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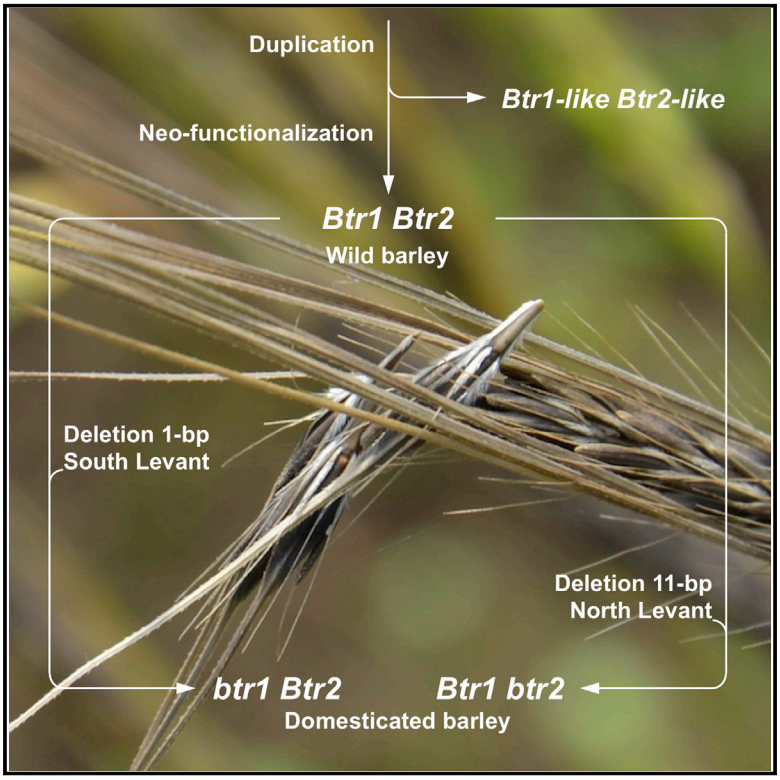
Evolution of the Grain Dispersal System in Barley

Graphical Abstract



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In Brief

Spatially and temporally independent selections of seeds with a non-brittle rachis were made during the domestication of barley by farmers in the southern and northern regions of the Levant, actions that made a major contribution to the emergence of early agrarian societies.

Highlights

- Wild barley spikes are brittle at maturity due to thin cell walls at rachis nodes
- This characteristic evolved by duplication and neo-functionalization of two genes, *Btr1* and *Btr2*
- During domestication, deletions in *Btr1* or *Btr2* converted the rachis to non-brittle
- The deletions happened twice: first in the South (*btr1*) and then in the North Levant (*btr2*)

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Evolution of the Grain Dispersal System in Barley

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SUMMARY

About 12,000 years ago in the Near East, humans began the transition from hunter-gathering to agriculture-based societies. Barley was a founder crop in this process, and the most important steps in its domestication were mutations in two adjacent, dominant, and complementary genes, through which grains were retained on the inflorescence at maturity, enabling effective harvesting. Independent recessive mutations in each of these genes caused cell wall thickening in a highly specific grain "disarticulation zone," converting the brittle floral axis (the rachis) of the wild-type into a tough, non-brittle form that promoted grain retention. By tracing the evolutionary history of allelic variation in both genes, we conclude that spatially and temporally independent selections of germplasm with a non-brittle rachis were made during the domestication of barley by farmers in the southern and northern regions of the Levant, actions that made a major contribution to the emergence of early agrarian societies.

INTRODUCTION

Grain crops represent the primary source of caloric intake that enabled mankind to move from hunter-gathering to cultivation and agriculture (Harlan and Zohary, 1966). Archaeological evidence indicates that this change occurred in the Fertile Crescent ~12,000 to 9,500 years ago, with the domestication process lasting several centuries (Tanno and Willcox, 2006; Weiss et al., 2006; Willcox, 2013; Willcox et al., 2008). Subsequently, farming spread throughout Europe, Asia, and Africa. Barley (*Hordeum vulgare* L. subsp. *vulgare*) was one of the first crops to be domesticated (Zohary et al., 2013). Its wild progenitor, *H. vulgare* subsp. *spontaneum* (C. Koch) Thell. (Harlan and Zohary, 1966; Tanno and Willcox, 2012; Weiss et al., 2006), was collected long before it was cultivated, as indicated by the presence of wild barley grains in several pre-agricultural pre-pottery Neolithic sites. The earliest evidence of such gathering comes from Ohalo II, a site located on the shore of the Sea of Galilee (Israel), where 23,000-year-old barley grain remnants have been found in large quantities (Kislev et al., 1992; Weiss et al., 2008). Throughout the process of barley domestication, the effect of human selection under cultivation resulted in a plant type that produced an ever increasing amount of harvestable grain. Loss of the natural mode of grain dispersal was perhaps the most important single event in this process.

