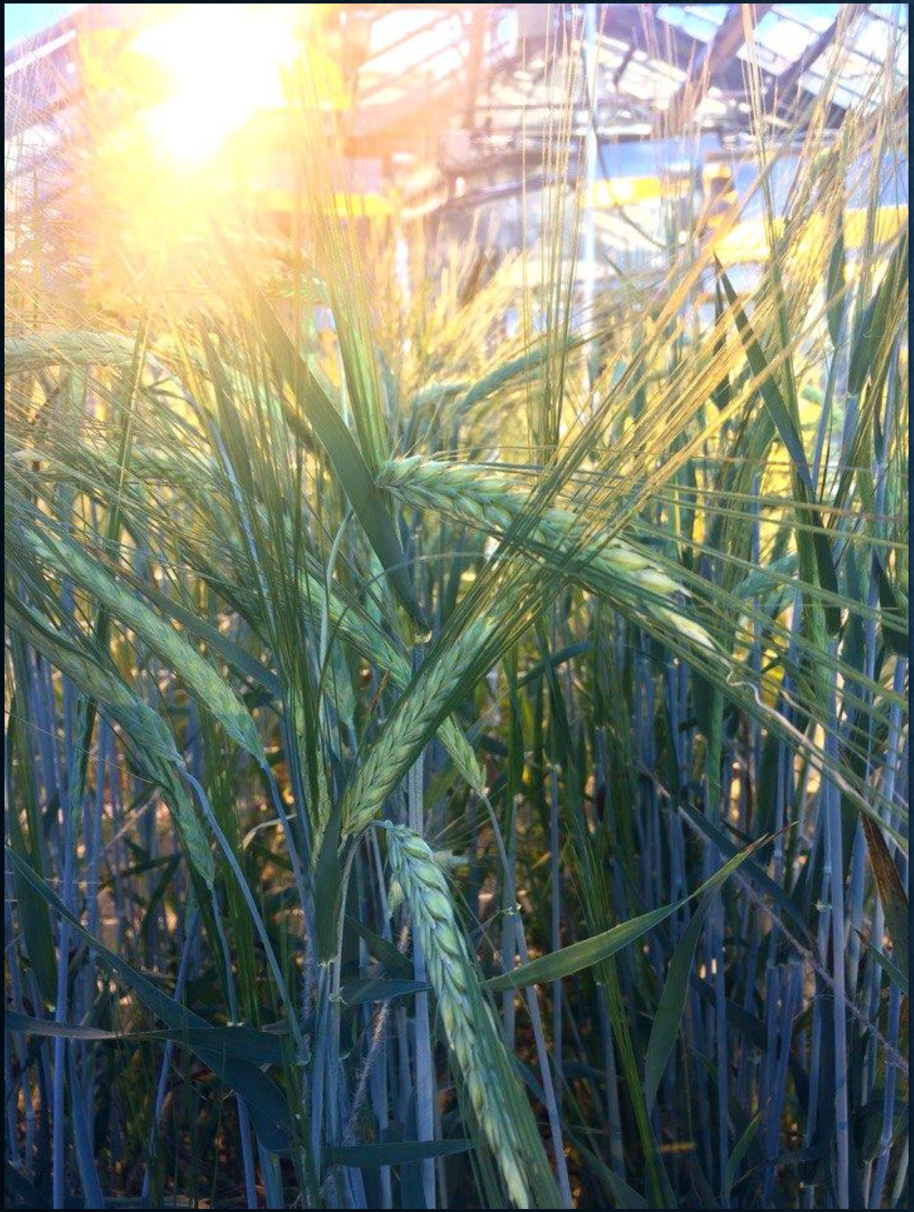

TARGETED KNOCKOUT OF THE *NUD* GENE IN SIBERIAN BARLEY

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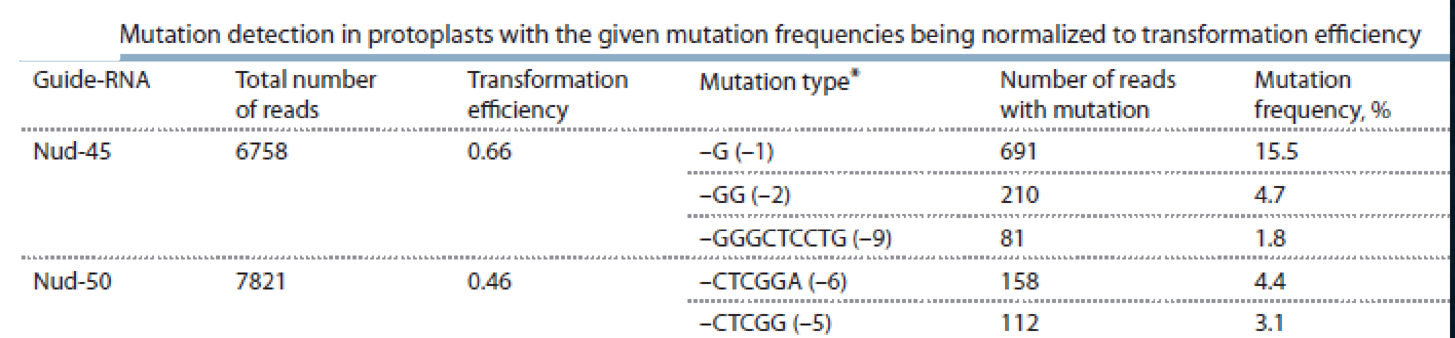
PURPOSE OF THE STUDY: TO OBTAIN A HULL PHENOTYPE BASED ON LOCAL ELITE BARLEY CULTIVARS.

The *NUDUM* (*NUD*) gene encodes a transcription factor involved in the formation of a lipidic layer at the grain surface of hulled barley. If the *NUD* gene function is lost, lemma and palea do no longer adhere to the grain's pericarp, which entails the non-adherent hull (i.e. naked) phenotype (nud).

