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1 **Title:**

2 **Selection of transformation efficient lines in the Oregon Promise barley mapping population based on**
3 ***TFA* (transformation amenability) haplotype**

4

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20

21 **Abstract**

22 Barley (*Hordeum vulgare*) cv. 'Golden Promise' is one of the most usefuland well-studied cultivars for
23 genetic manipulation. In a previous report, we identified several transformation amenability (*TFA*) loci
24 responsible for *Agrobacterium*-mediated transformation by using F_2 immature embryos, derived from
25 'Haruna Nijo' × Golden Promise, as explants. In this report, we describe higher density mapping of these
26 *TFA* regions with additional SNP markers using the same F_2 individuals. To demonstrate the robustness of
27 transformability alleles at the *TFA* loci, we genotyped 200 doubled haploid progeny from the cross Golden
28 Promise × 'Full Pint'. Based on SNP genotype, we selected lines having Golden Promise alleles at *TFA* loci
29 and used them for transformation. Of the successfully transformed lines, DH120366 came the closest to
30 achieving a level of transformation efficiency comparable to Golden Promise. The results validate that the
31 genetic substitution of *TFA* alleles from Golden Promise can facilitate the development of transformation
32 efficient lines from recalcitrant barley cultivars.

33

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

35 polymorphism, transformation

36

37 **Key message**

38 The genetic substitution of transformation amenability alleles from Golden Promise can facilitate the
39 development of transformation efficient lines from recalcitrant barley cultivars.

40

41 **Introduction**

42 The success of genetic transformation in plants is strongly genotype-dependent. For example, the Columbia
43 ecotype in *Arabidopsis thaliana* and cvs. 'Nipponbare' or 'Taichung 65' (T-65) in rice (*Oryza sativa*) are the
44 most capable genotypes at producing transgenic plants (Zhang et al. 2006; Nishimura et al. 2006). There is a
45 genetic component to transformation efficiency in plants, including attributes such as the ratio of
46 T-DNA/transgene integration, callus differentiation, and green shoot regeneration (Nam et al. 1997; Cogan et
47 al. 2004; Sparrow et al. 2004). There may be interactions between genetic factors (cultivars) and
48 experimental conditions, e.g. components of medium, temperature during tissue culture, and type of explants.
49 If the genes responding to these conditions are isolated and manipulated, more cultivars and/or breeding lines
50 would be amenable to genetic transformation.

51 Barley (*Hordeum vulgare*) transformation is currently possible with both biolistic and
52 *Agrobacterium*-mediated methods. After Tingay et al. (1997) developed the *Agrobacterium*-mediated method
53 for barley transformation, 'Golden Promise' has been used for the functional analysis of several genes of
54 scientific and economic interests (reviewed in Mrízová et al. 2014). Despite massive attempts at technical
55 improvements, barley transformation is still performed with a limited number of cultivars e.g. Golden
56 Promise and 'Igri' (Kumlehn et al. 2006; Hensel et al. 2008; Harwood 2012). These genotype-dependent
57 transformation systems limit the opportunities for complementation analysis when the transformed cultivar
58 has a non-functional allele at the target gene. For example, Deng et al. (2015) studied the gene cascades
59 related to a vernalization gene (*VRN1*) encoding a transcription factor using Golden Promise as a model.
60 However, Golden Promise does not require vernalization and is photoperiod insensitive. Therefore, wild type
61 Golden Promise was unsuitable for the transcriptome sequencing (RNA-seq) and chromatin
62 immunoprecipitation sequencing (ChIP-seq) to identify direct targets of *VRN1*. To produce a
63 vernalization-requiring Golden Promise, full-length versions of *VRN1* and *VRN2*, plus a functional copy of
64 *PHOTOPERIOD1* were introgressed into Golden Promise through three rounds of recurrent crossing with
65 marker assisted selection. As another example, Sato et al. (2016) developed a backcross-derived line in the cv.
66 Golden Promise background with substitutions of target segments carrying the *Qsd1* gene for seed dormancy
67 and used this substitution line for transformation experiments including complementation and RNAi. In both
68 examples, substantial time and resources were required to develop the necessary genetic stocks prior to

69 transformation. It is not prudent to directly attempt transformation of target genotypes without knowing their
70 degree of transformability.

