Title:

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

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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 F_2 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

63 complementation analysis when the transformed cultivar has a non-functional allele at the target gene.
64 For example, Deng et al. (2015) studied the gene cascades related to a vernalization gene (*VRN1*)
65 encoding a transcription factor using 'Golden Promise' as a model. However, 'Golden Promise' does
66 not require vernalization and is photoperiod insensitive. Therefore, wild type 'Golden Promise' was
67 unsuitable for the transcriptome sequencing (RNA-seq) and chromatin immunoprecipitation
68 sequencing (ChIP-seq) to identify direct targets of *VRN1*. To produce a vernalization-requiring
69 derivative of 'Golden Promise', full-length versions of *VRN1* and *VRN2*, plus a functional copy of
70 *PHOTOPERIOD1* were introgressed into 'Golden Promise' through three rounds of recurrent crossing
71 with marker assisted selection. As another example, Sato et al. (2016) developed a backcross-derived
72 line in the cv. 'Golden Promise' background with substitutions of target segments carrying the *Qsd1*
73 gene for seed dormancy and used this substitution line for transformation experiments including
74 complementation and RNAi. In both examples, substantial time and resources were required to
75 develop the necessary genetic stocks prior to transformation. It is not prudent to directly attempt
76 transformation of target genotypes without knowing their degree of transformability.
77 We recently reported a significant technical advancement for *Agrobacterium*-mediated
78 transformation efficiency in barley using progeny from the cross between cvs. 'Haruna Nijo', which
79 is recalcitrant for transformation, and 'Golden Promise' (Hisano and Sato 2016). By isolating 3,013
80 F_2 generation immature embryos and subsequent infection with *Agrobacterium*, we generated 60
81 independently transformed plants and genotyped them using genome-wide SNP markers. By mapping
82 regions distorted in favor of 'Golden Promise' alleles in these plants, we identified three major
83 transformation amenability (*TFA*) loci. In the same report we describe the use of progeny from the
84 cross of 'Morex' (recalcitrant) × 'Golden Promise'. Based on these results we proposed that
85 transformation could be used in any barley genotype into which favorable *TFA* alleles from 'Golden
86 Promise' were introgressed. To validate this *TFA*-based pre-selection strategy for candidate line
87 transformation, assessment of *TFA* alleles from 'Golden Promise' in other genetic backgrounds is
88 necessary.
89 Here we report validation of the effects of 'Golden Promise' *TFA* alleles using 200 doubled
90 haploid progeny from the cross of 'Golden Promise' × 'Full Pint' (the Oregon Promise mapping
91 population). This population was developed with the principal goal of assessing the contributions of
92 barley genotype to beer flavor. Those results will be reported elsewhere, as will QTLs and genes
93 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 F_2 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).

125 **Construction of linkage maps**

126 A genetic linkage map was constructed using genotyping data generated by SNP markers in tHN×GP
127 population. Grouping and ordering of markers was performed using the default settings of AntMap
128 (Iwata and Ninomiya, 2006) and the Kosambi mapping function. For the Oregon Promise population,
129 a framework genetic map was initially developed with the BeadExpress SNP markers using JoinMap
130 v4, which integrated 168 markers into nine linkage groups with chromosomes 1H and 6H fragmented
131 into two linkage groups. Addition of KASP markers generated a final genetic map with 251 markers,
132 of which 206 are non-redundant, with a genetic distance of 1,311 cM, using the Kosambi function,
133 over eight linkage groups (Supplemental Table S1). The majority of intervals between markers are
134 below 20 cM, with only four regions on chromosomes 3H, 6H, and 7H having regions above 20 cM.
135 Despite substantial effort, markers could not be developed to bridge the two linkage groups of
136 chromosome 1H. Collinearity was observed for all markers relative to the consensus genetic map of
137 barley (Close et al. 2009).
138
139 ***In silico*** **selection of Oregon Promise doubled haploids for transformability, based on *TFA***
140 **haplotype**

