	<u>CCGCTT</u> <b>T</b> TGCAGCCGC <b>A</b> GAAA--

(Braatz *et al.*, 2017)  
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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 T<sub>1</sub> plant was produced

gDNA of T<sub>1</sub> plant



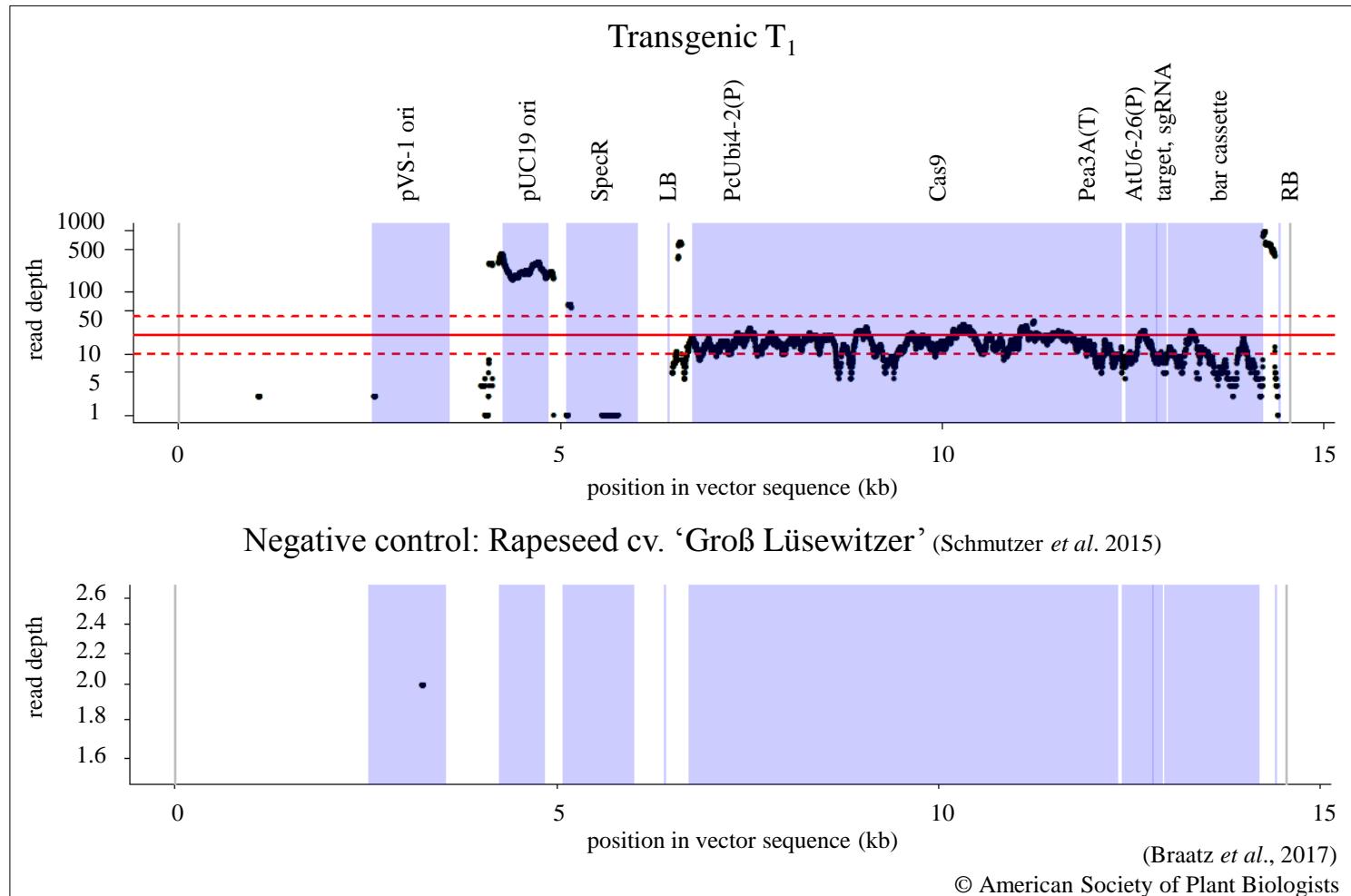
412 mio. raw data reads



Quality trimming and  
mapping against transformation vector  
and Darmor-*bzh* reference  
(BWA mem, SAMtools, Novosort, R)

