

Supplemental information

**A reference-guided TILLING by
amplicon-sequencing platform supports
forward and reverse genetics in barley**

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Supplemental Information (SI):

A reference-guided TILLING by amplicon-seq platform supports forward and reverse genetics in barley

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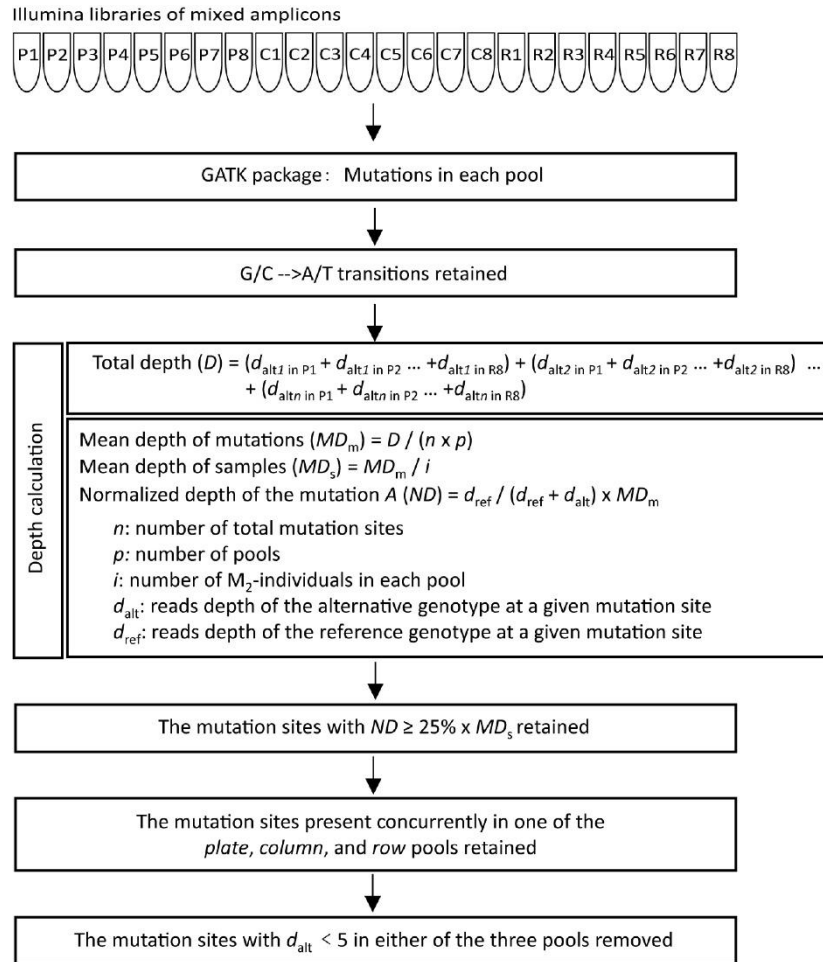
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Supplemental Figure 1. Examples of the range of phenotypic alterations observed in the M₂ population.

(A) Curly plant at the heading stage, (B) brittle stem, (C) many-noded dwarf, (D) light green leaves, (E) albostrain leaves, (F) yellow plant throughout its lifecycle, (G) disease-mimicking leaves, (H) *eceriferum*, loss of cuticle wax on the whole plant, (i) six-rowed spike, (J) dense spike, (K) spike wrapped by flag leaf sheath, (L) multiple awns.



Supplemental Figure 2. The flow-chart of data processing and mutations filtration in TILLING by amplicon-seq.

A



B

F ₁ families	F ₂ plants	Green F ₂ plants	Yellow F ₂ plants	3:1 ratio	T allele	A allele
F1-1	119	92	27	$\chi^2 = 0.339$	92	27
F1-2	61	46	15	$\chi^2 = 0.005$	46	15
F1-3	52	39	13	$\chi^2 = 0$	39	13
F1-4	38	29	9	$\chi^2 = 0.035$	29	9
F1-5	34	25	9	$\chi^2 = 0.039$	25	9
F1-6	76	61	15	$\chi^2 = 1.123$	61	15
F1-7	16	10	6	$\chi^2 = 1.333$	10	6
Total	396	302	94	$\chi^2 = 0.337$	302	94

Supplemental Figure 3. Genetic analysis of the chlorotic mutant M4009 in an F₂ population.