MATERIALS AND RESULTS

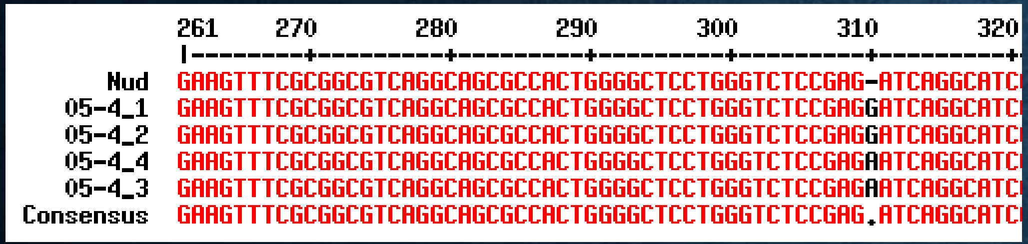
Loss-of-function mutations first were generated in protoplasts on cultivars Aley and Golden promise.

As a result of the transformation, a large number of mutations were obtained.
We allocated 5 mutant lines for the Nud gene, all lines have naked grain phenotype:

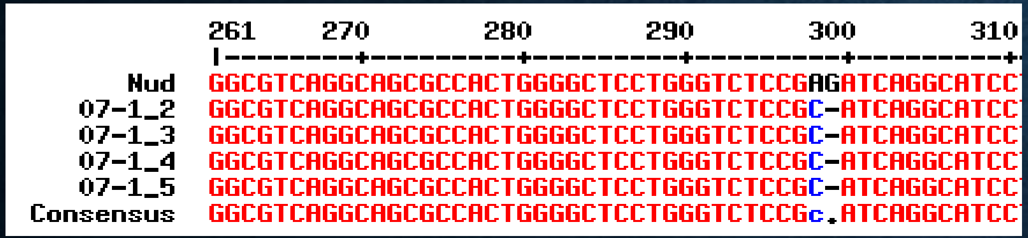


Mutation detection in protoplasts with the given mutation frequencies being normalized to transformation efficiency