Average 20x genome coverage  
Information about inserted sequences

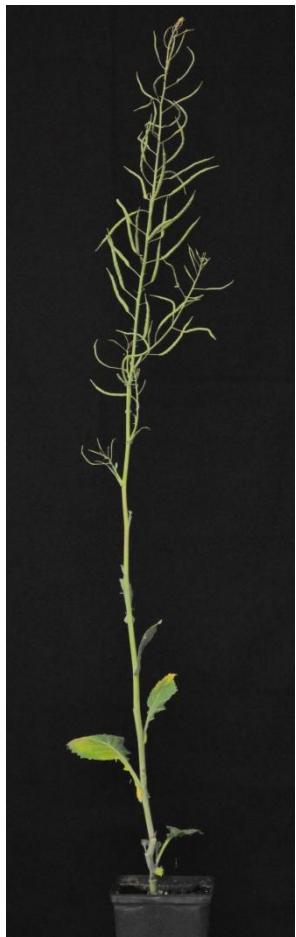
# The T<sub>1</sub> plant carried vector backbone insertions



Mapping of genome sequences against the transformation vector sequence

— average genome coverage,    - - - half & double of the coverage

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



$T_1$

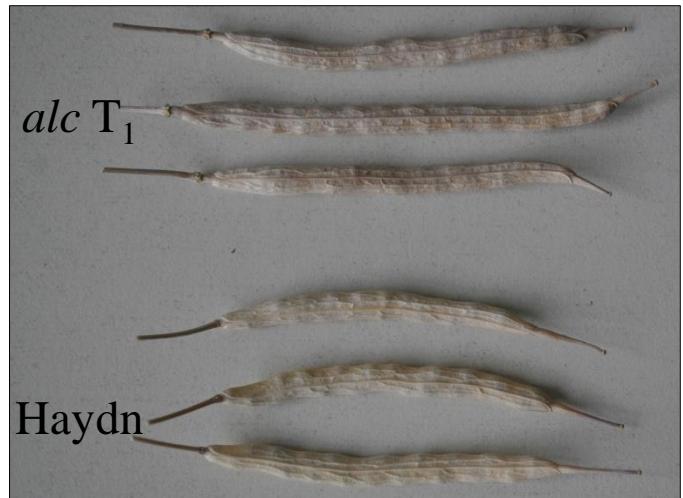


$T_2$

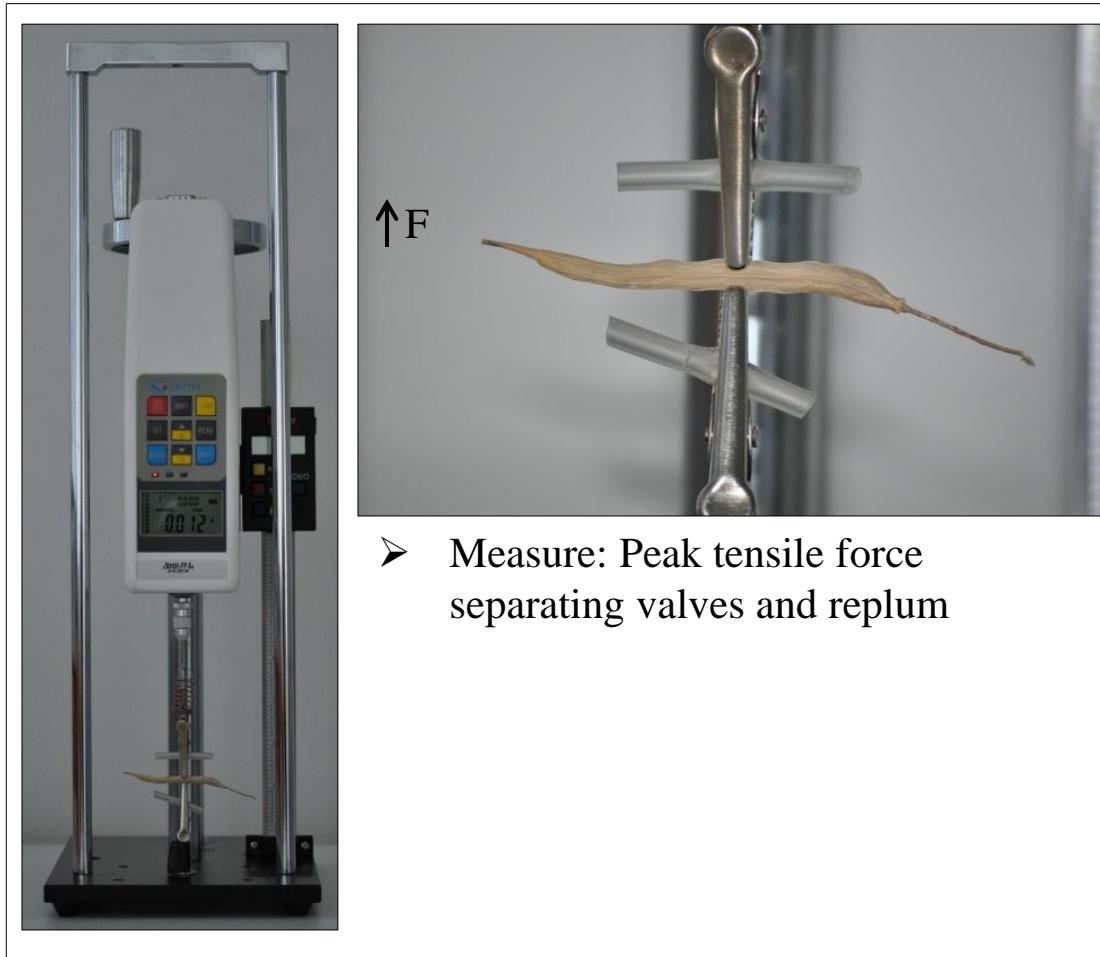


Haydn

*alc* mutants



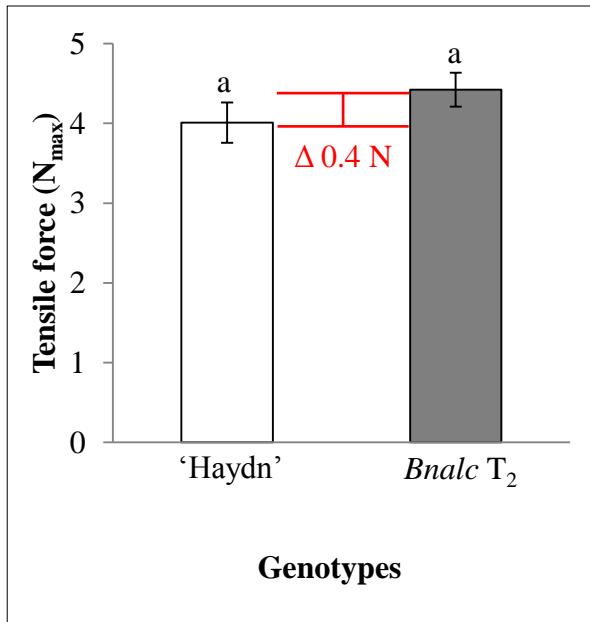
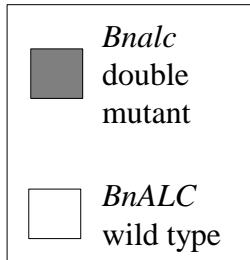
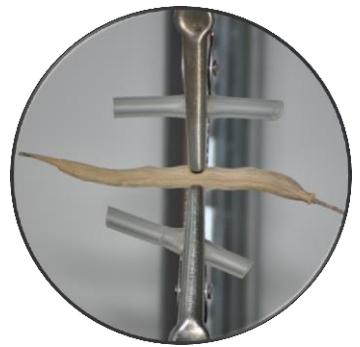
# Bench-top phenotyping of single siliques assesses shatter resistance



