Title:
Selection of transformation efficient barley genotypes based on TFA (transformation amenability) haplotype and higher resolution mapping of the TFA loci

Authors’ information:
Hiroshi Hisano1*, Brigid Meints2, Matthew J. Moscou3, Luis Cistue4, Begoña Echávarri4, Kazuhiro Sato1 and Patrick M.Hayes5

1. Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan
2. Dept. Crop and Soil Sciences, Washington State University, Mount Vernon, WA, USA
3. The Sainsbury Laboratory, Norwich Research Park, Norwich, UK
4. Dept. Genetica y Produccion Vegetal, Estacion Experimental de Aula Dei, Zaragoza, Spain
5. Dept. Crop and Soil Science, Oregon State University, Corvallis, OR, USA

*Corresponding author
Hiroshi Hisano
Institute of Plant Science and Resources, Okayama University,
2-20-1 Chuo, Kurashiki, Japan
Tel & Fax: +81-86-434-1243
E-mail: hiroshi.hisano@rib.okayama-u.ac.jp

Abstract
Barley (Hordeum vulgare) cv. ‘Golden Promise’ is one of the most useful and well-studied cultivars for genetic manipulation. In a previous report, we identified several transformation amenability (TFA) loci responsible for Agrobacterium-mediated transformation by using the F2 generation of immature embryos, derived from ‘Haruna Nijo’ × ‘Golden Promise’, as explants. In this report, we describe higher density mapping of these TFA regions with additional SNP markers using the same transgenic plants. To demonstrate the robustness of transformability alleles at the TFA loci, we genotyped 200 doubled haploid progeny from the cross ‘Golden Promise’ × ‘Full Pint’. Based on SNP genotype, we selected lines having ‘Golden Promise’ alleles at TFA loci and used them for transformation. Of the successfully transformed lines, DH120366 came the closest to achieving a level of transformation
efficiency comparable to ‘Golden Promise’. The results validate that the genetic substitution of TFA alleles from ‘Golden Promise’ can facilitate the development of transformation efficient lines from recalcitrant barley cultivars.

**Keywords** *Agrobacterium tumefaciens*, doubled haploid, *Hordeum vulgare* (barley), single nucleotide polymorphism, transformation

**Key message**
The genetic substitution of transformation amenability alleles from ‘Golden Promise’ can facilitate the development of transformation efficient lines from recalcitrant barley cultivars.

**Introduction**
The success of genetic transformation in plants is strongly genotype-dependent. For example, the Columbia ecotype in *Arabidopsis thaliana* and cvs. ‘Nipponbare’ or ‘Taichung 65’ (T-65) in rice (*Oryza sativa*) are the most capable genotypes at producing transgenic plants (Zhang et al. 2006; Nishimura et al. 2006). There is a genetic component to transformation efficiency in plants, including attributes such as the ratio of T-DNA/transgene integration, callus differentiation, and green shoot regeneration (Nam et al. 1997; Cogan et al. 2004; Sparrow et al. 2004). There may be interactions between genetic factors (cultivars) and experimental conditions, e.g. components of medium, temperature during tissue culture, and type of explants. If the genes responding to these conditions are isolated and manipulated, more cultivars and/or breeding lines would be amenable to genetic transformation. Recently, two transcription factors - *baby boom* (*BBM*) and *Wuschel2* (*WUS2*) - were identified as key players for transformation efficiency in monocot plants including maize, sorghum, sugarcane, and rice (Lowe et al. 2016).