(A) Representative phenotypes of the parental lines, their F₁ hybrid and F₂ segregants. (B) Segregation analysis of green and yellow F₂ lines. Genotyping was conducted using functional KASP marker.

Supplemental Table 1. Pseudomolecule statistics of the HTX assembly.

chromosome	Assembled length (Mb)	No. of contigs	N50 (Mb)	maximum contig length (Mb)	minimum contig length (Kb)
1H	472.1	269	3.1	10.6	200.4
2H	611.7	356	3.1	21.9	200.1
3H	571.1	336	3	15.4	201.1
4H	564	365	2.6	13.4	203.7
5H	535.2	342	2.8	13.3	205
6H	496.6	305	2.9	14	200.9
7H	578.5	332	3	17.6	201.7
Un-assigned	223.1	10359	0	6.8	0.1

Supplemental Table 2. The primers used for amplicon sequencing. (Separate Excel file)

Supplemental Table 3. Mutations revealed by amplicon sequencing in sub-panels of the TILLING population.

	Experiment 1		Experiment 2		Experiment 3
EMS concentration for mutagenesis (mM)	22	32	22	32	32
Number of tested plants	262	250	262	250	1,728
Number of amplicons	47		72		56
Total size of amplicons (bp) based on MorexV3	51,467		104,108		81,266
Number of generated NGS-sequencing libraries	24		24		36
Mean dataset size per library (Gb)	1.75		2.39		2.98
Sequencing depth (x)	531		359		255
Number of mutations	19	46	44	92	309
Number of mutations per Mb	1.41	3.58	1.61	3.53	2.31
% of mutations validated by Sanger sequencing	90.48% (57/63)				

Supplemental Table 4. The mutations initially called within each pool, with reads depth of the reference (Ref) and alternative (Alt) genotype and the normalized depth (ND). (Separate Excel file)

Supplemental Table 5. Mutants detected and validated at the *Nud* gene among 4,608 M₂ individuals by *Cel* I-digestion and capillary electrophoresis approach.

No.	M ₂ plant ID	Concentration of EMS treatments	Site on PCR amplicon (nt)	Site on CDS (nt)	Nucleotide change	Amino acids change
1	4535	22mM (0.28%)	477	180	AAG to AAA	synonymous (Lys)
2	4943	22mM (0.28%)	609	312	ATT to ATC	synonymous (Ile)
3	5787	22mM (0.28%)	708	411	AAG to AAA	synonymous (Lys)
4	6103	22mM (0.28%)	791	494	CCC to CTC	Pro->Leu
5	7048	32mM (0.40%)	488	191	CCC to CTC	Pro->Leu
6	7296	32mM (0.40%)	660	363	ACC->ACT	synonymous (Thr)
7	7697	32mM (0.40%)	405	108	ACC->ACT	synonymous (Thr)
8	7771	32mM (0.40%)	630	333	AAG->AAA	synonymous (Lys)
9	8293	32mM (0.40%)	527	230	CCA->CTA	Pro->Leu
10	8338	32mM (0.40%)	681	384	GAG->GAA	synonymous (Glu)
11	8651	32mM (0.40%)	473	176	GCC->GTC	Ala->Val

Supplemental Table 6. EMS-induced mutations revealed by whole-genome sequencing.

