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ORIGINAL PAPER

Analysis of the barley bract suppression gene *Trd1*

Kelly Houston · Arnis Druka · Nicky Bonar · Malcolm Macaulay · Udda Lundqvist · Jerome Franckowiak · Michele Morgante · Nils Stein · Robbie Waugh

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Abstract A typical barley (*Hordeum vulgare*) floret consists of reproductive organs three stamens and a pistil, and non-reproductive organs—lodicules and two floral bracts, abaxial called 'lemma' and adaxial 'palea'. The floret is subtended by two additional bracts called outer or empty glumes. Together these organs form the basic structural unit of the grass inflorescence, a spikelet. There are commonly three spikelets at each rachis (floral stem of the barley spike) node, one central and two lateral spikelets. Rare naturally occurring or induced phenotypic variants that contain a third bract subtending the central spikelets have been described in barley. The gene responsible for this phenotype was called the *THIRD OUTER GLUME1* (*Trd1*). The *Trd1* mutants fail to suppress bract growth and as a result produce leaf-like structures that subtend each rachis node in the basal portion of the spike. Also, floral development at the collar is not always suppressed. In rice and maize, recessive mutations in *NECK LEAF1* (*Nl1*) and *TASSEL SHEATH1* (*Tsh1*) genes, respectively, have been shown to be responsible for orthologous phenotypes. Fine mapping of the *trd1* phenotype in an F_3 recombinant population enabled us to position *Trd1* on the long arm of chromosome 1H to a 10 cM region. We anchored this to a conserved syntenic region on rice chromosome Os05 and selected a set of candidate genes for validation by resequencing PCR amplicons from a series of independent mutant alleles. This analysis revealed that a GATA transcription factor, recently proposed to be *Trd1*, contained mutations in 10 out of 14 independent *trd1* mutant alleles that would generate non-functional TRD1 proteins. Together with genetic linkage data, we confirm the identity of *Trd1* as the GATA transcription factor ortholog of rice *Nl1* and maize *Tsh1* genes.

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K. Houston · A. Druka · N. Bonar · M. Macaulay · R. Waugh (✉)
The James Hutton Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK
e-mail: Robbie.Waugh@hutton.ac.uk

K. Houston
e-mail: kelly.houston@hutton.ac.uk

U. Lundqvist
Nordic Genetic Resource Center, Smedjevägen 3, P.O. Box 41, 230 53 Alnarp, Sweden

J. Franckowiak
Agri-Science Queensland, Department of Employment, Economic Development and Innovation, Hermitage Research Facility, 604 Yangan Road, Warwick, QLD 4370, Australia

M. Morgante
University of Udine, 33100 Udine, Italy

N. Stein
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, 06466 Gatersleben, Germany

Introduction

During floral development, the spikelet meristem (SM) of the developing barley inflorescence differentiates into a central spikelet meristem (CSM) and two lateral spikelet meristems (LSMs). This is followed by differentiation of each spikelet meristem into a floral meristem (FM) and a pair of glume primordia (GPs). Further development leads to a mature barley spikelet consisting of the rachis (floral stem) and sessile spikelets attached at the rachis nodes