71 We recently reported a significant technical advancement for *Agrobacterium*-mediated
72 transformation efficiency in barley using progeny from the cross between cvs. 'Haruna Nijo', which is
73 recalcitrant for transformation, and Golden Promise (Hisano and Sato, in press). Through isolating immature
74 embryos from 3,013 F_2 individuals and subsequent infection with *Agrobacterium*, we generated 60
75 independently transformed plants and genotyped them using genome-wide SNP markers. By mapping
76 regions distorted in favor of Golden Promise alleles in these plants, we identified three major transformation
77 amenability (*TFA*) loci. In the same report we describe the use of progeny from the cross of 'Morex'
78 (recalcitrant) × Golden Promise. Based on these results we proposed that transformation could be used in any
79 barley genotype into which favorable *TFA* alleles from Golden Promise were introgressed. To validate this
80 *TFA*-based pre-selection strategy for candidate line transformation, assessment of *TFA* alleles from Golden
81 Promise in other genetic backgrounds is necessary.

82 Here we report validation of the effects of Golden Promise *TFA* alleles using 200 doubled haploid
83 progeny from the cross of Golden Promise × 'Full Pint' (the Oregon Promise mapping population). This
84 population was developed with the principal goal of assessing the contributions of barley genotype to beer
85 flavor. Those results will be reported elsewhere, as will QTLs and genes associated with agronomic and
86 disease resistance traits. Of immediate applicability to *TFA* allele validation, Full Pint is recalcitrant to
87 *Agrobacterium*-mediated transformation, and the Oregon Promise population has been extensively genotyped
88 for QTL mapping, allowing for the inspection of haplotypes and selection of target *TFA* alleles. In addition,
89 we added markers to the Haruna Nijo × Golden Promise map in order to better delimit the *TFA* QTLs and
90 facilitate map alignment with anchor markers.
91
92 **Materials and Methods**
93
94 **Plant materials**

95 Sixty transgenic plants (tHN×GP) previously generated using F_2 immature embryos from the cross of Haruna
96 Nijo × Golden Promise (Hisano and Sato, in press) were used for higher resolution mapping by genotyping
97 with SNP markers. The 202 doubled haploids comprising the Oregon Promise doubled haploid population
98 were developed using anther culture, following the protocols described by Cistué et al. (1994) and Echávarri
99 and Cistué (2016). The Oregon Promise population was developed jointly by the Cistue lab at the Dept.
100 Genetica y Produccion Vegetal, Estacion Experimental de Aula Dei, Zaragoza, Spain and the Hayes lab in the
101 Department of Crop and Soil Science, Oregon State University, Corvallis, Oregon, USA.
102

103 **Genotyping**

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

111

112 **Construction of linkage maps**

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

124

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

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

130

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

132 The ORF fragment of the *enhanced green gluorescent protein* (*EGFP*) gene (Clontech laboratories, USA)
133 was amplified by PCR using PrimeSTAR Max DNA Polymerase (Takara, Japan) with the specific primer
134 pair (EGFP-F1-SacI, 5'-GAGCTCACCATGGTGAGCAAGGGCGAGGAG-3' and EGFP-R1-BamHI,
135 5'-GGATCCTTACTTGTACAGCTCGTCCATGCC-3'). The program involved: an initial denaturation step
136 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
137 extension step at 72 °C for 1 min, and a final extension at 72°C for 10 min. The PCR fragment of *EGFP* gene

138 and pBUH3 (Nigorikawa et al. 2012) was digested with the restriction enzymes *Sac*I and *Bam*HI and ligated
139 using the Quick ligation kit (New England Biolabs, USA).
140

141 **_Agrobacterium_-mediated transformation of selected Oregon Promise doubled haploids and EGFP**
142 **detection**

