Supplemental information

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

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Supplemental Information (SI):

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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.
Supplemental Figure 3. Genetic analysis of the chlorotic mutant M4009 in an F$_2$ population.

(A) Representative phenotypes of the parental lines, their F$_1$ hybrid and F$_2$ segregants.

(B) Segregation analysis of green and yellow F$_2$ 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           |
| Number of tested plants      | 262          | 250          | 262          |
| 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           |
| Number of mutations per Mb   | 1.41         | 3.58         | 1.61         |
| % 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. | M2 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  | Homozygous  | Homozygous  | Homozygous  |
|               |                   |                  |                             | mutations   | mutations/Mb** | mutations   | 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**   |                   |                  |                             | **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. | M2-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 | 460                  | Ala→Thr             |
| 263129883               | G                         | A                       | HORVU.MOREX.r3.1HG0041400  | Ubiquitin C-terminal hydrolases superfamily protein | 332                  | Ser→Asn             |
| 299519042               | C                         | T                       | HORVU.MOREX.r3.1HG0045800  | Gag polyprotein | 2435                | Ala→Val             |
| 308667455               | T                         | A                       | HORVU.MOREX.r3.1HG0047060  | Protochlorophyllide reductase | 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-wt   | GAAGGTGACCAAGTTCATGCTGTCTGGGAGCTCGAGGA            | 58.19   | 107               | KASP genotyping |
| 1HG0047060-4009 | GAAGGTGAGATCAAGGCTGTCTGGGAGCTCAGGA                      | 57.21   | 175               | VIGS gene      |
| 1HG0047060-R-com| CCGAACTTGGGAGGAAAT                                       | 59.96   | 284               | silencing      |
| 1HG0047060-VIGS-F| GCTGGCGAAGTACTCGAGGAT                                   | 64.47   | 175               | qRT-PCR        |
| 1HG0047060-VIGS-R| CCGAAGTACTCGAGGAT                                        | 64.13   | 284               |              |
| 1HG0047060-ex4-F2 | CAGTCCGGTATCGGAGGAC                                      | 63.04   | 175               |              |
| 1HG0047060-3UTR-R | CAGTCCGGTATCGGAC                                      | 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.