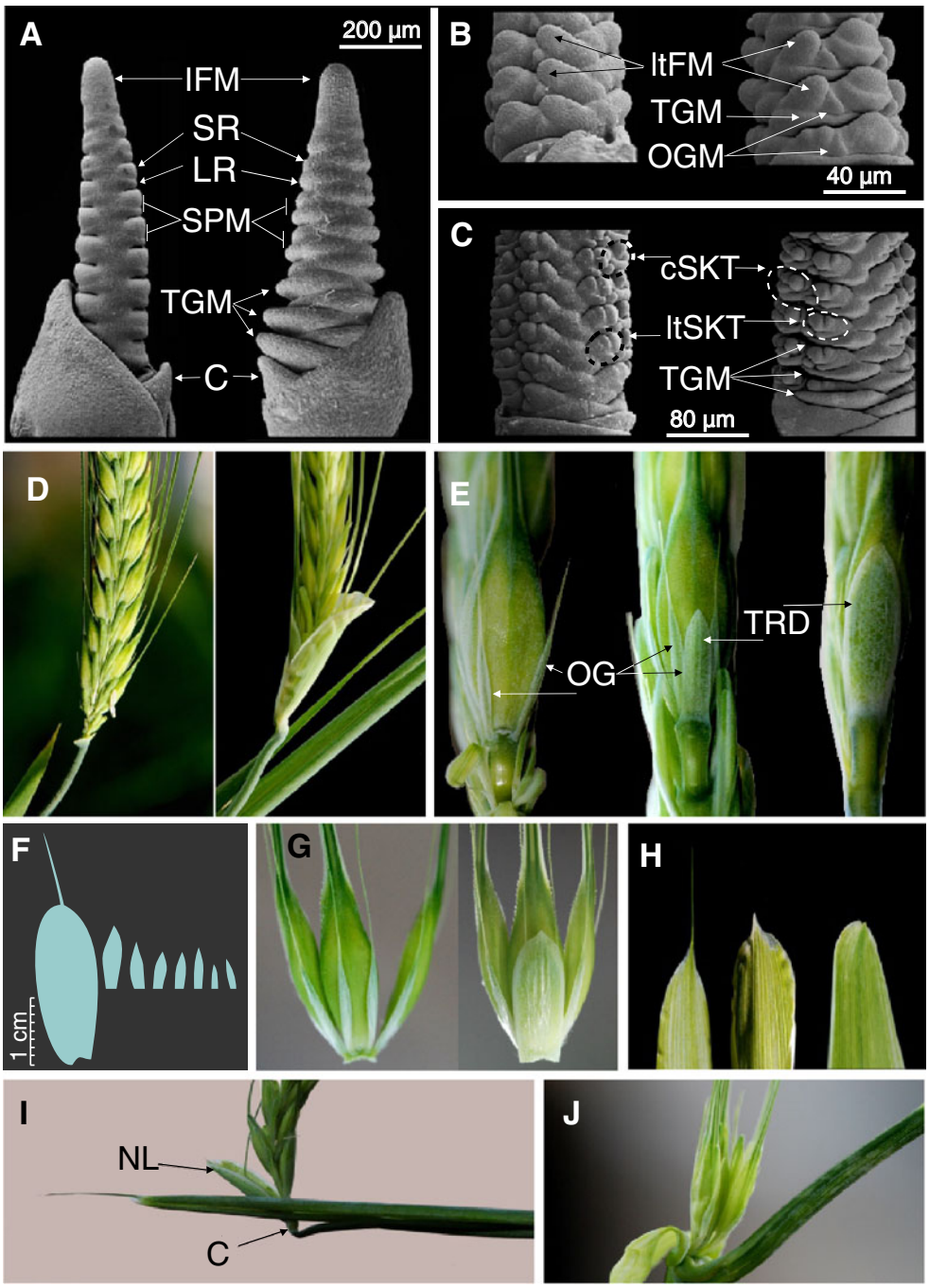

Fig. 1 Development and features of barley spikes carrying recessive alleles of the *Trd1* gene. **a–c** Scanning electron micrographs of the developing inflorescences from the cv. Bowman (*left*) and *trd1* isogenic line BW069 (*right*) at 10–14 days (**a**), 3 weeks (**b**) and 4 weeks (**c**) after imbibition. **d** Spikes from the cv. Bowman (*left*) and isogenic line BW069 (*right*) 3 weeks after fertilization. Size variation of the third outer glume, **e** exposed third spikelet from the bottom of the spike of the cv. Bowman (*left*), equivalent spikelet from the isoline BW069 (*middle*) and the second spikelet from the bottom of the BW069 spike (*right*). **f** Outlines of dissected third outer glumes from the BW069. **g–j** Some features of the *trd1* plants, third outer glume in the six-rowed background (**g** on the right, left cv. Morex), variation in the tip shape of different third outer glumes (**h**), effect of the *Trd1* gene on the development of the flag leaf (**i**) and development of the floral structures at the peduncle node (**j**). *IFM* inflorescence meristem, *SPM* spikelet meristem, *TGP* third glume primordia, *C* collar, *NL* neck leaf, *SR* spikelet ridge, *LR* leaf ridge, *OGP* outer glumeprimordia, *ltFM* lateral floret meristem, *cSKT* central spikelet primordium, *ltSKT* lateral spikelet primordium

(Fig. 1a). Recessive mutations in the *THIRD OUTER GLUME1* (*Trd1*) gene results in a leaf-like structure arising from the lower half of the rachis nodes of the spike. These structures are called 'third outer glumes' (Fig. 1b, d, e) and typically persist for the first six rachis nodes of the inflorescence, decreasing in size up the spike (Fig. 1c). Even though the third outer glume morphologically more resembles leaf, it has been classed as a floral bract (Schmidt and Ambrose 1998). The *trd1* mutation have been categorised as a transitional mutation between the reproductive and vegetative region of the plant (Bossinger et al. 1992).

The third outer glume phenotype was first recorded by Vavilov in a black hulled six row cultivar that was classified as *Hordeum vulgare* var. *afghanicum* (Ivanova 1937). The third outer glume persisted to the top of the spike in these specimens, and the phenotype was recessive. Konzak (1953) also identified individuals with a third outer glume as a spontaneous mutation in the barley cultivar Valki. The *trd1* mutants are also referred to as *bracteatum* (*bra*) mutants in the literature as they appear to be the result of mutations in the same gene (Franckowiak and Lundqvist 1997). This conclusion is supported by the work of Tsuchiya (1974) who conducted complementation tests and found that *trd1* was allelic to *bra-c.1*. Pozzi et al. (2003) proposed that *bra-d.7* was also allelic to *trd1* as both alleles mapped on the bottom of chromosome 1H. However, allelism studies by Lundqvist and Franckowiak (2007) indicated that the *bra-d.7* and *bra-c.1* mutations were in separate genes. The approximate position of *Trd1* has been determined as 11.4 cM proximal to the *EARLY MATURITY 8* (*eam8 or mat-a*) locus (Konzak 1953), and 16.0 cM distal to the *BLACK LEMMA AND PERICARP* (*Blp1*) locus on chromosome 1H (Takahashi and Yasuda 1971).

Previously, we refined the likely location of *Trd1* to an inferred position at 132.5 cM on the reference map of barley chromosome 1H (Close et al. 2009) using a gene prediction model based on synteny with rice *Nl1* gene (Druka et al. 2011). However, the precise map position of *Trd1* has not been determined. Here, we describe the phenotype and provide a high resolution mapping analysis of the locus in barley. We also phenotyped and resequenced *Trd1* gene in 30 barley mutant lines previously described as containing *trd1* mutations (Lundqvist et al. 1997; Franckowiak and Lundqvist 2002; Druka et al. 2011). We describe the sequence analysis of the 14 lines that in our hands expressed the third outer glume 1 phenotype, along with *Trd1* amplified from the original parental lines. This allowed us to identify unambiguously seven previously unknown *Trd1* sequence variants. These may be important for detailed molecular studies on the functions of *Trd1*.

Materials and methods

Plant material

Initially, 30 *trd1* mutant lines obtained from several genetic stock collections were grown to confirm their phenotype. For each line, 3–10 seeds were grown. Plants were grown individually in 12.5 cm pots in a glasshouse maintained between 16 and 24°C with natural light supplemented with high pressure sodium lamps to maintain a 16 h light and 8 h dark conditions. Plants were irrigated regularly and sprayed with fungicide when necessary. These lines comprised five cv. Bowman backcross-derived lines (Druka et al. 2011), four *trd1* lines (GSHO227, GSHO1695, GSHO2060 and GSHO2061) representing two of the *trd1* alleles (*trd1.b* and *bra-c.1*) that were recently described by Whipple et al. (2010) from the National Small Grains Collection, Aberdeen, USA (http://www.ars.usda.gov/pwa/aberdeen) and 24 original mutant lines from the NordGen collection, Sweden (http://www.nordgen.org). Donor parents were also included in the phenotyping and resequencing; they originated from the stocks at The James Hutton Institute, except for Valki, which was provided by Harold Bockelman from the National Small Grains Collection, Aberdeen, USA (http://www.ars.usda.gov/pwa/aberdeen). For mapping *Trd1*, three F2 populations were generated by crossing the Bowman backcross-derived line BW069 with the barley cultivars Morex, Barke and Bowman. Details of all the lines including mutagens used to generate them and their donor parent cultivar can be found in Online Resource 1.

Characterisation of the phenotype

Plants were grown as described above. At approximately 2, 3 and 4 weeks after imbibition, the inflorescences of BW069 (*trd1.1*) and Bowman were dissected and fixed in 2.5% glutaraldehyde in PIPES pH 7.2 buffer for 24 h. These samples were washed twice for 15 min in 0.2 M PIPES pH 7.2 buffer followed by washing twice in distilled water for 15 min. Samples were dehydrated through a graded ethanol series (50, 70, 90, 100% ethanol) and transferred to 100% acetone for 15 min. A Bal-Tec CPD030 critical point drier was used to dehydrate the samples further before mounting on aluminium stubs for sputter coating. Samples were covered in 40 nm of gold using a Cressington 208HR sputter coater and then viewed using a Philips XL30 environmental scanning electron microscope (ESEM). Twenty weeks after germination the presence or absence of bracts were recorded for all lines, the donor lines and cv. Bowman. Mature inflorescences were photographed using a Nikon D80 camera with EX DG Macro 105 mm F2.8 (Sigma) lens.

DNA extraction

For all samples DNA was extracted using a Nucleoplex™ Plant DNA Kit (Product No. 33300, Tepnel Life Sciences Plc, UK) run on a Nucleoplex™ robot (Tepnel Life Sciences Plc, UK). The standard lysis protocol and the main plant protocol for DNA isolation were used. DNA was eluted in 100 µl of 10 mM Tris typically yielding concentration of 40 ng/µl.