Guide-RNA	Total number of reads	Transformation efficiency	Mutation type*	Number of reads with mutation	Mutation frequency, %
Nud-45	6758	0.66	–G (–1)	691	15.5
			–GG (–2)	210	4.7
			–GGGCTCCTG (–9)	81	1.8
Nud-50	7821	0.46	–CTCGGA (–6)	158	4.4
			–CTCGG (–5)	112	3.1



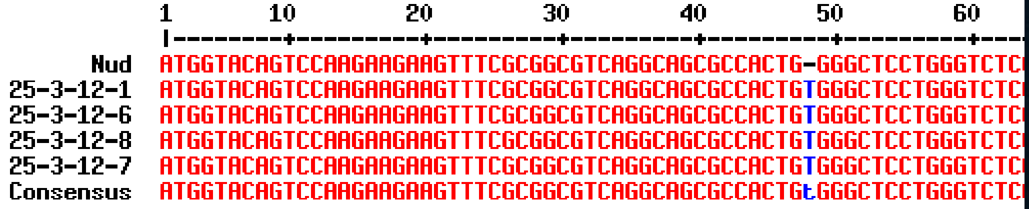
```
           261      270       280       290       300       310       320
           |--------+---------+---------+---------+---------+---------+
       Nud GAAGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGAG-ATCAGGCATC
    05-4_1 GAAGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGAGGATCAGGCATC
    05-4_2 GAAGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGAGGATCAGGCATC
    05-4_4 GAAGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGAGAATCAGGCATC
    05-4_3 GAAGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGAGAATCAGGCATC
 Consensus GAAGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGAG.ATCAGGCATC
```



```
           261      270       280       290       300       310
           |--------+---------+---------+---------+---------+
       Nud GGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGAGATCAGGCATCC
    07-1_2 GGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGC-ATCAGGCATCC
    07-1_3 GGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGC-ATCAGGCATCC
    07-1_4 GGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGC-ATCAGGCATCC
    07-1_5 GGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGC-ATCAGGCATCC
 Consensus GGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGc.ATCAGGCATCC
```

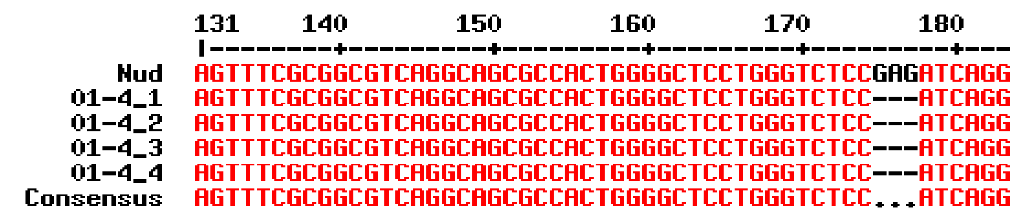
```
           1        10        20        30        40        50        60
           |--------+---------+---------+---------+---------+---------+--
       Nud ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTG-GGGCTCCTGGGTC
 25-3-10-5 ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGTGGGCTCCTGGGTC
 25-3-10-7 ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGTGGGCTCCTGGGTC
25-3-10-13 ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGTGGGCTCCTGGGTC
 25-3-10-8 ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGTGGGCTCCTGGGTC
 Consensus ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGtGGGCTCCTGGGTC
```

Reading frame shift



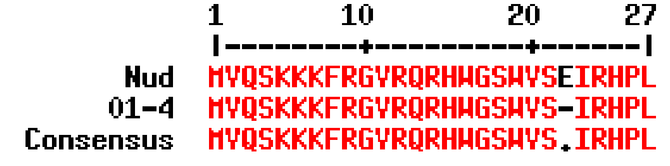
```
           1        10        20        30        40        50        60
           |--------+---------+---------+---------+---------+---------+----
       Nud ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTG-GGGCTCCTGGGTCTC
 25-3-12-1 ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGTGGGCTCCTGGGTCTC
 25-3-12-6 ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGTGGGCTCCTGGGTCTC
 25-3-12-8 ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGTGGGCTCCTGGGTCTC
 25-3-12-7 ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGTGGGCTCCTGGGTCTC
 Consensus ATGGTACAGTCCAAGAAGAAGTTTCGCGGCGTCAGGCAGCGCCACTGtGGGCTCCTGGGTCTC
```