141 Based on markers in common between the tHN×GP and Oregon Promise populations, three Oregon
142 Promise lines, DH120366, DH120536, and DH120543 were selected for the favorable allele haplotype
143 at three major *TFA* loci: *TFA1*, *TFA2* and *TFA3*, reported by Hisano and Sato (2016). In DH120536
144 there were 'Full Pint' alleles at some of the marker loci defining the target haplotypes at *TFA2* and
145 *TFA3*.
146
147 **Construction of the binary vector pBUH3-EGFP carrying the *EGFP* gene**

148 The ORF fragment of the *enhanced green gluorescent protein* (*EGFP*) gene (Clontech laboratories,
149 USA) was amplified by PCR using PrimeSTAR Max DNA Polymerase (Takara, Japan) with the
150 specific primer pair (EGFP-F1-SacI, 5'-GAGCTCACCATGGTGAGCAAGGGCGAGGAG-3' and
151 EGFP-R1-BamHI, 5'-GGATCCTTACTTGTACAGCTCGTCCATGCC-3'). The program involved:
152 an initial denaturation step at 98°C for 2 min, 30 cycles of denaturation step at 95°C for 10 sec,
153 annealing step at 55°C for 20 sec and extension step at 72 °C for 1 min, and a final extension at 72°C
154 for 10 min. The PCR fragment of *EGFP* gene and pBUH3 (Nigorikawa et al. 2012) was digested with

the restriction enzymes *Sac*I and *Bam*HI and ligated using the Quick ligation kit (New England Biolabs, USA).

Agrobacterium-mediated transformation of selected Oregon Promise doubled haploids and EGFP detection

The procedure for *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 *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 *hygromycin phosphotransferase* (*HPT*) and *EGFP* genes (EGFP-F1; 5'-gacgacggcaactacaagac-3' and EGFP-R2; 5'-gactgggtgctcaggtagtg-3').

Identification and *in silico* mapping of the barley *BBM* and *WUS2* genes

Barley *BBM* and *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 - *BBM* (CS155772) and *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).

Results

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 _TFA_s 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,

217 respectively, were the highest BLAST hits. According to the barley consensus map and genome (IBSC
218 2012), the barley *BBM* gene is at cM 67.35 on chromosome 2H, and the barley *WUS2* gene is at cM
219 90.23 on chromosome 3H. The nearest SNP markers were 8889-842 (cM 50.06 on 2H in the tHN×GP
220 linkage map; between 2580-1456 and 2809-271) for the barley *BBM* gene and 8020-87 (cM 83.16 on
221 3H) for the barley *WUS2* gene (supplemental Table S2). The putative positions are plotted in Fig. 1.
222 In DH120536, the *BBM* gene is located in the region lacking the GP-allele at *TFA2*.
223
224 **Transformation of selected Oregon Promise DH lines**
225 Immature embryos of the three selected lines were used for infection by *Agrobacterium* carrying the
226 pBUH3-EGFP vector (Supplemental Figure S2) with four to six replications. All the lines showed
227 transient EGFP expression in co-cultivated immature embryos at 10 days after infection. The
228 efficiency of transformation of these Oregon Promise selections and their parents is summarized in
229 Table 1. During selection by hygromycin, EGFP expression was observed in the resistant callus of
230 DH120366 and DH120543. However, only one tiny clump of resistant callus was observed for
231 DH120536 and it did not show green fluorescence. EGFP expression in resistant callus of DH120543
232 is shown in Fig 3. Regeneration of green shoots and development of roots were observed from resistant
233 calli for both DH120366 and DH120543 (Fig. 3). 'Full Pint' did not show any resistant green shoots,
234 nor did DH120536. From DH120366 and DH120543, respectively, 53 and 15 independent transgenic
235 barley plants were generated from 252 and 193 immature embryos. Transformation efficiencies were
236 23.7% (53/224) and 15.5% (15/97), respectively. For this calculation, we omitted the number of calli
237 which died during *Agrobacterium* infection as we consider this a technical error caused by physical
238 damage to explants. Transgenes were detected by PCR using specific primers for *HTP* and *EGFP*
239 genes in all the regenerated plants and a representative result is shown in Fig. 4.
240
241 **Discussion**
242
243 **Validating *TFAs* using the tHNxGP and Oregon Promise populations**
244 Hisano and Sato (2016) identified three putative transformation amenability (*TFA*) loci, with large
245 effects, in 60 transformed F_2 plants from the cross of 'Haruna Nijo' and 'Golden Promise' (tHN×GP)
246 based on genotyping with 124 SNPs derived from a 384 barley oligonucleotide pooled assay (BOPA,
247 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_3F_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 *TFA*s