Mapping *Trd1* phenotype

The third outer glume phenotype was mapped using 182 homozygous recessive individuals from a population of 960 Barke × BW069 F3 plants using two custom Taqman genotyping assays designed to differentiate polymorphic SNPs between Barke and BW069. These Taqman assays were designed by amplifying SNP markers 1_11481 at 126.5 cM and 1_10443 at 140.0 cM (Close et al. 2009) using OLAD802F (5′-TCAAGAACTACGGCATCTGG3′), OLAD802R (5′-TCTTCGAGTTGTGGAACTGC-3′), and OLAD808F (5′-CGAACTTGATGGATGCTGAC-3′), OLAD 808R (5′-TAGGTTGGTTGGTTGGTTGG-3′), respectively. Sequences for the Taqman assays were edited to contain only one polymorphism between Barke and BW069, checked for repetitive regions using RepeatMasker (http://www.repeatmasker.org/) and submitted to the Applied Biosystems custom assay program for design and production (https://www5.appliedbiosystems.com/tools/cadt/). 10 µl reactions were used containing 15 ng DNA, 1× Taqman Genotyping Mastermix (Applied Biosystems 4371355) and 1× Assay (Applied Biosystems, non-human custom). PCR was performed on StepOnePlus using the recommended program [60°C, 30 s pre-PCR read; 95°C, 10 min, 40 cycles (95°C, 15 s; 60°C, 1 min); 60°C, 30 s post-PCR read]. The analysis was performed automatically using the default parameters. The resulting genotypic data were used to calculate recombination frequencies between these markers and *Trd1*.

Candidate gene identification

To identify a set of candidate genes we focused on gene annotations for a region on chromosome Os05 in rice, which is syntenic to the region on barley chromosome 1H where the introgression containing *Trd1* has been located (Druka et al. 2011). Our candidate gene analysis strategy included identification of homologous barley genes in EST libraries using BLAST searches, then designing primers to barley sequences followed by resequencing of a small set of wild type and mutant alleles to see if there were any polymorphisms that were potentially causal to the third outer glume phenotype (specific to mutant only and leading to non-conserved amino acid change) and/or can be used for mapping. We focused primarily on transcription factors, along with genes involved in processes such as auxin signalling as candidates for *Trd1*. One of the genes from this set, a GATA transcription factor (GU722205) has recently been proposed as a candidate gene for barley *Trd1* (Whipple et al. 2010).

Sequencing *Trd1*

Candidate genes were resequenced in BW069, BW880, Bowman, Bonus (donor parent of BW069), Morex and Barke to identify polymorphisms unique to the mutant lines, and to allow us to identify polymorphisms that could be used for mapping in F_2 populations (BW069 × Barke, BW069 × Bowman, BW069 × Morex). The only gene where we observed polymorphisms unique to the mutant line was a GATA transcription factor (GU722205) (LOC_Os05g50270). Nucleotide BLAST searches of 30× cv. Bowman and 30× cv. Morex coverage next generation survey (NGS) sequence assemblies (accessible on request) allowed us to obtain genomic sequence both upstream and downstream from the coding sequence of *Trd1*. We located a potential promoter region including a TATA box 35 bp upstream from the transcription start. Primers were designed 595 bp upstream and 521 bp downstream of the start of transcription to include the promoter region. Four pairs of primers, OLAD847F (5′-GCAGGCAGTTTCCCAACC-3′), OLAD847R (5′-CTGTGGTCGTCGTAGGAGTAGG-3′), OLAD 848F (5′-CAGTGCGTGGTGTAGGTACG-3′), OLAD848R (5′-CCCTAGCGAGTGTGGATGG-3′), OLAD 849F (5′-CATTTAAACTCGCCCATGC-3′), OLAD849R (5′-CTCAATTCGAACCCAAGAGC-3′), OLAD 851F (5′GCGTCTCGGTTACTACATTGC-3′) and OLAD851R (5′-CACTGACGAAGTGAGCATGG-3′) were used to amplify *Trd1* and adjacent sequences (GB accession GU722205, Whipple et al. 2010). 40 ng DNA was amplified using 0.6 units HotStarTaq (Qiagen 203205), 1× PCR Buffer (supplied), 2 mM dNTPs (Roche 11051440001, 11051458001, 11051466001, 11051482001) in a 25 µl reaction on a Perkin Elmer 9700 PCR machine using a 55°C touchdown program (this was amended to 58°C touchdown if multiple bands were obtained at lower temperature). 5 µl PCR reactions were run on 1% agarose gels (1 × TBE) for 30 min at 100 V alongside size marker (Invitrogen 1 Kb plus) to confirm amplification and band size. PCR cycle details are as follows: 98°C for 2 min; 10 cycles of 98°C for 15 s, 65°C for 20 s, 72°C for 1 min with a touchdown on the primer annealing step of −1°C/cycle; then 30 cycles of 98°C for 15 s, 56°C for 20 s, 72°C for 1 min; followed by 72°C for 2 min. To purify the PCR product, a 10 µl reaction mix containing 5 µl of PCR product and 2 µl of ExoSAP-IT (GE Healthcare US78201)

was used with the following conditions in a thermocycler: 37°C, 15 min; 80°C, 15 min. The big dye terminator kit version 3.1 was used in 10 µl reaction mix with the following volumes of reagents; 5 µl ExoSAP product, 0.25 µl Big Dye, and 1.88 µl buffer. Samples were sequenced on an ABI3730 and sequences aligned in Geneious Pro version 5.0.2 (Drummond et al. 2011).

Mapping *Trd1* gene

To map *Trd1,* it was first PCR-sequenced from the parental lines of several barley reference mapping populations: Steptoe × Morex (Kleinhofs et al. 1993), Morex × Barke (Close et al. 2009), Oregon Wolfe Barley dominant (OWBd) × Oregon Wolfe Barley recessive (OWBr) (Costa et al. 2001) and Golden Promise × Morex (unpublished). A microsatellite polymorphism in intron 1 distinguished the parents of the OWBd × OWBr population. In OWBd the microsatellite repeat was $(CA)_2$ and in OWBr, $(CA)_3$. The region of *Trd1* containing this polymorphism was sequenced from the 96 individuals of the OWB reference population using a pair of primers OLAD826F (5′-AGCATGGTGGCAGGGGCGG-3′) and OLAD826R (5′-GCTTGTTCGACGATAATGCCA GACA-3′) using reaction conditions as described above. AntMap version 1.2 (Iwata and Ninomiya 2006) was used to construct the genetic linkage map. Default settings were used, with the exception of the locus ordering criterion, which was changed to SARF, to minimise the sum of adjacent recombination fractions.