143 The procedure for *Agrobacterium*-mediated transformation method was identical to that reported by Hisano
144 and Sato (in press). Briefly, the protocol was follows: barley immature embryos were infected with
145 *Agrobacterium tumefaciens* strain AGL1 carrying pBUH3-EGFP and co-cultivated for 3 days. Surviving
146 immature embryos were then (1) incubated on callus-induction medium without selection for a week and (2)
147 selected on selection-medium containing hygromycin under dark condition for 4 weeks. Calli resistant to
148 hygromycin were transferred onto regeneration-medium and incubated under a 16 h light/8 h dark
149 photoperiod regime. Regenerated shoots were transferred to rooting-medium. The expression of EGFP was
150 detected using LED blue light and an Orange Filter for GFP (Optocode, Japan). For confirming transgene
151 presence, touch down PCR was performed by the method described in Hisano and Sato (in press) with
152 specific primers for the *hygromycin phosphotransferase* (*HPT*) and *EGFP* genes (EGFP-F1;
153 5'-gacgacggcaactacaagac-3' and EGFP-R2; 5'-gactgggtgctcaggtagtg-3').
154

155 **Identification and _in silico_ mapping of the barley _BBM_ and _WUS2_ genes**

156 Barley *BBM* and *WUS2* genes were identified by searching the barley full-length cDNAs at the IPK Barley
157 BLAST Server (http://webblast.ipk-gatersleben.de/barley/) using the maize homologues - *BBM*
158 (CS155772) and *WUS2* (EA275154) - as query sequences. The linkage map positions of these genes, and the
159 nearest SNP markers, were estimated using the barley genome sequence (IBSC 2012).
160
161

162 **Results**
163

164 **Genotyping of transgenic tHN×GP plants and reconfirmation of the _TFA_ regions**

165 The 10K-SNP Illumina Infinium HD assay of the population of 60 transgenic HN×GP barley plants
166 (tHN×GP) resulted in 1,131 SNP markers defining the haplotype of these plants. Among these SNP markers,
167 124 were previously used (Hisano and Sato, in press) and 1,007 were newly developed in the present study
168 for tHN×GP. Using these genotype data, a genetic linkage map consisting of 1,131 SNPs was constructed for
169 the tHN×GP population. The Kosambi cM distances between these makers are shown in supplemental Table
170 S1, along with the Chi-square values calculated for 1:2:1 and 3:1 ratios at each marker locus. The Chi-square
171 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, in press). 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. 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 (in press) 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. 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

206 EGFP expression in co-cultivated immature embryos at 10 days after infection. The efficiency of
207 transformation of these Oregon Promise selections and their parents is summarized in Table 1. During
208 selection by hygromycin, EGFP expression was observed in the resistant callus of DH120366 and DH120543.
209 However, only one tiny clump of resistant callus was observed for DH120536 and it did not show green
210 fluorescence. EGFP expression in resistant callus of DH120543 is shown in Fig 3. Regeneration of green
211 shoots and development of roots were observed from resistant calli for both DH120366 and DH120543 (Fig.
212 3). Full Pint did not show any resistant green shoots, nor did DH120543. From DH120366 and DH120543,
213 respectively, 53 and 15 independent transgenic barley plants were generated from 252 and 193 immature
214 embryos. Transformation efficiencies were 23.7% (53/224) and 15.5% (15/97), respectively. For this
215 calculation, we omitted the number of calli which died during *Agrobacterium* infection as we consider this a
216 technical error caused by physical damage to explants. Transgenes were detected by PCR using specific
217 primers for *HTP* and *EGFP* genes in all the regenerated plants and a representative result is shown in Fig. 4.
218
219 **Discussion**
220
221 **Validating *TFAs* using the tHNxGP and Oregon Promise populations**
222 Hisano and Sato (in press) identified three putative transformation amenability (*TFA*) loci, with large effects,
223 in 60 transformed F_2 plants from the cross of Haruna Nijo and Golden Promise (tHN×GP) based on
224 genotyping with 124 SNPs derived from a 384 barley oligonucleotide pooled assay (BOPA, Close et al.
225 2009). For this report, we performed an Infinium HD assay to develop additional markers for the tHN×GP
226 population with the goal of increasing marker density and the number of markers in common with the
227 Oregon Promise population. The Oregon Promise population was genotyped with an array of markers,
228 including BOPA-derived SNPs, allowing for direct map alignment with tHN×GP. We placed the *TFA* loci on
229 the Oregon Promise linkage map using markers in common between the two populations and/or the position
230 information of BOPA markers in the consensus barley map (IBGS 2012). Higher marker density was
231 achieved by the addition of new SNP markers to the tHN×GP population and this provided greater resolution
232 of *TFA3*. In the near future, the *TFA* loci will be targets for map-based cloning, requiring even higher marker
233 resolution. Maximum population size is required for high resolution mapping. However, because producing
234 large numbers of transformed plants may not be feasible, identification of transformation-inefficient
235 segments on a high resolution map may be an alternative strategy for narrowing down each *TFA* QTL to a
236 candidate gene.
237
238 **The identification of transformation-efficient lines in the Oregon Promise population**
239 In prior research, we developed only two transformed plants from a cross of Morex × Golden Promise