- Measure: Peak tensile force separating valves and replum

Tensile force measurement

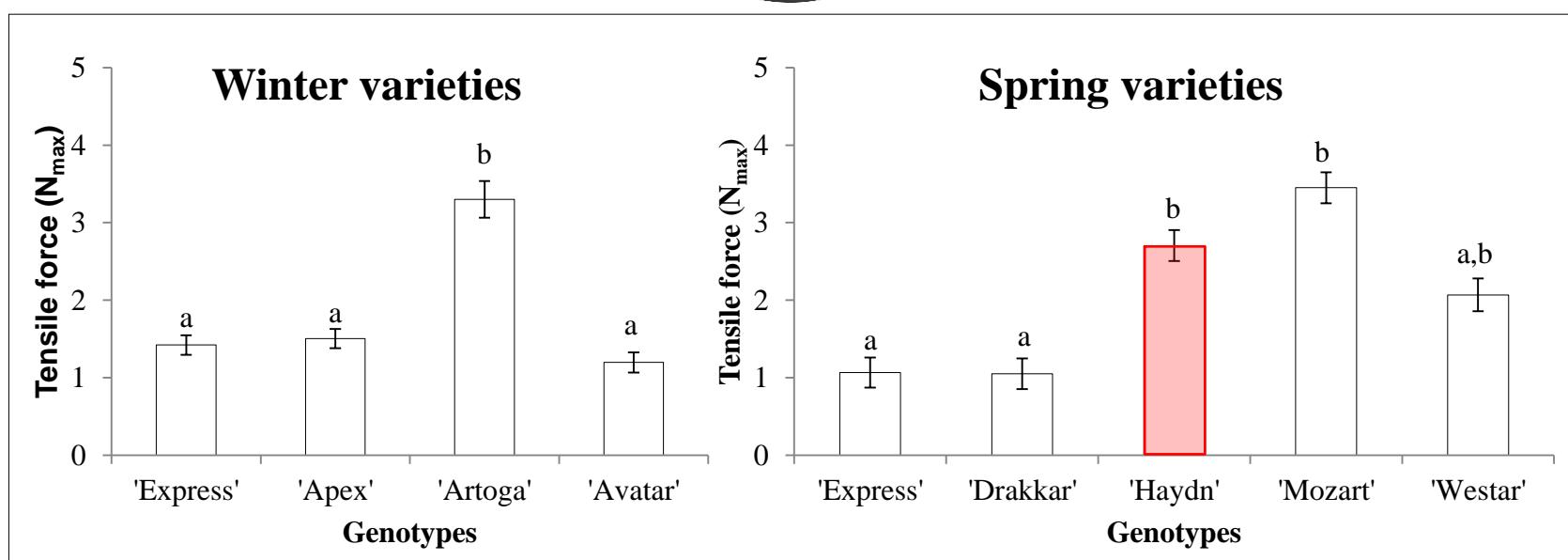
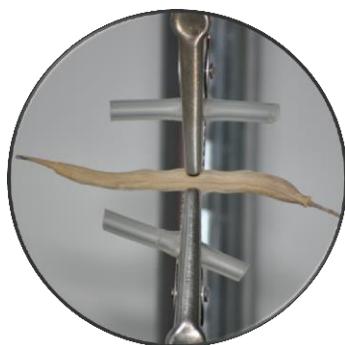
# Cas9-induced *Bnalc* shatter resistance was masked by transformed genotype



Tensile force measurements

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 ( $p \geq 0.05$ )