Changing the amino acid composition of the protein

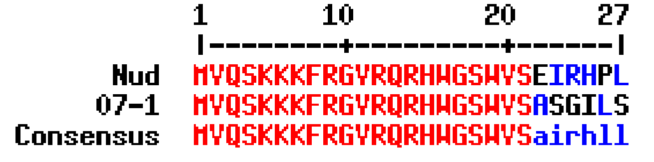


```
           131      140       150       160       170       180
           |--------+---------+---------+---------+---------+---
       Nud AGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCCGAGATCAGG
    01-4_1 AGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCC---ATCAGG
    01-4_2 AGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCC---ATCAGG
    01-4_3 AGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCC---ATCAGG
    01-4_4 AGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCC---ATCAGG
 Consensus AGTTTCGCGGCGTCAGGCAGCGCCACTGGGGCTCCTGGGTCTCC...ATCAGG
```

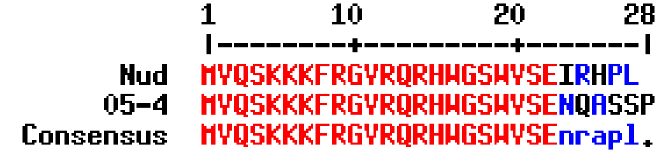
Disruption Nud amino acid sequences of mutants



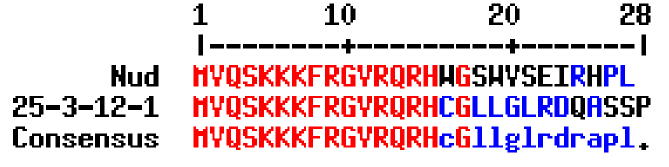
```
           1       10        20     27
           |--------+---------+------|
      Nud  MVQSKKKFRGVRQRHWGSWVSEIRHPL
     01-4  MVQSKKKFRGVRQRHWGSWVS-IRHPL
Consensus  MVQSKKKFRGVRQRHWGSWVS.IRHPL
```



```
           1       10        20     27
           |--------+---------+------|
      Nud  MVQSKKKFRGVRQRHWGSWVSEIRHPL
     07-1  MVQSKKKFRGVRQRHWGSWVSASGILS
Consensus  MVQSKKKFRGVRQRHWGSWVSairhll
```



```
           1       10        20      28
           |--------+---------+-------|
      Nud  MVQSKKKFRGVRQRHWGSWVSEIRHPL
     05-4  MVQSKKKFRGVRQRHWGSWVSENQASSP
Consensus  MVQSKKKFRGVRQRHWGSWVSEnrapl.
```



```
           1       10        20      28
           |--------+---------+-------|
      Nud  MVQSKKKFRGVRQRHWGSWVSEIRHPL
25-3-12-1  MVQSKKKFRGVRQRHCGLLGLRDQASSP
Consensus  MVQSKKKFRGVRQRHcGllglrdrapl.
```

It was demonstrated that the naked grain phenotype of these mutant lines is not accompanied by any modifications of agronomically relevant properties.

Statistical analysis (Mann–Whitney *U* test) of measured plant parameters and yield (mutant and control line comparison):