384-plex genotyping

All mutant and parent lines listed in the Online Resource 1 were genotyped using a custom 384-SNP Illumina GoldenGate oligo pool assay (Illumina Inc.) (Moragues et al. 2010) on a BeadXpress platform according to the Manufacturer's protocol and viewed using GenomeStudio V2010.3 (Illumina). Polymorphism at 384 SNP sites located in coding gene sequences more or less equally distributed along the seven chromosomes of barley was examined. These were a subset of the 3,072 SNPs described by Close et al. (2009). Details of the SNPs in this assay can be found in Online Resource 2. Cluster analysis was carried out using the PAST 1.91 software suite (Hammer et al. 2001).

Results

Development of the third outer glume in barley

To describe the development of the third outer glume, we selected one of the Bowman backcross-derived lines, BW069 (*bra-c.1* reassigned *trd1.1*), as a reference and compared the morphology of the inflorescence with its recurrent parent Bowman at four developmental stages—double ridge, triple mound, floral organ primordium and mature spike (Kirby and Appleyard 1987; Waddington et al. 1983). Although the phenotype of *trd1* has been characterised before (Ivanova 1937; Konzak 1953; Franckowiak and Lundqvist 2010a; Whipple et al. 2010), here we provide a more detailed description of the phenotype at several stages of development. Ten to 14 days after imbibition, the shoot apical meristem had differentiated into an inflorescence reaching the double ridge stage, with typical features comprising the spikelet ridge, leaf ridge and collar structures. Clear structural differences in spike development between the wild type and the mutant line could be observed at this stage—in the mutant, spikelet meristems appear more similar to leaf primordia (Fig. 1a). Three weeks after imbibition (Fig. 1b), the cells of the spikelet meristem had differentiated into a central and two lateral spikelet meristems (the triple mound stage), and a pair of central outer glume primordia became clearly visible. In *trd1.1*, the third outer glume primordia slow their development compared to other structures, and a decrease in the size of the third outer glume primordia from the base to the tip of the spike was observed. At the onset of the floral organ primordium stage, 4 weeks after imbibition, pairs of sterile glumes had developed, and surrounded the reproductive structures of the barley spikelet (Fig. 1c) in the wild type and *trd1.1* mutant. The cells that will form the lemma, anthers, and carpel for each spikelet were present in both the wild type plant and *trd1.1*. At this stage in *trd1.1*, the size differences between the top and bottom of the third outer glumes were more obvious. Three weeks after fertilization, the characteristics defining the *trd1* mutant line are the presence of the third outer glumes, which decreases in size moving up the spike, subtending the lower spikelets of the mature spike, and a large, fully developed bract at the collar. Bract development at the collar and subsequent rachis nodes is completely suppressed in the wild type cv. Bowman (Fig. 1d–g). Both the size and the shape of the third outer glume vary between mutant lines and depend upon its position on the spike. The tips of some third outer glumes show morphological variation, some are awned, and others have pointed or rounded tips (Fig. 1h). The *trd1* phenotype includes the development of a "neck leaf" from the collar (Fig. 1i). In several individuals, the development of spikelet-like structures at the collar node, between the collar leaf and the rachis has been observed (Fig. 1j). Development of such structures at the collar node was not observed in Bowman.

Mapping *trd1* phenotype

To fine map *trd1*, we developed three populations consisting of 960 F_3 recombinant families each by crossing

cvs. Barke, Morex and Bowman with BW069. One hundred and eighty two F_3 plants that had third outer glume phenotype (hence homozygous recessive for *Trd1* gene) were selected from the BW069 × Barke population and genotyped with markers 1_1481 and 1_0443 that previously have been positioned on the barley consensus map at positions 126.48 and 140.53 cM, respectively (Close et al. 2009) (Fig. 2). Twenty lines with recombination in this interval were identified. Eleven contained recombination between the proximal marker and the third outer glume phenotype, and nine recombinants were distal to the phenotype, positioning *trd1* between 134.7 and 135.1 cM (a 0.4 cM interval) on 1H based on 362 meiotic events (Fig. 2).

Candidate gene identification

Twenty candidate genes from a conserved syntenic region on chromosome Os05 in rice were identified (Fig. 3). A homolog of the *Antirrhinum RADIALIS* (*Rad*) gene, rice LOC_Os05g50340 and barley unigene 32_10890 (*HvRadialis*) was selected as one of the candidate genes. Corley et al. (2005) had shown that *Rad* interacted with other genes to regulate floral symmetry, and the rice homolog was in a syntentic position based on the introgressions identified for *Trd1* (Druka et al. 2011), therefore making it a suitable candidate. Re-sequencing EST marker u32_10890 identified a polymorphism in BW880 which was used to map the gene in F3 recombinant lines. Genotyping *HvRadialis* in 190 recessive homozygous *trd1* F3 lines from the BW069 × Bowman population revealed two recombination events (Fig. 3) discounting it as *Trd1*, but providing a closely linked marker for high resolution genetic analysis. Other candidate genes included two copies of TAG lipase gene (LOC_Os05g49830 and LOC_Os05g49840). Previously, the TAG lipase *Eg1* was shown to be responsible for the extra glume phenotype in rice (Li et al. 2009). However, resequencing barley