Barley (*Hordeum vulgare*) transformation is currently possible with both biolistic and *Agrobacterium*-mediated methods (Lü et al. 2015). After Tingay et al. (1997) developed the *Agrobacterium*-mediated method for barley transformation, ‘Golden Promise’ has been used for the functional analysis of several genes of scientific and economic interests (reviewed in Mrízová et al. 2014). Despite massive attempts at technical improvements, barley transformation is still performed with a limited number of cultivars e.g. ‘Golden Promise’ and ‘Igri’ (Kumlehn et al. 2006; Hensel et al. 2008; Harwood 2012). These genotype-dependent transformation systems limit the opportunities for
complementation analysis when the transformed cultivar has a non-functional allele at the target gene. For example, Deng et al. (2015) studied the gene cascades related to a vernalization gene \( VRN1 \) encoding a transcription factor using ‘Golden Promise’ as a model. However, ‘Golden Promise’ does not require vernalization and is photoperiod insensitive. Therefore, wild type ‘Golden Promise’ was unsuitable for the transcriptome sequencing (RNA-seq) and chromatin immunoprecipitation sequencing (ChIP-seq) to identify direct targets of \( VRN1 \). To produce a vernalization-requiring derivative of ‘Golden Promise’, full-length versions of \( VRN1 \) and \( VRN2 \), plus a functional copy of \( PHOTOPERIOD1 \) were introgressed into ‘Golden Promise’ through three rounds of recurrent crossing with marker assisted selection. As another example, Sato et al. (2016) developed a backcross-derived line in the cv. ‘Golden Promise’ background with substitutions of target segments carrying the \( Qsd1 \) gene for seed dormancy and used this substitution line for transformation experiments including complementation and RNAi. In both examples, substantial time and resources were required to develop the necessary genetic stocks prior to transformation. It is not prudent to directly attempt transformation of target genotypes without knowing their degree of transformability.

We recently reported a significant technical advancement for \( Agrobacterium \)-mediated transformation efficiency in barley using progeny from the cross between cvs. ‘Haruna Nijo’, which is recalcitrant for transformation, and ‘Golden Promise’ (Hisano and Sato 2016). By isolating 3,013 F2 generation immature embryos and subsequent infection with \( Agrobacterium \), we generated 60 independently transformed plants and genotyped them using genome-wide SNP markers. By mapping regions distorted in favor of ‘Golden Promise’ alleles in these plants, we identified three major transformation amenability (\( TFA \)) loci. In the same report we describe the use of progeny from the cross of ‘Morex’ (recalcitrant) × ‘Golden Promise’. Based on these results we proposed that transformation could be used in any barley genotype into which favorable \( TFA \) alleles from ‘Golden Promise’ were introgressed. To validate this \( TFA \)-based pre-selection strategy for candidate line transformation, assessment of \( TFA \) alleles from ‘Golden Promise’ in other genetic backgrounds is necessary.

Here we report validation of the effects of ‘Golden Promise’ \( TFA \) alleles using 200 doubled haploid progeny from the cross of ‘Golden Promise’ × ‘Full Pint’ (the Oregon Promise mapping population). This population was developed with the principal goal of assessing the contributions of barley genotype to beer flavor. Those results will be reported elsewhere, as will QTLs and genes associated with agronomic and disease resistance traits. In addition these lines will be used for future
complementation or genome editing work. Of immediate applicability to TFA allele validation, ‘Full Pint’ is recalcitrant to Agrobacterium-mediated transformation, and the Oregon Promise population has been extensively genotyped for QTL mapping, allowing for the inspection of haplotypes and selection of target TFA alleles. In addition, we added markers to the ‘Haruna Nijo’ × ‘Golden Promise’ map in order to better delimit the TFA QTLs and facilitate map alignment with anchor markers.

Materials and Methods

Plant materials
Sixty transgenic plants (tHN×GP) previously generated using F2 immature embryos from the cross of ‘Haruna Nijo’ × ‘Golden Promise’ (Hisano and Sato 2016) were used for higher resolution mapping by genotyping with SNP markers. The 200 doubled haploids comprising the Oregon Promise doubled haploid population were developed using anther culture, following the protocols described by Cistué et al. (1994) and Echávarri et al. (2016). The Oregon Promise population was developed jointly by the Cistue lab at the Dept. Genetica y Produccion Vegetal, Estacion Experimental de Aula Dei, Zaragoza, Spain and the Hayes lab in the Department of Crop and Soil Science, Oregon State University, Corvallis, Oregon, USA. The population was generated for the principal purpose of mapping genetic determinants of barley contributions to beer flavor. Those results will be reported elsewhere. For the purposes of this research, a doubled haploid population was needed that involved a transformation-recalcitrant genotype crossed with Golden Promise.