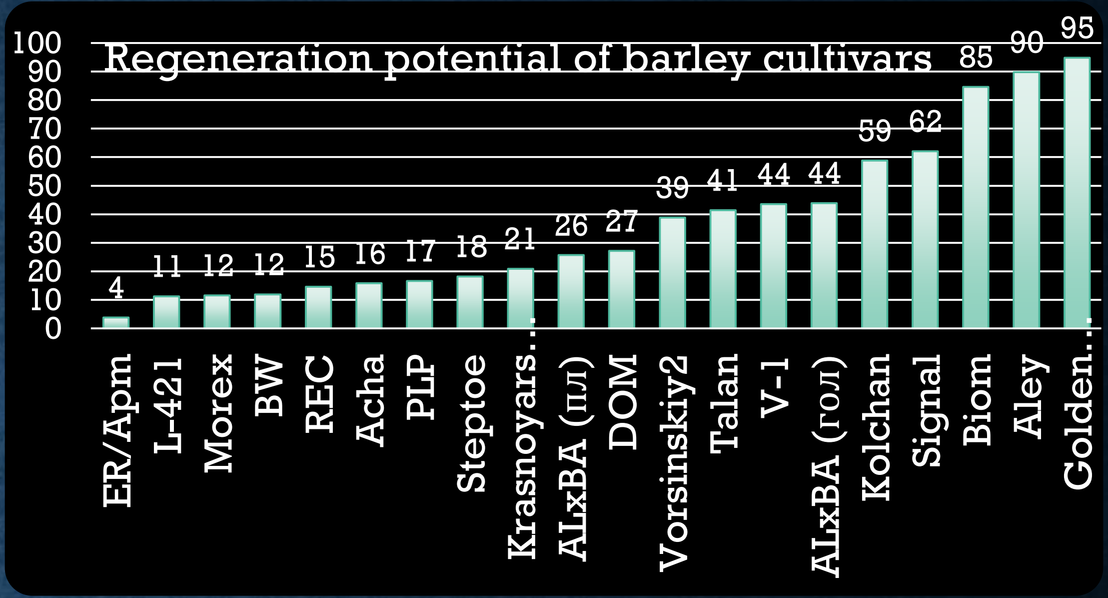
Parameter of the sheaf analysis	**Plant height**	**Spike density**	**Awns length**	**Total tillering**	**Productive tillering**	**Spikes length**	**Main spikes length**	**Number of grains per plant**	**Number of grains from the main spike**	**Weight of 1000 grains**	**Weight of grains per plant**	**The mass of grains from the main spike**
u_{emp} value for 01	18,5	40,5	35	42	46,5	11↓	27,5	50	47,5	19	38	22
u_{emp} value for 05	41	44,5	39,5	46,5	49,5	12↓	45	49	26,5	28	49	40,5
u_{emp} value for 07	34	36,5	43,5	25,5	29,5	23,5	44	47,5	15,5↑	11,5↓	46	41

Interpretation: The difference is significant Zone of uncertainty The difference is not significant

A panel of barley cultivars (20 including the control one – Golden promise) was tested for the *in vitro* regeneration potential of immature embryo explants:

Biom
Talan
Vorsinsky2
Aley
Acha
Signal
L-421
Kolchan
V-1
Krasnoyarsky91

Golden Promise
ER/Apm
ALxBA (пл)
ALxBA (гол)
PLP
REC
Morex
DOM
BW
Steptoe



The cultivars Aley, Biom and Signal performed best in the regeneration screen. Upon establishment of a protoplast transfection protocol, it was shown that mutations can be triggered in the *NUD* gene of cv. Aley, thereby confirming that the used *cas9*/gRNA vectors work effectively in this genetic background as well.

Attempts to achieve site-directed mutagenesis at the whole-plant level using *Agrobacterium*-mediated and ballistic DNA transfer to embryo explants of cultivars Aley and Biom are in progress.

References:
1. Gerasimova, S.V., Hertig, C., Korotkova, A.M. *et al.* Conversion of hulled into naked barley by Cas endonuclease-mediated knockout of the *NUD* gene. *BMC Plant Biol* **20,** 255 (2020).
2. Gerasimova SV, Korotkova AM, Hertig C, Hiekel S, Hoffie R, Budhagatapalli N, et al. Targeted genome modifcation in protoplasts of a highly regenerable Siberian barley cultivar using RNA-guided Cas9 endonuclease. Vavilov J Genet Breed. 2019;22:1033–9.

Acknowledgement: The study is supported by the Russian Science Foundation (project No 21-66-00012) and by the ICG Kurchatov Genomics Center (075-15-2019-1662).