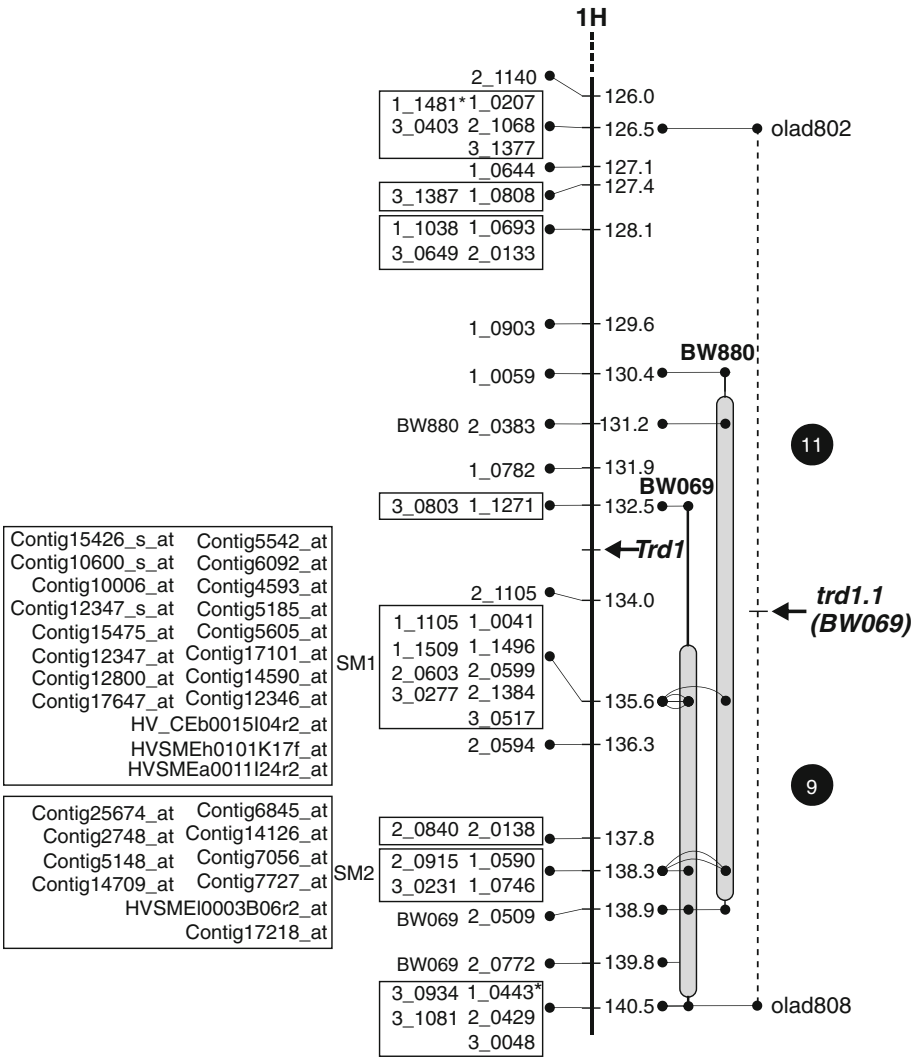

Fig. 2 The Barley consensus map of chromosome 1H including the location of the overlapping introgression of the two Bowman backcross-derived lines, BW069 and BW880, which contain the mutant alleles of *Trd1 trd1.1 and trd1.3*, respectively. The *lines* extending from the *bars* representing BW069 and BW880 indicate the region which includes the breakpoint between the introgression and the cv. Bowman part of 1H, showing the maximum potential region included in each introgression. The map position obtained by mapping *Trd1* in the OWB reference population is shown at 133.25 cM. The two Taqman genotyping assays, olad802 and olad808, designed to the markers 1_1481 and 1_0443, highlighted using *, were used to map genotype BarkexBW069 F3 homozygous recessive individuals. The number of recombinants observed between each marker and *trd1.1* in the 182 homozygous recessive lines genotyped with these two assays are shown in *black circles* positioned between the marker and the location of *trd1.1*. This data were used to map *trd1.1* to between 134.7 and 135.1 cM on 1H

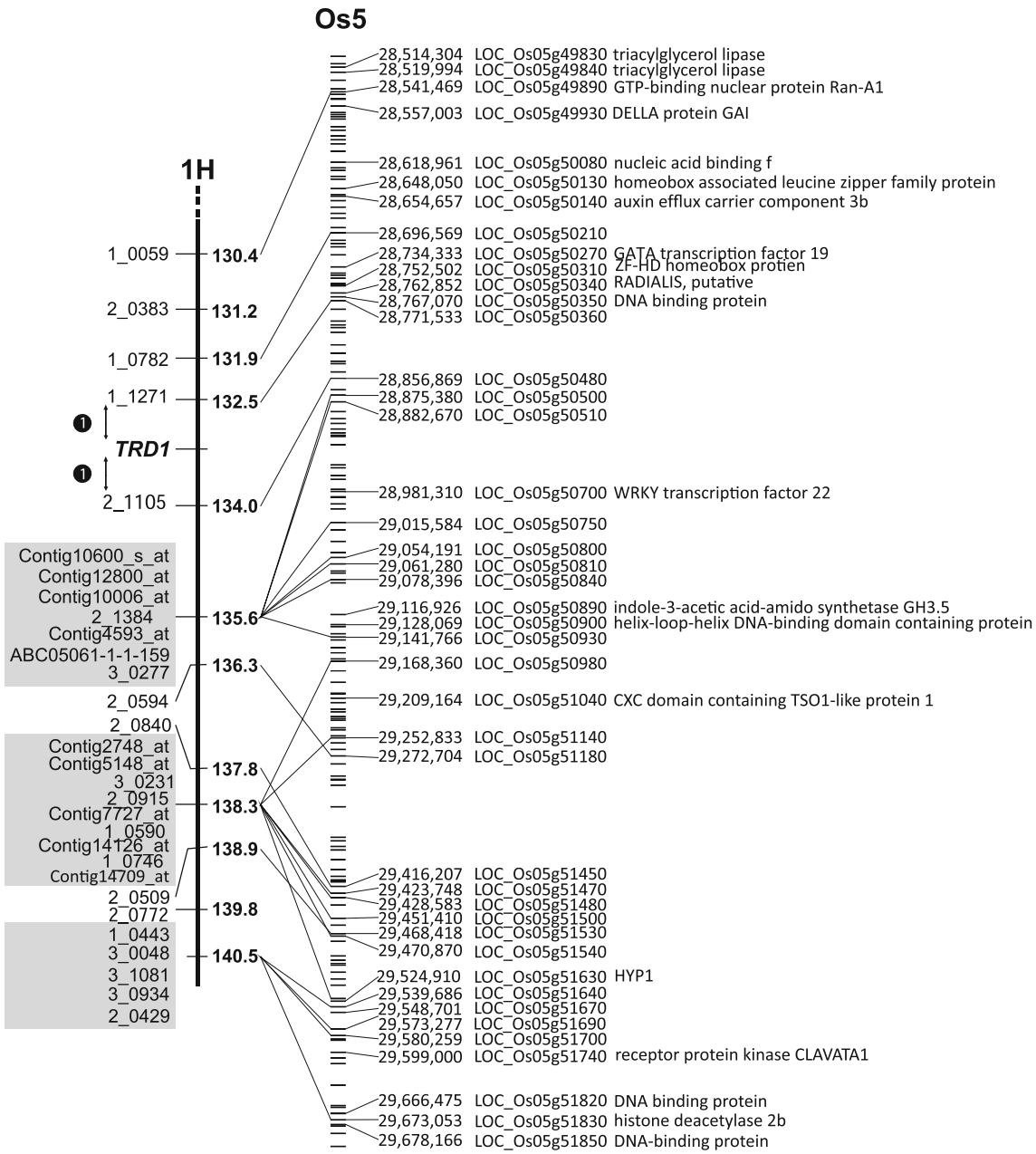

Fig. 3 Anchoring the barley *Trd1* locus to the rice physical map and selection of candidate genes based on annotations. A subset of markers from the consensus map (Fig. 2) that have rice homologs chromosome Os5 are shown, those that are boxed are from the same barley genetic locus. *Bars* on the left indicate 5′ positions of the rice genes, position and gene id are shown for homologs used to anchor the rice physical map to the barley genetic map. Rice genes for which annotations are provided are those selected as *Trd1* candidate genes. The number of recombinants observed between *Trd1* and the markers that define the interval it was mapped to in the OWB population of 94 doubled haploid individuals are indicated in *black circles* positioned between the marker and the location of *Trd1*

unigenes 21_2629 and 21_2631, homologs of LOC_Os05g49830 and LOC_Os05g49840, respectively, also failed to reveal any polymorphisms. Other attractive candidates included several homeobox domain containing genes because at least two of this class of gene have been implicated in regulating spikelet morphology in barley, including *SIX-ROWED SPIKE 1* (Komatsuda et al. 2007) and *HOODED* (Müller et al. 1995). For several of these genes we were not able to find barley homologs in EST collections and these were given a lower priority. Ultimately, resequencing a candidate GATA transcription factor (GU722205) revealed polymorphisms that were unique to the mutant lines.