Genotyping
For increasing marker density in the tHN×GP population map, additional SNP markers were developed using a 10K Infinium HD assay (Illumina, USA) including oligo sets of BOPA1 (Close et al. 2009). Genotyping of the Oregon Promise population was performed using a custom Illumina BeadExpress 384-plex based on previously characterized SNPs with a high minor allele frequency (Close et al. 2009). A total of 171 BeadExpress SNP markers were polymorphic. KASP markers were developed from SNPs in the designs of the POPA/BOPA and OPA 9K to bridge fragmented linkage groups and ensure markers were present at distal positions of chromosome arms (Close et al. 2009; Comadran et al. 2012).
Construction of linkage maps

A genetic linkage map was constructed using genotyping data generated by SNP markers in tHN×GP population. Grouping and ordering of markers was performed using the default settings of AntMap (Iwata and Ninomiya, 2006) and the Kosambi mapping function. For the Oregon Promise population, a framework genetic map was initially developed with the BeadExpress SNP markers using JoinMap v4, which integrated 168 markers into nine linkage groups with chromosomes 1H and 6H fragmented into two linkage groups. Addition of KASP markers generated a final genetic map with 251 markers, of which 206 are non-redundant, with a genetic distance of 1,311 cM, using the Kosambi function, over eight linkage groups (Supplemental Table S1). The majority of intervals between markers are below 20 cM, with only four regions on chromosomes 3H, 6H, and 7H having regions above 20 cM. Despite substantial effort, markers could not be developed to bridge the two linkage groups of chromosome 1H. Collinearity was observed for all markers relative to the consensus genetic map of barley (Close et al. 2009).

In silico selection of Oregon Promise doubled haploids for transformability, based on TFA haplotype

Based on markers in common between the tHN×GP and Oregon Promise populations, three Oregon Promise lines, DH120366, DH120536, and DH120543 were selected for the favorable allele haplotype at three major TFA loci: TFA1, TFA2 and TFA3, reported by Hisano and Sato (2016). In DH120536 there were ‘Full Pint’ alleles at some of the marker loci defining the target haplotypes at TFA2 and TFA3.

Construction of the binary vector pBUH3-EGFP carrying the EGFP gene

The ORF fragment of the enhanced green fluorescent protein (EGFP) gene (Clontech laboratories, USA) was amplified by PCR using PrimeSTAR Max DNA Polymerase (Takara, Japan) with the specific primer pair (EGFP-F1-SaeI, 5’-GAGCTCACCATGGTGAGCAAGGGCGAGGAG-3’ and EGFP-R1-BamHI, 5’-GGATCCCTTTACTTGTACAGCTCGTCCATGCCG-3’). The program involved: an initial denaturation step at 98°C for 2 min, 30 cycles of denaturation step at 95°C for 10 sec, annealing step at 55°C for 20 sec and extension step at 72 °C for 1 min, and a final extension at 72°C for 10 min. The PCR fragment of EGFP gene and pBUH3 (Nigorikawa et al. 2012) was digested with
the restriction enzymes \( \text{Sac} \)I and \( \text{Bam} \)HI and ligated using the Quick ligation kit (New England Biolabs, USA).