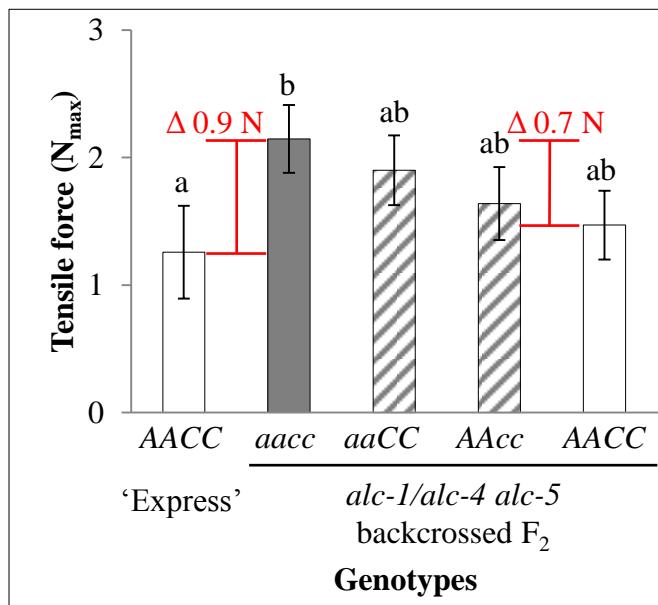
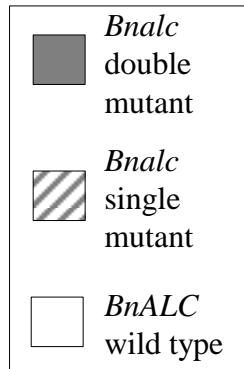
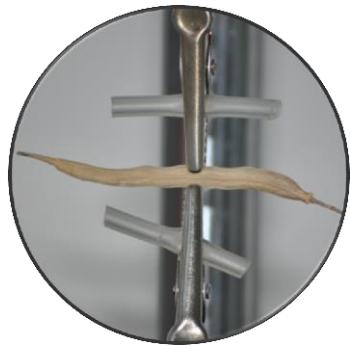
# ‘Haydn’ shows high shatter resistance



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 ( $p \geq 0.05$ ) (Braatz *et al.*, under review)<sup>14</sup>

# EMS mutants confirmed the Cas9-induced *Bnalc* shatter resistance



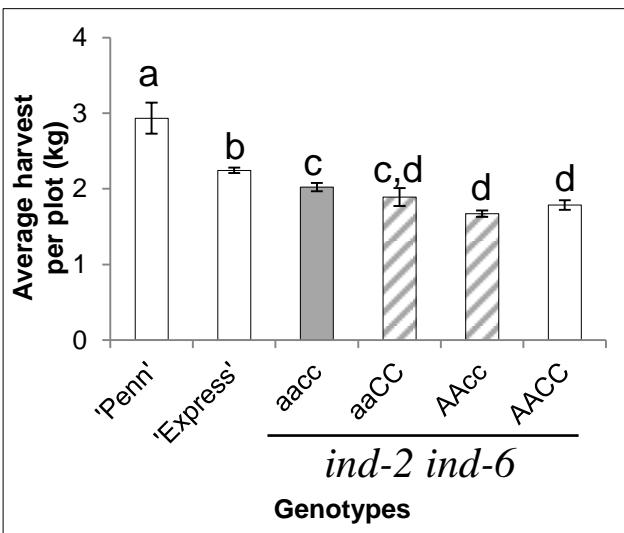
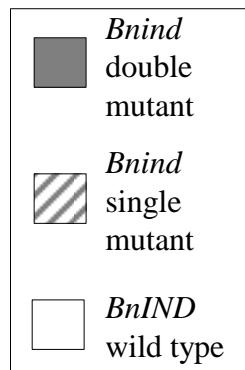
Tensile force measurements

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 ( $p \geq 0.05$ )

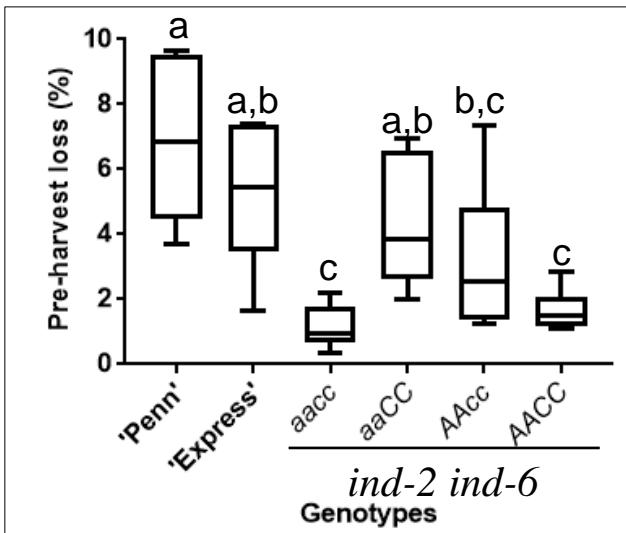
# Preliminary field data support *Bnind* shatter resistance