Allelic variants of *Trd1*

After growing 3–10 seeds from all 30 lines as described above and phenotyping the spikes, we found that only 14 exhibited the third outer glume trait. All lines regardless of phenotype were used for resequencing *Trd1*, but only details of those with the *trd1* phenotype are presented in Table 1. BLAST analysis of NGS sequence data from the cultivars Morex and Bowman (access available on request) identified a contig of 8,711 nucleotides which contained *Trd1*. We amplified and re-sequenced 2,214 nucleotides of this contig, containing the entire *Trd1* gene (1,139 nt), 558 nucleotides upstream of the transcription start site and 506 nucleotides downstream of the polyadenylation site, from the 30 lines previously described as having the *trd1* allele, and their donor lines (Online resource 1). All sequences can be found in GenBank under accession numbers JQ421103–JQ421135. For the 14 lines which expressed the mutant phenotype sequencing this region revealed 15 mutations in 11 of them (Table 2; Fig. 4). We did not find any mutations within *Trd1* in the lines carrying *trd1.12, trd1.13 and trd1.14* alleles. Mutations that were identified in the allelic lines did not occur in either the donor lines or cv. Bowman. No mutations where found in the lines that did not have the mutant phenotype and no mutations were identified in the putative promoter region of *Trd1*. Several lines shared the same DNA polymorphisms, *trd1.8* and *trd1.9*, and, *trd1.4* and *trd1.*5.

Sequence analysis of cvs. Bonus and Valki (original parents of the *trd-1695*, *trd-2061* and *trd-2060* alleles, reassigned as *trd1.5*, *trd1.1* and *trd1.3*, respectively) showed no differences when compared to cv. Bowman, confirming that the observed sequence changes in these alleles, initially reported in Whipple et al. (2010), were induced mutations. In addition, we were able to show that *trd-227* (*trd1.2*) carries a mutation changing a TGG codon (tryptophan) to an AGG codon (arginine). Exactly the same mutation was found in *trd-2061*, which has a different background, suggesting the possibility of erroneous assignment of one of the alleles, differences in expression levels of these alleles, or errors in line identification that could have occurred during backcrossing.

To test the possibility of incorrect assignment of the alleles, we genotyped all 30 lines and five cultivars included in this study using a 384-SNP Illumina GoldenGate oligo pool assay. The resulting SNP data were used to construct a dendrogram which confirmed that the genotype of each line corresponded to that of the correct donor line (Online Resource 3). The only exceptions were *bra-c.8*, *bra-c.4* and *trd0011*. *Bra-c.8* was recorded as having Kristina background, however, our analysis showed that it clustered with lines with Bonus background, and contained the same mutations as *trd1.4* and *trd1.5*. The *bra-c.4* mutant had a Foma background but clustered with Bonus and 12 lines which all have a Bonus background. The *trd0011* mutant has a Bonus background but clusters with Kristina and another line with a Kristina background. To allow for independent verification of the results two sets of all lines were grown and used for analysis. These data lead

Table 1 Details of the *Trd1* allelic lines included in this study, including donor cultivar of the line, the mutagen used to generate the mutant line, the number of times this line has been backcrossed the locus and which authors have described this line

Allele	Accession	Previous nomenclature	Donor	Mutagen	Reference
trd1.1	BW069, GSHO2061	*bra-c.1, trd-2061,*	Bonus	X-ray	Franckowiak and Lundqvist (1997), Whipple et al. (2010), Druka et al. (2011)
trd1.2	GSHO227	*trd-227*	Valki	Spontaneous	Franckowiak and Lundqvist (1997), Whipple et al. (2010), Druka et al. (2011)
trd1.3	BW880, GSHO2060	*trd1.b, trd-2060*	Valki	Spontaneous	Franckowiak and Lundqvist (1997) Whipple et al. (2010), Druka et al. (2011)
trd1.4	BW070	*bra-d.7*	Foma	Ethylene imine	Druka et al. (2011)
trd1.5	NGB114303, GSHO1695	*trd-1695*	Bonus	X-rays	Whipple et al. (2010)
trd1.6	NGB114305	*bra-c.3*, *trd3*	Bonus	Ethylene imine	This study
trd1.7	NGB114307	*bra-c.5*, *trd5*	Bonus	Gamma-rays	This study
trd1.8	NGB114314	*trd.9*	Bonus	Ethyl methane sulfonate	This study
trd1.9	NGB114315	*trd.10*	Bonus	Ethyl methane sulfonate	This study
trd1.10	NGB114316	*trd.11*	Bonus	Sodium azide	This study
trd1.11	NGB114317	*trd.13*	Bonus	Sodium azide	This study
trd1.12	NGB114308	*bra-c.6*, *trd6*	Foma	Neutrons	This study
trd1.13	NGB114304	*bra-c.2*, *trd2*	Bonus	Neutrons	This study
trd1.14	NGB114310	*trd-d.7*	Foma	Ethylene imine	This study

Previous nomenclature, accession, and new allele designation are shown

For the full list see Online Resource1

Table 2 Nucleotide polymorphisms in *Trd1* and the resulting amino acid changes are summarised

Allele	Position	Location	Nucleotide	Amino acid	Effect
trd1.1	499	First exon	T > A	W > R	Decrease hydrophobicity
trd1.2	499	First exon	T > A	W > R	Decrease hydrophobicity
trd1.3	188	First exon	A > C	Q > P	Increase hydrophobicity, decrease polarity
trd1.3	222	First exon	C > G	Y > STOP	Translation termination
trd1.3	342	First exon	T > G	P > P	Same amino acid
trd1.3	529	Intron	Deletion > CA4	N/A	Not known
trd1.3	632	Intron	A > T	N/A	Not known
trd1.4	203	First exon	AGGAAGCT > C	EEAA > C	Frameshift
trd1.5	203	First exon	AGGAAGCT > C	EEAA > C	Frameshift
trd1.6	466	First exon	T > A	C > S	Zinc binding
trd1.7	514	First exon	C > -	R > A	Frameshift
trd1.8	310	First exon	C > T	L > F	Decrease isoelectric point
trd1.9	310	First exon	C > T	L > F	Decrease isoelectric point
trd1.10	102	5′ UTR	G > A	N/A	Not known
trd1.11	192	First exon	G > A	W > STOP	Translation termination

Only lines that differed from the wild type sequence of *Trd1* are included Details provided in this table include the location (base number and exon/intron in relation to GU722205) of each mutation in *Trd1* and the subsequent effect concerning changes in hydrophobicity, polarity and translation of the protein for each *trd1* allele