\textit{Agrobacterium-mediated transformation of selected Oregon Promise doubled haploids and EGFP detection}

The procedure for \textit{Agrobacterium}-mediated transformation method was identical to that reported by Hisano and Sato (2016). Briefly, the protocol was follows: barley immature embryos were infected with \textit{Agrobacterium tumefaciens} strain AGL1 carrying pBUH3-EGFP and co-cultivated for 3 days. Surviving immature embryos were then (1) incubated on callus-induction medium without selection for a week and (2) selected on selection-medium containing hygromycin under dark condition for 4 weeks. Calli resistant to hygromycin were transferred onto regeneration-medium and incubated under a 16 h light/8 h dark photoperiod regime. Regenerated shoots were transferred to rooting-medium. The expression of EGFP was detected using LED blue light and an Orange Filter for GFP (Optocode, Japan). For confirming transgene presence, touch down PCR was performed by the method described in Hisano and Sato (2016) with specific primers for the \textit{hygromycin phosphotransferase} (\textit{HPT}) and \textit{EGFP} genes (\textit{EGFP}-F1; 5’-gacgacggcaactacaagac-3’ and \textit{EGFP}-R2; 5’-gactgggtgctcaggtagtg-3’).

\textit{Identification and in silico mapping of the barley BBM and WUS2 genes}

Barley \textit{BBM} and \textit{WUS2} genes were identified by searching the barley full-length cDNAs at the IPK Barley BLAST Server (http://webblast.ipk-gatersleben.de/barley/) using the maize homologues - \textit{BBM} (CS155772) and \textit{WUS2} (EA275154) - as query sequences. The linkage map positions of these genes, and the nearest SNP markers, were estimated using the barley genome sequence (IBSC 2012).

\textbf{Results}

\textbf{Genotyping of transgenic tHN×GP plants and reconfirmation of the TFA regions}

The 10K-SNP Illumina Infinium HD assay of the population of 60 transgenic HN×GP barley plants (tHN×GP) resulted in 1,131 SNP markers defining the haplotype of these plants. Among these SNP markers, 124 were previously used (Hisano and Sato 2016) and 1,007 were newly developed in the present study for tHN×GP. Using these genotype data, a genetic linkage map consisting of 1,131 SNPs...
was constructed for the tHN×GP population. The Kosambi cM distances between these makers are shown in supplemental Table S2, along with the Chi-square values calculated for 1:2:1 and 3:1 ratios at each marker locus. The Chi-square values of each marker are plotted in linkage map order in Supplemental Figure S1. Significant (<0.01) levels of segregation distortion defined the same chromosomal positions for TFA1, TFA2 and TFA3 per our previous report (Hisano and Sato 2016). Fig. 1 shows the linkage maps of chromosomes 2H and 3H and the regions showing significant distortion of marker segregation that define the TFA1, TFA2 and TFA3 loci. At TFA1, the markers between NIASHv1109O03_00000798_3H and 8984-579 showed significant (<0.01) distortion for 1:2:1 and 1:3 (GP:[hetero+HN]) ratios. At TFA2, markers between FLOubaf102I04_00000319_2H and FLOubaf138j23_00000441_2H showed significant (<0.01) distortion for 1:2:1 and 1:3 (GP:[hetero+HN]) ratios. At TFA3, markers between FLOubaf138j23_00000441_2H and NIASHv3092H0900000823_2H showed significant (<0.01) distortion for a 3:1 (GP+hetero]:HN) ratio. For chromosome 2H and 3H, 143 and 164 new markers were developed respectively, in this study. The addition of new markers at the distal region of chromosome 2HL expanded the region of TFA3.

In silico mapping of TFAs in the Oregon Promise population

Based on allele type at the TFA loci reported in Hisano and Sato (2016) the map-ordered marker data for the Oregon Promise population were reviewed and lines DH120366, DH120536, and DH120543 were selected as explant genotypes for the Agrobacterium-mediated transformation experiment (Supplemental Figure S1 and Figure S2). Selection of the Oregon Promise candidates for Agrobacterium-mediated transformation was based on markers in common between the two populations. The graphical genotypes of three selected lines and tentative positions of TFA1, TFA2, and TFA3 are shown in Fig. 2. According to marker haplotype, DH120366 and DH120543 have complete ‘Golden Promise’ alleles in the TFA regions, but there are ‘Full Pint’ alleles at portions of TFA2 and TFA3 in DH120536 (Fig. 2).