240 (Hisano and Sato, in press). We also tried to select transformation amenable lines from BC_3F_8 recombinant
241 chromosome substitution lines derived from the cross of Golden Promise × Haruna Nijo (with Haruna Nijo
242 as the recurrent parent) but did not obtain any transgenic plants from 4,661 immature embryos. In the present
243 study, the Oregon Promise doubled haploids were useful in validating the effects of favorable alleles at the
244 *TFA* loci and in showing that transformation-efficient lines can be developed in the
245 transformation-recalcitrant background of cv. Full Pint. These findings may encourage barley researchers to
246 introgress *TFA* alleles from Golden Promise into any genotype to transform target genes.

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

262

263 **Importance of *TFA2* and *TFA3***

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

269 To obtain transgenic plants with *Agrobacterium*-mediated transformation, essential factors include
270 (i) *Agrobacterium*-plant cell interaction including T-DNA integration, (ii) activity of propagation of callus
271 during selection, and (iii) regeneration from callus (Cheng et al. 2004). Recently these factors were defined at
272 the molecular levels in certain dicot plants. Anand et al. (2007) reported that a host gene, *VIP2*, was essential
273 for *Agrobacterium*-mediated transformation and was involved in T-DNA integration into the host genome.

274 Ikeuchi et al. (2013, 2016) reviewed the genes related to callus induction and regeneration in *Arabidopsis* and
275 related species and reported that several hormone-related genes and/or their transcription factors - such as
276 *baby boom* (*BBM*) and *Wuschel2* (*WUS2*) - were important for callus differentiation. More recently Lowe et
277 al. (2016) reported that co-overexpression of the maize *BBM* and maize *WUS2* genes improved efficiency of
278 transformation in monocot plants including maize, sorghum, sugarcane, and rice. These genes could
279 stimulate proliferation of transgenic callus resulting also improving regeneration efficiency.

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

289

290 **Conclusion**

291 Here we demonstrated the *TFA*-based selection method for transformation amenability using doubled haploid
292 barley lines derived from a cross between cvs. Golden Promise and Full Pint. Transgenic plants were
293 generated from two selected lines that have complete Golden Promise allele sets at *TFA1*, *TFA2,* and *TFA3*.
294 No transgenic plants were generated from a line that had the Golden Promise allele at *TFA1,* but a portion of
295 Full Pint alleles at *TFA2* and *TFA3*. These results suggested that our *TFA*-based method was efficient for
296 selecting donor plants for transformation experiments, e.g. complementation test, and that *TFA2*/*TFA*3 was
297 necessary for successful transformation as well as the most major loci, *TFA1*.

298

299

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

303

304

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317
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424 **Tables**

425

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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.