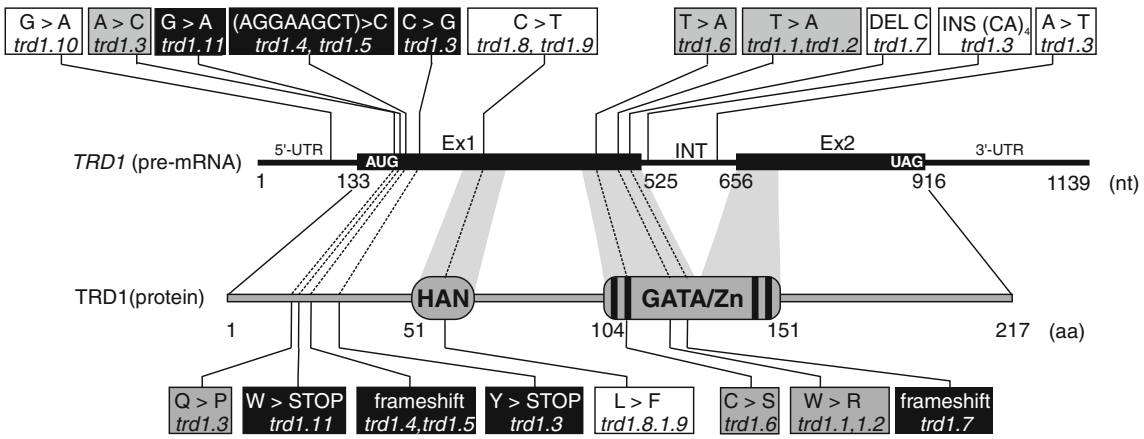

Fig. 4 Genomic and protein structure of *Trd1*. The 5′ and 3′ untranslated regions (*UTR*), exons (*EX1* and *EX2*), and intron (*INT*) are illustrated, as well as the codon where translation is initiated (*AUG*) and terminated (*UAG*) on the line representing the nucleotide (*nt*) sequence. Position of mutations in the genomic DNA sequence in relation to GU722205 and the effect these mutations have on the putative amino acid sequence are indicated. On the line representing the amino acid (*aa*) sequence the conserved HAN and GATA zinc finger domains are shown, and the position of the four conserved cysteine residues are represented by *vertical black bars* within the GATA zinc finger

us to conclude that these anomalies are due to a mix up in seed handling at some point. Therefore, *bra-c.8*, *bra-c.4* and *trd0011* were omitted from further analysis.

To map *Trd1*, we used a polymorphism in microsatellite length between OWBd and OWBr. This was mapped in 94 DHL population derived from the OWBr and OWBd cross (Costa et al. 2001) to 133.75 cM on chromosome 1H. This position is within the introgression of the Bowman back-cross derived line BW880 (*trd1.3*) and the overlapping introgression of the BW069 (*trd1.1*) (Fig. 2, Druka et al. 2011), coinciding with the position of the *trd1* phenotype we mapped in BW069 × Barke population.

Discussion

Mutations controlling extra bract development have been previously described in rice (Nagao et al. 1963) and maize (Briggs 1992; McSteen and Hake 2001), and more recently the genes have been identified (Wang et al. 2009; Whipple

et al. 2010). The *extra glume1* (*eg1*) and *neck leaf 1* (*nl1*) mutants of rice produce a very similar phenotype to *trd1*, each developing an extra glume outside of the lemma (Li et al. 2009). In *nl1* mutants, bracts develop in the panicle and base of the primary branches. In wild type plants, the growth of these bracts is suppressed (Wang et al. 2009). In mutants of *TASSEL SHEATH1* (*Tsh1)*, the maize ortholog of *Nl1*, the bracts subtend branches and spikelet pairs in both in tassel and ear (Whipple et al. 2010). Five *Tsh* genes affecting extra bract suppression have been identified in maize (Whipple et al. 2010; Chuck et al. 2010). Apart from *Tsh1*, only *Tsh4* has been cloned (Chuck et al. 2010). *Trd1* was proposed as an ortholog of maize *Tsh1* and rice *Nl1* based on evidence from conservation of synteny, mRNA expression patterns and PCR-resequencing of two mutant and a non-related wild type alleles (Whipple et al. 2010). One mutant allele clearly affected the function of the putative protein due to a non-synonymous substitution and premature stop codon towards the N-terminus, while the other was a non-synonymous substitution of a highly conserved amino acid residue (tryptophan to arginine) (Whipple et al. 2010). By resequencing additional alleles in 14 lines and all the relevant parent lines, we confirmed that the GATA transcription factor (GU722205) is *Trd1*.

As we used similar mutant stocks, we confirmed the two previously described mutant alleles of *Trd1* (Whipple et al. 2010) and identified nine new alleles. Interestingly, several of these mutations were at the same codon as mutations in maize *Tsh1* alleles, and produced the same amino acid. Like *tsh1-3* in maize, *trd1.8* and *trd1.9* contain a substitution of leucine with phenylalanine within the *HAN* motif. In both species, these lines were mutagenized by ethyl methanesulphonate (EMS) thus partially explaining such conservation between species. Presently, we cannot provide a good explanation to this finding although we are aware that certain DNA structures can provide hotspots for mutations (Rogozin and Pavlov 2003; Zhao et al. 2010). Despite no mutations in the *HAN* motif having been identified in *Arabidopsis han* mutants, the mutations found in *tsh1-3*, *trd1.8* and *trd1.9* would suggest that the conserved *HAN* motif could play an important role in molecular function of *Trd1* underlying bract suppression.

The class B GATA transcription factors, which include *Trd1*, *Nl1*, *Tsh1*, and their *Arabidopsis* homolog *HANABA TARANU* (*Han)* share a number of characteristics. Generally, they have two or three exons and a single zinc finger with the DNA sequence encoding the zinc finger split between two exons. The central feature of the zinc finger loop consists of 18 residues and contain cysteine molecules in the following configuration, $CX_2CX_{18}CX_2C$, that are stabilized by the zinc atom. This configuration is characteristic of subfamily II of GATA transcription factors (Reyes et al. 2004). The mutations identified in the allelic series potentially exhibit a range of effects on the structure of TRD1. Mutations in the GATA zinc finger, especially its highly conserved cysteine residues, are expected to disrupt DNA binding and therefore affect transcription of target genes. Altogether, four of the *trd1* alleles: *trd1.1*, *trd1.2*, *trd1*.6 and *trd1.7* had mutagen-induced nucleotide sequence changes that could lead to a truncated TRD1 protein with a missing DNA-binding domain (Fig. 4). The GATA DNA-binding region of the zinc finger of *trd1.6* contains an adenine to thymine substitution at base 466 resulting in a cysteine to serine change in the protein. The cysteine molecules of all zinc finger transcription factors are highly conserved (Liu et al. 1999). These molecules bind to zinc ions, which stabilise the folds of the zinc finger allowing it to bind to DNA. The CDS of *trd1.1* and *trd1.2* also contain a mutation within the conserved GATA region. An adenine to thymine substitution at base 499 produces arginine instead of tryptophan. This tryptophan molecule is essential for preserving the structure of the zinc binding region (Omichinski et al. 1993) and is highly conserved across GATA zinc fingers. The mutation in *tsh1-1* in maize occurred at the same codon as the tryptophan to arginine mutation in *trd1.1* and *trd1.2*. These mutant lines were generated using different methods; *trd1.1* by X-rays, *trd1.2* was a spontaneous mutation and *tsh1-1* by EMS. In addition to disrupting the GATA zinc finger, the tryptophan to arginine mutation in *trd1.1* and *trd1.2*, and the cysteine to serine mutation in *trd1.6* decrease hydrophobicity of the protein. The fourth allele, *trd1.7*, with a mutation in the GATA region has a deletion of a cytosine at base 514 causing a frameshift mutation. A further *TSH1* allele, *tsh1-2*, is due to a mutation within the GATA zinc finger domain and has a proline substituted for serine (Whipple et al. 2010). A substitution of arginine with glycine is present in the GATA zinc finger domain of *nl1-2* (Wang et al. 2009).