In silico mapping of the barley BBM and WUS2 genes

The candidate barley homologues of the BBM and WUS2 genes (AK364030 and AK370947, respectively) were found from barley full-length cDNA sequences. The genomic sequences morex_contig_58483 and morex_contig_66485, harboring the barley BBM and WUS2 genes,
respectively, were the highest BLAST hits. According to the barley consensus map and genome (IBSC 2012), the barley BBM gene is at cM 67.35 on chromosome 2H, and the barley WUS2 gene is at cM 90.23 on chromosome 3H. The nearest SNP markers were 8889-842 (cM 50.06 on 2H in the tHN×GP linkage map; between 2580-1456 and 2809-271) for the barley BBM gene and 8020-87 (cM 83.16 on 3H) for the barley WUS2 gene (supplemental Table S2). The putative positions are plotted in Fig. 1. In DH120536, the BBM gene is located in the region lacking the GP-allele at TFA2.

**Transformation of selected Oregon Promise DH lines**

Immature embryos of the three selected lines were used for infection by *Agrobacterium* carrying the pBUH3-EGFP vector (Supplemental Figure S2) with four to six replications. All the lines showed transient EGFP expression in co-cultivated immature embryos at 10 days after infection. The efficiency of transformation of these Oregon Promise selections and their parents is summarized in Table 1. During selection by hygromycin, EGFP expression was observed in the resistant callus of DH120366 and DH120543. However, only one tiny clump of resistant callus was observed for DH120536 and it did not show green fluorescence. EGFP expression in resistant callus of DH120543 is shown in Fig 3. Regeneration of green shoots and development of roots were observed from resistant calli for both DH120366 and DH120543 (Fig. 3). ‘Full Pint’ did not show any resistant green shoots, nor did DH120536. From DH120366 and DH120543, respectively, 53 and 15 independent transgenic barley plants were generated from 252 and 193 immature embryos. Transformation efficiencies were 23.7% (53/224) and 15.5% (15/97), respectively. For this calculation, we omitted the number of calli which died during *Agrobacterium* infection as we consider this a technical error caused by physical damage to explants. Transgenes were detected by PCR using specific primers for HTP and EGFP genes in all the regenerated plants and a representative result is shown in Fig. 4.

**Discussion**

**Validating TFA4s using the tHNxGP and Oregon Promise populations**

Hisano and Sato (2016) identified three putative transformation amenability (TFA) loci, with large effects, in 60 transformed F2 plants from the cross of ‘Haruna Nijo’ and ‘Golden Promise’ (tHN×GP) based on genotyping with 124 SNPs derived from a 384 barley oligonucleotide pooled assay (BOPA, Close et al. 2009). For this report, we performed an Infinium HD assay to develop additional markers.
for the tHN×GP population with the goal of increasing marker density and the number of markers in common with the Oregon Promise population. The Oregon Promise population was genotyped with an array of markers, including BOPA-derived SNPs, allowing for direct map alignment with tHN×GP. We placed the *TFA* loci on the Oregon Promise linkage map using markers in common between the two populations and/or the position information of BOPA markers in the consensus barley map (IBGS 2012). Higher marker density was achieved by the addition of new SNP markers to the tHN×GP population and this provided greater resolution of *TFA3*. In the near future, the *TFA* loci will be targets for map-based cloning, requiring even higher marker resolution. Maximum population size is required for high resolution mapping. However, because producing large numbers of transformed plants may not be feasible, identification of transformation-inefficient segments on a high resolution map may be an alternative strategy for narrowing down each *TFA* QTL to a candidate gene.