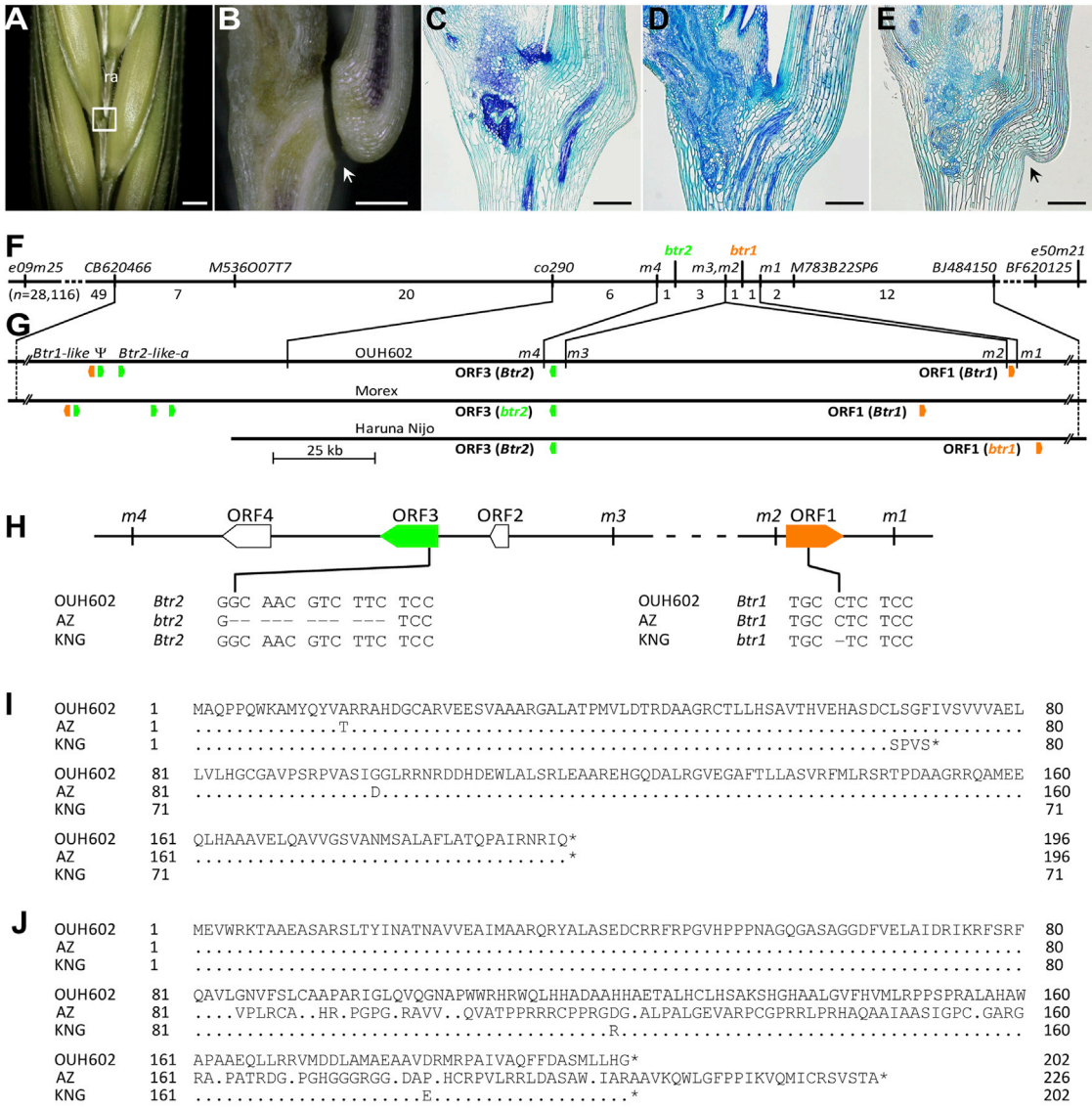


Figure 1. The Isolation of *Btr1* and *Btr2*

(A and B) The morphology of the rachis junction in *H. vulgare* subsp. *spontaneum* OUH602. (A) The connection between adjacent rachises at the milk stage. (B) The separation layer at the rachis node at the hard dough stage.