Overview of field trial in full bloom



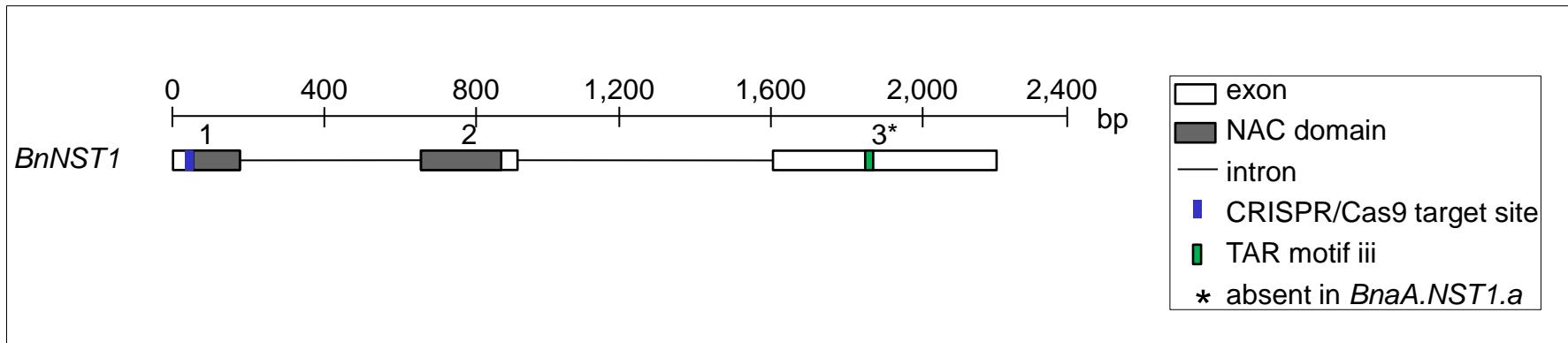
Seed collection tray



Conditions: 2016/17, Rheinbach (Bonn), randomized block design, three repetitions, 3 x 3 m plots, 11 cm row distance, 55 seeds/m<sup>2</sup>, backcrossed F<sub>3</sub> *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 m<sup>2</sup> central plot

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



Cas9 target upstream of the NAC domain

- Indel mutations in all four gene copies of primary transformant
- Multiple alleles per gene → chimeric mosaic *T<sub>1</sub>* plant
- Inheritance to *T<sub>2</sub>* currently under investigation
- Phenotyping pending

*T<sub>1</sub>* plant

(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 *Bnalc* and *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



Prof. Dr. Christian Jung, Dr. Hans-Joachim Harloff, and all colleagues of the  
**Plant Breeding Institute, University of Kiel**

**Zoological Institute, Functional Morphology  
and Biomechanics, University of Kiel**

Prof. Dr. Stanislav Gorb

Dr. Lars Heepe

**Leibniz Institute of Plant Genetics and  
Crop Plant Research (IPK), Gatersleben**

Dr. Martin Mascher

Dr. Nils Stein

Dr. Axel Himmelbach

**Variation statistics, University of Kiel**

Dr. Mario Hasler

**IKMB, University of Kiel**

Sanger sequencing lab

**Formerly Saaten-Union BioTec, Leopoldshöhe**

Dr. José Orsini

**Institute of Crop Science and Resource Conservation, INRES,  
University of Bonn**

Prof. Dr. Jens Léon

Winfried Bungert

Karin Woitol

**Karlsruhe Institute of Technology, Karlsruhe**

Prof. Dr. Holger Puchta

**NPZ Innovation, Hohenlith**

Dr. Gunhild Leckband

Dr. Amine Abbadi

Dr. Steffen Rietz

**Funding**

Stiftung Schleswig-Holsteinische Landschaft

**And you for your attention!**