**The identification of transformation-efficient lines in the Oregon Promise population**

In prior research, we developed only two transformed plants from a cross of ‘Morex’ × ‘Golden Promise’ (Hisano and Sato 2016). We also tried to select transformation amenable lines from BC$_3$F$_8$ recombinant chromosome substitution lines derived from the cross of ‘Golden Promise’ × ‘Haruna Nijo’ (with ‘Haruna Nijo’ as the recurrent parent) but did not obtain any transgenic plants from 4,661 immature embryos. In the present study, the Oregon Promise doubled haploids were useful in validating the effects of favorable alleles at the *TFA* loci and in showing that transformation-efficient lines can be developed in the transformation-recalcitrant background of cv. ‘Full Pint’. These findings may encourage barley researchers to introgress *TFA* alleles from ‘Golden Promise’ into any genotype to transform target genes. Cultivars that have low amenability for transformation could be used, provided optimization of the conditions required to improve efficiency. However for particularly recalcitrant cultivars, genetic approaches like those used in this study may be required for success.

According to the segregation in doubled haploid lines, the accumulation of favorable alleles at the three *TFA* loci would be expected at a frequency of 1/8 (12%). We selected only 3 lines (from 202 lines) based on haplotypes at the three *TFA* loci (less than 1% of the population) and two of these were successfully transformed. These results suggest that screening by genotyping for *TFA* alleles was efficient in ensuring successful transformation in Oregon Promise lines. We reached efficiencies of transformation of 23.7% and 15.5% in DH120366 and DH120543, respectively, whereas efficiency was more than 30% in ‘Golden Promise’. In our previous research we reported 7 minor-effect *TFAs*
(TFA4 to TFA10) in tHN×GP (Hisano and Sato 2016). Among them, 4 ‘Golden Promise’ alleles (TFA5, TFA6, TFA8 and TFA10) might affect transformation efficiency. Currently, we cannot evaluate if both ‘Haruna Nijo’ and ‘Full Pint’ have the same alleles at these loci. DH120366 and DH120543 may lack favorable alleles at these minor-effect loci and as a result have lower transformation-amenability than ‘Golden Promise’. It would be possible to develop transformation-efficient near-isogenic lines by several rounds of backcrossing and marker-assisted selection using ‘Golden Promise’ and ‘Full Pint’. However this would require periodic phenotypic validation in order to assure introgression of the as yet uncharacterized TFA regions.

The genetic factors for transformation amenability and related genes

Oregon Promise lines DH120366 and DH120543 are transformation-amenable but DH120536 is not amenable. All three lines have the ‘Golden Promise’ allele at TFA1, but DH120536 has the ‘Full Pint’ alleles at portions of the predicted TFA2 and TFA3 haplotypes. It is likely that DH120536 will be of assistance in delimiting the TFA2 and TFA3 critical regions in order to isolate the genes involved in transformation amenability. We suggest that introgression of the ‘Golden Promise’ allele at TFA1 is necessary, but not sufficient, for transformation amenability in barley.

To obtain transgenic plants with Agrobacterium-mediated transformation, essential factors include (i) Agrobacterium-plant cell interaction including T-DNA integration, (ii) activity of propagation of callus during selection, and (iii) regeneration from callus (Cheng et al. 2004). Recently these factors were defined at the molecular levels in certain dicot plants. Anand et al. (2007) reported that a host gene, VIP2, was essential for Agrobacterium-mediated transformation and was involved in T-DNA integration into the host genome. Ikeuchi et al. (2013, 2016) reviewed the genes related to callus induction and regeneration in Arabidopsis and related species and reported that several hormone-related genes and/or their transcription factors - such as baby boom (BBM) and Wuschel2 (WUS2) - were important for callus differentiation. More recently Lowe et al. (2016) reported that co-overexpression of the maize BBM and maize WUS2 genes improved efficiency of transformation in monocot plants including maize, sorghum, sugarcane, and rice. These genes could stimulate proliferation of transgenic callus resulting also improving regeneration efficiency.