In *trd1.11* the substitution of adenine with guanine produces a stop codon at base 192 resulting in truncated TRD1 protein. Additional mutations were identified in *trd1.3* that influences the putative protein sequence. Base 188 in the first exon of *trd1.3* contains a non-synonymous base substitution of cytosine with adenine, resulting in the production of glutamine instead of proline. A further mutation is found in *trd1.3*, a synonymous substitution of thymine with guanine. This is clearly non consequential as it is after the stop codon and is shared with OWBd and OWBr which have the wild type phenotype. While no mutations were found within the putative promoter region, our analysis cannot exclude the possibility of regulatory polymorphisms generating the phenotype observed in *trd1.12-trd1.14*. The CDS of the donor parents for all

allelic lines and Bowman, the recurrent parent, were the same as the wild type (GU722205).

The genotypic cluster analysis using 384 SNPs provided confidence in the origin of the *trd1* lines analysed in this study and allowed us to remove potentially mislabelled lines. Anastasio et al. (2011) used a similar technique to identify potentially mislabelled accessions of *A. thaliana*. A group of 5,965 natural accessions or laboratory strains were genotyped with 149 SNPs and analysed. As these lines were expected to conform to isolation by distance it was simple to identify outliers that could be the result of misidentification or mislabelling. In total 286 accessions (5%) from their dataset are thought to have been mislabelled. This type of information helps to prevent the perpetuation of future mistakes and misinterpretation.

Currently, a single gene has been sequenced in barley which suppresses bract development (here and Whipple et al. 2010). Previously, the spike morphology of semi bracteatum lines (*bra-a* and *bra-b*) has been described in the same way as *trd1* by some authors (Lundqvist and Franckowiak 2002; Franckowiak and Lundqvist 2010b) (Online Resource 1) and therefore were treated as *trd1* mutant lines. However, all potential semi bracteatum lines examined here showed suppression of bract and collar leaf development, and lacked mutations in *Trd1*. It is likely that *bra-a* and *bra-b* are regulated by a gene other than *Trd1* as SNP genotyping data from Druka et al. (2011) position introgressions for these two genes on 7H, and 2H and 3H, respectively. The development of the third outer glumes is poor in the *bra-a* Bowman backcross derived lines (Lundqvist and Franckowiak 2002), which could explain why this characteristic was not observed in these lines when grown in this study. For maize, Whipple et al. (2010) identified five separate genes that regulate bract development, *Tsh1*–*Tsh*5. In addition to *Tsh1*, *Tsh4* has been cloned and identified as an SBP box transcription factor (Chuck et al. 2010). In rice, two genes have been identified which suppress the development of the neck (collar) leaf phenotype, *Nl1* and *Nl*2 (Khush 1987). Wang et al. (2009) identified, sequenced and described *Nl1*. No detailed study has been carried out on *Nl*2. Possibly more genes regulating the *tsh* phenotype are present in maize because the maize genome underwent ancient genome duplication. Alternatively it could be that these genes are yet to be identified in barley and rice. *Tsh4* (Chuck et al. 2010) is located on maize chromosome 5, and is not within a gene block that is a duplicate of the gene block containing *Tsh1*. In barley, four of the lines previously considered allelic to *Trd1* based on past allelism tests produced the phenotype but did not contain a mutation in the coding DNA sequence. Like the semi bracteatum lines discussed above, either these lines contain mutations in a regulatory region or the phenotype is the result of a mutation in a different gene(s).

Whipple et al. (2010) illustrated how effective conservation of synteny can be when looking for orthologs between different species. Mayer et al. (2011) used rice, *Brachypodium* and sorghum to anchor 21,766 barley genes to the physical map based on ordering genes assigned to syntenous positions. The importance of using multiple models to anchor genes to the barley genome was illustrated by the finding that the position of 20, 13 and 14.5% of barley genes were supported exclusively by *Brachypodium*, sorghum and rice, respectively. In addition, *Brachypodium* was found to have a higher number of conserved syntentic loci (14,422) than rice (12,093) or sorghum (11,887). Generally *Brachypodium* is considered to be a very good model species for cereals and is considered to be similar to rice in terms of micro-collinearity with barley (Bossolini et al. 2007). Several other well-described mutant loci at the telomeric region of chromosome 1H are yet to be cloned, including *EARLY MATURITY 8* (*eam8*), and *BLACK LEMMA AND PERICARP* (*Blp1*). The information we provide here for the map position of *Trd1* may enhance their identification, for example, using double crosses to select recombinant lines based on simple morphological characters determined by recessive alleles of these genes.

Suppression of the third outer glume phenotype appears to be conserved across the grass family (Whipple et al. 2010). The current study identified mutations in *Trd1* which are also present in its homologs. The combination of a large allelic series of *Trd1* (mostly) induced mutants but the phenotype being rarely observed in domesticated and wild grasses suggest strong evolutionary selection against this character. The bractless condition could have evolved to allow better pollen dispersal for wind pollinated species. However, as domesticated barley *H. vulgare* ssp. *vulgare* is derived from predominately inbreeding species, *H. vulgare* ssp. *spontaneum* which itself is likely to have evolved from a wind pollinated grass; bract suppression would have had to occur in a prior ancestor or due to a different reason. Suppression of such bracts could result in different resource partitioning within the plant, with implications for growth or development. This was illustrated with the *LEAFY (LFY)* gene which regulates bract development in *Arabidopsis* (Schultz and Haughn 1991). One feature of *LFY* mutants of *Arabidopsis* is the development of bracts on more than twice as many nodes compared to wild type plants typically in wild type individual's bracts develop on the bottom three nodes of the inflorescence. In this case, in addition to the development of bracts, *LFY* mutant lines showed slower floral meristem development than wild type individuals. This is potentially because wild type plants utilise the cells that are destined to develop bracts in mutant plants, to develop floral meristems instead (Coen and Nugent 1994).

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