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444 **Figure legends**
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446 **Fig. 1.** Genetic linkage maps of barley chromosomes 2H and 3H and position of the *TFA1*, *TFA2* and *TFA3*
447 *loci*.
448 The genetic linkage map was constructed using AntMap (Iwata and Ninomiya 2006) using SNP genotyping
449 data of transgenic HN×GP barley plants. The regions showing significant segregation distortion are marked
450 by color bars. Significant segregation distortion was identified using chi-square tests for GP:hetero:HN=1:2:1
451 (df=2, green), GP:[hetero+HN]=1:3 (df=1, orange) and [GP+hetero]:HN=3:1 (df=1, blue). Significance
452 levels are represented by line thickness (thin, $p<0.05$; middle, $p<0.01$; thick, $p<0.001$). A red square shows
453 the expanded region (between 8293-202 and FLOUbaf102a14_00001505_2H) of *TFAs* found by the Infinium
454 HD assay of HN×GP plants described in this report.
455
456 **Fig. 2.** Graphical genotypes of Oregon Promise doubled haploid barley lines used in this study.
457 Three lines, DH120366, DH120536 and DH120543, were selected based on allele composition at the
458 *Transformation amenability (TFA)* for evaluation of *Agrobacterium*-mediated transformation amenability.
459 The green thin lines show the boundaries of the *TFAs* (as estimated by significant segregation distortion) and
460 the thick green lines show the peaks of *TFAs* (as estimated by maximum segregation distortion).
461
462 **Fig. 3.** Stable expression of EGFP in doubled haploid barley lines.
463 (A)(B) Hygromycin-resistant callus of DH120543, regeneration from callus of (C) DH120543 and of (D)
464 DH120366, (E)(F) magnified pictures of regeneration shoot from callus of DH120366, (G)(H)
465 root-developed regeneration plants of DH120543 (left) and DH120366 (right). (A)(C)(D)(E)(G) were
466 photographed under white light, and (B)(F)(H) were photographed under blue light with an orange-colored
467 filter.

468

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

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

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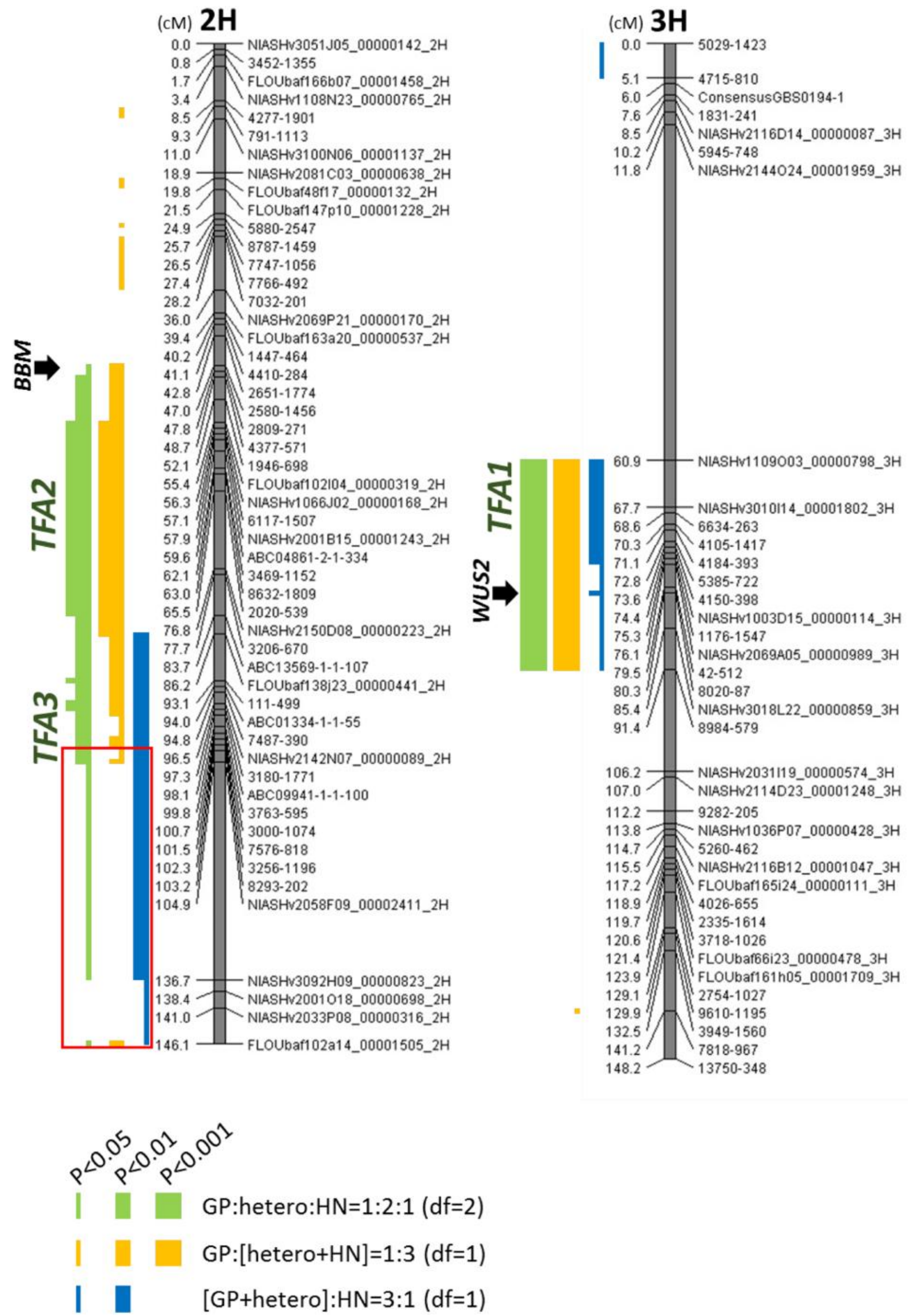