(*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*/*TFA*3 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.

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Compliance with ethical standards

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

Reference

Anand A, Krichevsky A, Schornack S, Lahaye T, Tzfira T, Tang Y, Citovsky V, Mysore KS (2007) *Arabidopsis* VIRE2 INTERACTING PROTEIN2 is required for *Agrobacterium* T-DNA integration in Plants. Plant Cell 19:1695-1708

Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. In Vitro Cell Dev Biol Plant 40:31-45

Cistué L, Ramos A, Castillo AM, Romagosa I (1994) Production of large number of doubled haploid plants from barley anthers pretreated with high concentrations of mannitol. Plant Cell Reports 13:709-712

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao S, Varshney RK, Szűcs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A and Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics, 10:1-13

Cogan NOI, Newbury HJ, Oldacres AM, Lynn JR, Kearsey MJ, King GJ, Puddephat, IJ (2004) Identification and characterization of QTL controlling Agrobacterium-mediated transient and stable transformation of *Brassica oleracea*. Plant Biotechnol J 2:59-69.

Comadran J, Kilian B, Russell J, Ramsay L, Stein N, Ganal M, Shaw P, Bayer M, Thomas W, Marshall D, Hedley P, Tondelli A, Pecchioni N, Francia E, Korzun V, Walther A and Waugh R (2012) Natural variation in a homolog of *Antirrhinum CENTRORADIALIS* contributed to spring growth habit and

372 environmental adaptation in cultivated barley. Nature Genetics, 44:1388-1392.
373
374 Deng W, Casao MC, Wang P, Sato K, Hayes PM, Finnegan EJ, Trevaskis B (2015) Direct links
375 between the vernalization response and other key traits of cereal crops. Nat Commun 6:5882
376
377 Echávarri B, Cistué L (2016) Enhancement in androgenesis efficiency in barley (Hordeum vulgare L.)
378 and bread wheat (Triticum aestivum L.) by the addition of dimethyl sulfoxide to the mannitol
379 pretreatment medium. Plant Cell Tiss Organ Cult 125:11-22
380
381 Harwood WA (2012) Advances and remaining challenges in the transformation of barley and wheat. J
382 Exp Bot 63:1791-1798
383
384 Hensel G, Valkov V, Middlefell-Williams J, Kumlehn, J (2008) Efficient generation of transgenic
385 barley: The way forward to modulate plant–microbe interactions. J Plant Physiol 165:71-82
386
387 Hisano H, Sato K (2016) Genomic regions responsible for amenability to Agrobacterium-mediated
388 transformation in barley. Sci Rep 6:37505
389
390 Ikeuchi M, Sugimoto K, Iwase A (2013) Plant callus: mechanisms of induction and repression. The
391 Plant Cell 25:3159-3173
392
393 Ikeuchi M, Ogawa Y, Iwase A, Sugimoto K (2016) Plant regeneration: cellular origins and molecular
394 mechanisms. Development 143:1442-1451
395
396 Iwata H, Ninomiya S (2006) AntMap: Constructing genetic linkage maps using an ant colony
397 optimization algorithm. Breed Sci 56:371-377
398
399 Kumlehn J, Serazetdinova L, Hensel G, Becker D, Loerz H (2006) Genetic transformation of barley
400 (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*.
401 Plant Biotechnol. J 4:251-261
402