Focusing on the barley genome, we identified a homologue of the maize BBM gene near SNP marker 8889-842 at cM 50.06 on 2H of the tHN×GP linkage map and this is located in the region lacking the GP-allele at TFA2 in DH120536. That could explain the lack of transformability in this
line. This chromosome region in barley is close to that which Yeo et al. (2014) found responsible for transformation efficiency in ‘Golden Promise’. We also found a barley homologue of WUS2 in TFA1, with the closest marker being 8020-87 (cM 83.16) on 3H in the tHN×GP linkage map. It is notable that the homologues of BBM and WUS2 genes are in TFA2 and TFA1, respectively. However, the gene locations do not correspond with regions of maximum segregation distortion (cM 60.42 on 2H for TFA2 and cM 73.93 on 3H for TFA1). No candidate gene homologue was found in TFA3.

**Conclusion**

Here we demonstrated the TFA-based selection method for transformation amenability using doubled haploid barley lines derived from a cross between cvs. ‘Golden Promise’ and ‘Full Pint’. Transgenic plants were generated from two selected lines that have complete ‘Golden Promise’ allele sets at TFA1, TFA2, and TFA3. No transgenic plants were generated from a line that had the ‘Golden Promise’ allele at TFA1, but a portion of ‘Full Pint’ alleles at TFA2 and TFA3. These results suggested that our TFA-based method was efficient for selecting donor plants for transformation experiments, e.g. complementation test, and that TFA2/TFA3 was necessary for successful transformation as well as the most major loci, TFA1.

**Author contribution statement**
P.M.H. and K.S. designed research. H.H., B.M., M. M., L. C. and B. E. conducted experiments. H.H. and P.M.H. analyzed the data. H.H., K.S. and P.M.H. wrote the paper. All authors read and approved the manuscript.

**Acknowledgements**

We thank Tanya Filichkin, and Laura Helgerson for their part in developing the Oregon Promise population, Yuka Motoi (IPSR, Okayama Univ.) for technical assistance and Dr. Yukihiro Ito (Tohoku Univ., Japan) for providing the pBUH3 vector. This work was supported by the Spanish Ministry of Science and Innovation (project AGL2015 - 69435-C3-2-R) to L.C., the Gatsby Foundation and BBSRC Institutional Strategic Programme (BB/J004553/1) to M.M., and JSPS KAKENHI, grant numbers 24880025 and 16K18634 to H.H.
Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest

Reference


environmental adaptation in cultivated barley. Nature Genetics, 44:1388-1392.


**Tables**

**Table 1. Summary of transformation results in Oregon Promise DH lines and their parents**

<table>
<thead>
<tr>
<th>Lines</th>
<th>Replications</th>
<th>No. of IE for survived co-cultivation with <em>Agrobacterium</em></th>
<th>No. of IE survived after co-cultivation with <em>Agrobacterium</em></th>
<th>No. of co-resistant callus after co-cultivation with <em>Agrobacterium</em></th>
<th>No. of resistant callus to hygromycin</th>
<th>No. of callus regenerated to green shoots</th>
<th>Efficiency of transformation (B/A, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golden Promise</td>
<td>1</td>
<td>55</td>
<td>52</td>
<td>-</td>
<td>4</td>
<td>7.7</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>59</td>
<td>51</td>
<td>-</td>
<td>24</td>
<td>47.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>57</td>
<td>54</td>
<td>-</td>
<td>24</td>
<td>44.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>36</td>
<td>28</td>
<td>-</td>
<td>7</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>65</td>
<td>23</td>
<td>-</td>
<td>12</td>
<td>52.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>64</td>
<td>40</td>
<td>-</td>
<td>23</td>
<td>57.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>total</strong></td>
<td><strong>336</strong></td>
<td><strong>248</strong></td>
<td>-</td>
<td><strong>94</strong></td>
<td><strong>37.9</strong></td>
<td></td>
</tr>
<tr>
<td>Full Pint</td>
<td>1</td>
<td>31</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>---</td>
<td>----</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>54</td>
<td>38</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>DH120366</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>16</td>
<td>16</td>
<td>2</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>45</td>
<td>44</td>
<td>11</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30</td>
<td>24</td>
<td>7</td>
<td>3</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>39</td>
<td>32</td>
<td>16</td>
<td>12</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>95</td>
<td>93</td>
<td>54</td>
<td>38</td>
<td>40.9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>27</td>
<td>15</td>
<td>9</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>total</td>
<td>252</td>
<td>224</td>
<td>99</td>
<td>53</td>
<td></td>
<td>23.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>DH120536</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>30</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>41</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>total</td>
<td>104</td>
<td>63</td>
<td>1</td>
<td>0</td>
<td></td>
<td>0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>DH120543</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>62</td>
<td>44</td>
<td>17</td>
<td>6</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>31</td>
<td>21</td>
<td>7</td>
<td>7</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>27</td>
<td>22</td>
<td>12</td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>49</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>16.7</td>
</tr>
<tr>
<td>total</td>
<td>193</td>
<td>97</td>
<td>38</td>
<td>15</td>
<td></td>
<td>15.5</td>
</tr>
</tbody>
</table>