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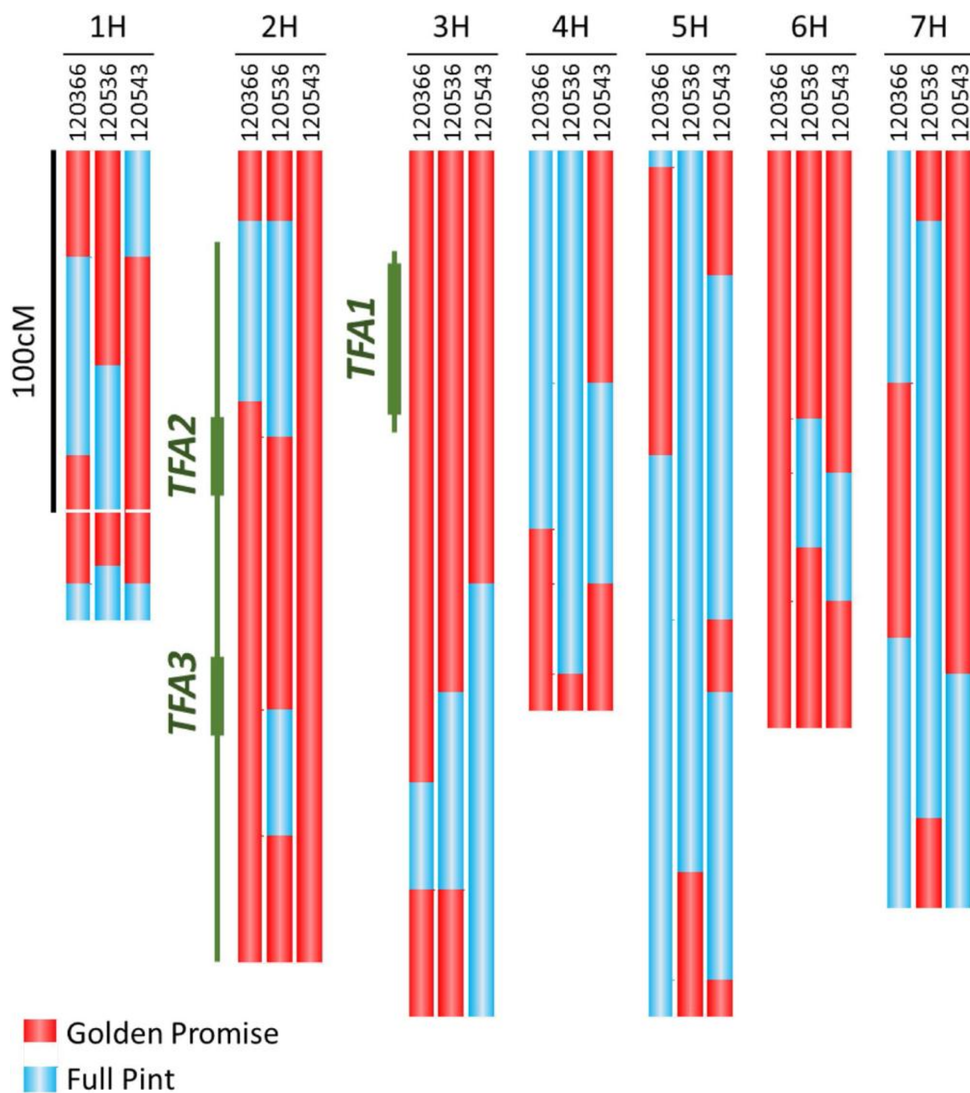