Samples	EMS concentration	Clean bases (Gb)	Genome region mapped (Gb)*	Genome-wide		Gene region	
				Homozygous mutations	Homozygous mutations/Mb**	Homozygous mutations	Homozygous mutations/Mb
HTX-2-8-1	22 mM	43.43	3.81	16,587	4.35	231	2.20
HTX-2-8-2	22 mM	47.05	3.83	13,231	3.45	196	1.87
HTX-2-8-3	22 mM	49.23	3.83	12,764	3.33	221	2.10
HTX-4-1	32 mM	46.28	3.82	27,965	7.32	677	6.45
HTX-4-2	32 mM	48.98	3.84	12,169	3.17	210	2.00
HTX-4-3	32 mM	49.52	3.84	6,880	1.79	128	1.22
Average mutations/Mb					3.90		2.64

* The HTX genome reference was used for read mapping and variant calling. ** The homozygous mutation rate was calculated for each sample as the number of homozygous SNPs divided by the cumulative size of the genomic region mapped with high-quality reads.

Supplemental Table 7. Mutations within a 1054-bp fragment of *HvBRI1* detected across 2240 M₂ individuals.

No.	M ₂ -plant ID	Mutation in amplicon (bp)	Mutation in CDS (bp)	Ref	Alt	Mutation in protein	Predicted effect*
1	8,015	190	2,460	G	A	Synonymous	–
2	7,640	191	2,461	G	A	A821T	Deleterious (–3.733)
3	9,396	194	2,464	C	T	pre-stop, Q822*	Null
4	7,046	339	2,609	G	A	G870D	Deleterious (–5.756)
5	7,332	437	2,707	G	A	A903T	Neutral
6	8,419	461	2,731	G	A	A911T	Neutral
7	8,292	502	2,772	G	A	Synonymous	–
8	7,443	511	2,781	C	T	Synonymous	–
9	6,945	588	2,858	G	A	R953K	Deleterious (–2.750)
10	8,236	903	3,173	C	T	A1058V	Neutral
11	7,494	1,010	3,280	G	A	A1094T	Neutral
12	6,649	1,023	3,293	G	A	S1098N	Neutral

* *In silico* prediction using PROVEAN (Choi and Chan, 2015)

Supplemental Table 8. The four SNPs in the mutant bulk that located in the coding sequence regions on chromosome 1H.

SNPs position (MorexV3)	Genotype in wild-type HTX	Genotype in mutant bulk	Gene ID (MorexV3 reference)	Gene annotation	SNP position on gene	Change on amino acid
70381118	C	T	HORVU.MOREX.r3.1HG0019700	Retrovirus-related Pol polyprotein from transposon opus Ubiquitin C-terminal hydrolases superfamily protein	460	Ala→Thr
263129883	G	A	HORVU.MOREX.r3.1HG0041400	Gag polyprotein	332	Ser→Asn
299519042	C	T	HORVU.MOREX.r3.1HG0045800	Protochlorophyllide reductase	2435	Ala→Val
308667455	T	A	HORVU.MOREX.r3.1HG0047060		1168	Pre-stop

Supplemental Table 9. Primers used for analysis of the causal gene in the chlorotic mutant M4009.

Primer	5'-3' nucleotide	Tm (°C)	Product size (bp)	Purpose
1HG0047060-F-wt	GAAGGTGACCAAGTTCATGCTGTCTGGGAGCTCAGCGAGA	58.19	107	KASP genotyping
1HG0047060-F-4009	GAAGGTGCGGAGTCAACGGATTGTCTGGGAGCTCAGCGAGT	57.21		
1HG0047060-R-com	CGCAACTTGGTGCGGAAT	59.96	284	VIGS gene silencing
1HG0047060-VIGS-F	TTTAAACCACCACCAGGGCCGAGAAGCTCACTGCCATCCGC	64.47		
1HG0047060-VIGS-R	CTTCCGTTTCTAAGGAAGGCCTTAGGACGCCGGGTTGCTGTC	64.13	175	qRT-PCR
1HG0047060-ex4-F2	CTGGCGCAGGTCGTCAGC	63.04		
1HG0047060-3UTR-R	CAGTCGGTGATCATGCGAGC	62.01		

The blue and green marked nucleotides at the 5'-end of allele-specific forward primers are the adaptors for FAM- and VIC-fluorescence in the assay, respectively. The underlines nucleotides at the 5'-end of the VIGS primers are the adaptors for the ligation independent cloning.