IE; Immature embryo, n.a.; not available.

* Checking by PCR, all the individuals were proved as positive transgenic plants.

Figure legends

**Fig. 1.** Genetic linkage maps of barley chromosomes 2H and 3H and position of the *TFA1, TFA2* and
TF A3 loci, and putative positions of BBM and WUS2 genes.

The genetic linkage map was constructed using AntMap (Iwata and Ninomiya 2006) using SNP genotyping data of transgenic HN×GP barley plants. The regions showing significant segregation distortion are marked by color bars. Significant segregation distortion was identified using chi-square tests for GP:hetero:HN=1:2:1 (df=2, green), GP:[hetero+HN]=1:3 (df=1, orange) and [GP+hetero]:HN=3:1 (df=1, blue). Significance levels are represented by line thickness (thin, p<0.05; middle, p<0.01; thick, p<0.001). A red square shows the expanded region (between 8293-202 and FLOUba102a14_00001505_2H) of TFAs found by the Infinium HD assay of HN×GP plants described in this report. The BBM and WUS2 genes were plotted by in silico mapping.

**Fig. 2.** Graphical genotypes of Oregon Promise doubled haploid barley lines used in this study.

Three lines, DH120366, DH120536 and DH120543, were selected based on allele composition at the Transformation amenability (TFA) for evaluation of Agrobacterium-mediated transformation amenability. The green thin lines show the boundaries of the TFAs (as estimated by significant segregation distortion) and the thick green lines show the peaks of TFAs (as estimated by maximum segregation distortion).

**Fig. 3.** Stable expression of EGFP in doubled haploid barley lines.

(A)(B) Hygromycin-resistant callus of DH120543, regeneration from callus of (C) DH120543 and of (D) DH120366, (E)(F) magnified pictures of regeneration shoot from callus of DH120366, (G)(H) root-developed regeneration plants of DH120543 (left) and DH120366 (right). (A)(C)(D)(E)(G) were photographed under white light, and (B)(F)(H) were photographed under blue light with an orange-colored filter.

**Fig. 4.** PCR analysis of transgenic plants.

PCR was performed for detecting transgenes, i.e. hygromycin phosphotransferase (HPT, 375 bp) and enhanced green fluorescent protein (EGFP, 311 bp) genes in regenerated plants and non-transgenic plants (DH120366 and DH120543) as negative controls. TP-DH120366-EGFP and TP-DH120543-EGFP were representative transgenic plants derived from DH120366 and DH120543, respectively. Marker; 100 bp ladder marker, Plasmid; pBUH3-EGFP as a positive control template, TP-GP-EGFP; transgenic plant carrying pBUH3-EGFP derived from ‘Golden Promise’ for another positive control.
Supplemental information

Figure S1. Distortion analysis of markers in HN×GP transgenic barley.

Figure S2. A schematic map of pBUH3-EGFP.

Table S1. Information of markers used for constructing map of Oregon Promise

Table S2. Information of markers used for tHN×GP analysis
Fig. 2.
Fig. 4.