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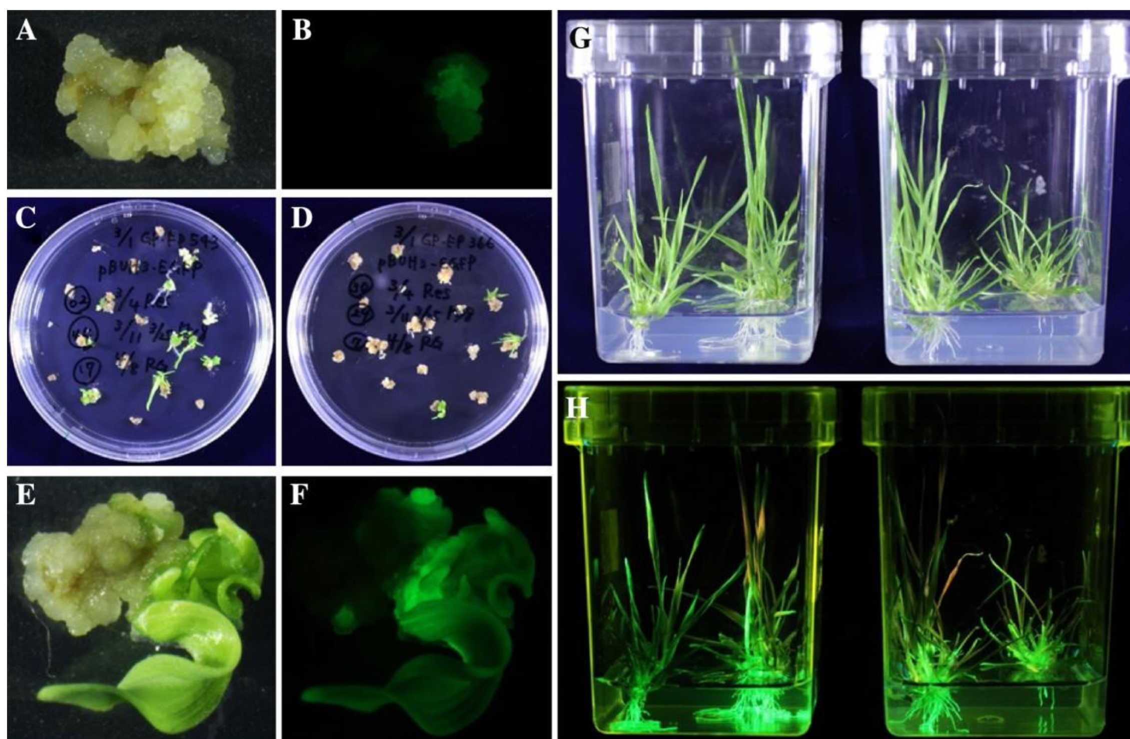
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507 **Supplemental information** (only available in the Journal Website)

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

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511 **Figure S2.** A schematic map of pBUH3-EGFP.

512

513 **Table S1.** Information of markers used for tHN×GP analysis