403 Lowe K, Wu E, Wang N, Hoerster G, Hastings C, Cho M-J, Scelonge C, Lenderts B, Chamberlin M,
404 Cushatt J, Wang L, Ryan L, Khan T, Chow-Yiu J, Hua W, Yu M, Banh J, Bao Z, Brink K, Igo E,
405 Rudrappa B, Shamseer PM, Bruce W, Newman L, Shen B, Zheng P, Bidney D, Falco SC, RegisterIII
406 JC, Zhao Z-Y, Xu D, Jones TJ, Gordon-Kamm WJ (2016) Morphogenic regulators *Baby boom* and
407 *Wuschel* improve monocot transformation. Plant Cell 28:1998-2015
408
409 Lü B, Wu J-J, Fu D-l (2015) Constructing the barley model for genetic transformation in Triticeae.
410 Journal of Integrative Agriculture 14:453-468
411
412 Mrízová K, Holasková E, Öz MT, Jiskrová E, Frébort I, Galuszka P (2014) Transgenic barley: A
413 prospective tool for biotechnology and agriculture. Biotechnol Adv 32:137-157
414
415 Nam J, Matthysse AG, Gelvin SB (1997) Differences in susceptibility of *Arabidopsis* ecotypes to
416 crown gall disease may result from a deficiency in T-DNA integration. Plant Cell 9:317-333.
417
418 Nigorikawa M, Watanabe A, Furukawa K, Sonoki T, Ito Y (2012) Enhanced saccharification of rice
419 straw by overexpression of rice exo-glucanase. Rice 5:14
420
421 Nishimura A, Aichi I, Matsuoka M (2007) A protocol for Agrobacterium-mediated transformation in
422 rice. Nat Protocols 1:2796-2802
423
424 Sato K, Yamane M, Yamaji N, Kanamori H, Tagiri A, Schwerdt JG, Fincher GB, Matsumoto T, Takeda
425 K, Komatsuda T (2016) Alanine aminotransferase controls seed dormancy in barley. Nat Commun
426 7:11625
427
428 Sparrow PAC, Townsend TM, Arthur A., Dale PJ, Irwin JA (2004) Genetic analysis of *Agrobacterium*
429 *tumefaciens* susceptibility in *Brassica oleracea*. Theor Appl Genet 108:644-650
430
431 The International Barley Sequencing Consortium (IBSC) (2012) A physical, genetic and functional
432 sequence assembly of the barley genome. Nature 491:711-716
433

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R. (1997) *Agrobacterium tumefaciens*-mediated barley transformation. Plant J 11:1369-1376

Yeo FKS, Hensel G, Vozábová T, Martin-Sanz A, Marcel TC, Kumlehn J, Niks RE (2014) Golden SusPtrit: a genetically well transformable barley line for studies on the resistance to rust fungi. Theor Appl Genet 127:325-337

Zhang X, Henriques R, Lin S-S, Niu Q-W, Chua N-H (2006) Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nat Protocols 1:641-646

Tables

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

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

	2	23	20	1	0	0.0
	total	**54**	**38**	**1**	**0**	0.0
DH120366	1	16	16	2	0	0.0
	2	45	44	11	0	0.0
	3	30	24	7	3	12.5
	4	39	32	16	12	37.5
	5	95	93	54	38	40.9
	6	27	15	9	0	0.0
	total	**252**	**224**	**99**	**53**	*** 23.7**
DH120536	1	30	19	0	0	0.0
	2	8	5	0	0	0.0
	3	25	13	1	0	0.0
	4	41	26	0	0	0.0
	total	**104**	**63**	**1**	**0**	**0.0**
DH120543	1	62	44	17	6	13.6
	2	24	4	1	0	0.0
	3	31	21	7	7	33.3
	4	27	22	12	1	4.5
	5	49	6	1	1	16.7
	total	**193**	**97**	**38**	**15**	*** 15.5**

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

TFA3 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 FLOUbaf102a14_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