(C–E) Longitudinal sections of the rachis node (junction of two rachises) at anthesis stage. (C) cv. KNG. (D) cv. AZ. (E) F_1 resulting from hybridization of KNG and AZ.

(F) The genetic map of the *Btr* region based on segregation in the cvs. AZ × KNG and OUH602 × cv. KNG populations. *m1*: *M238J23-124046* (*co384*), *m2*: *M238J23-122853* (*co384*), *m3*: *M238J23-36088* (*co138*), *m4*: *M238J23-31195* (*co5*). The numbers beneath the line indicate the number of recombinants recovered. One recombination corresponds to a genetic distance of 0.0036 cM.

(G) The physical map of the *Btr* region in OUH602, Morex, and Haruna Nijo (GenBank: KR813335, KR813336 and KR813337). *Btr1* and *Btr1-like* are marked by orange arrows, *Btr2* and *Btr2-like* by green arrows, Ψ is a pseudogene sharing some homology with the *Btr2* sequence.

(H) The *btr1* and *btr2* alleles differ from their wild-type alleles *Btr1* and *Btr2* by a 1-bp and an 11-bp deletion respectively located in the coding sequences.

(I and J) Amino-acid sequence alignment for parental lines. (I) BTR1. (J) BTR2. *Stop codon. Scale bars, 1 mm (A), 250 μm (B–E).

See also Figure S1 and Table S1.

In barley, the architecture of the inflorescence is classified as a "spike" (Forster et al., 2007; Komatsuda et al., 2007). The individual dispersal unit is comprised of a central fertile spikelet (setting a single grain) along with two sterile lateral spikelets; this structure is attached to the node of the central floral axis, which is referred to as the rachis (Figures 1A and 1B). At maturity, the spike in wild (i.e., ancestral) barley forms "constriction grooves" (Ubisch, 1915) (Figure 1B) and disarticulates at each rachis node allowing mature grain to disperse freely. This phenotype is referred to as "brittle rachis." Classical genetic studies

have established that a mutation in either of two complementary and tightly linked genes on barley chromosome 3H, *Non-brittle rachis 1* (*btr1*) or *Non-brittle rachis 2* (*btr2*), converts the brittle rachis into a non-brittle type. Wild-type dominant alleles of both genes are required to produce the brittle rachis that is ubiquitous among wild barleys (Komatsuda et al., 2004; Schiemann, 1921; Senthil and Komatsuda, 2005; Takahashi and Hayashi, 1964). The genotype of non-brittle cultivated barleys is either *btr1Btr2* (hereafter *btr1*-type, Figure 1C) or *Btr1btr2* (*btr2*-type, Figure 1D) (Takahashi and Hayashi, 1964; Ubisch, 1915). F_1 plants (*Btr1btr1Btr2btr2*) produce a brittle rachis with constriction grooves (Figure 1E). A double-recessive (*btr1btr2*) cultivar has not as yet been identified (Komatsuda et al., 2004; Takahashi, 1955). In this study, we identify *Btr1* and *Btr2* genes and elucidate the mechanism underlying disarticulation of the wild-type barley spike. We explore the evolution of brittle rachis and its subsequent conversion to a non-brittle type during the process of domestication. Using comparative DNA sequence information and archaeo-botanical data (Tanno and Willcox, 2012) our data are consistent with independent origins of barley domestication.

RESULTS

Microdeletions Generated *btr1*, *btr2*, and the Non-brittle Rachis Phenotype

To identify the *btr1* and *btr2* genes, the cultivars (cv.) Kanto Nakate Gold (cv. KNG, *btr1*-type) and Azumamugi (cv. AZ, *btr2*-type) (Komatsuda and Mano, 2002) (Figures 1C and 1D) were intercrossed to produce a population segregating at both *btr* loci. Based on 10,084 F_2 individuals, it was possible to map the two genes to within a 0.19 cM region on chromosome 3H flanked by the markers *CB620466* and *BJ484150* (Figure 1F). This interval was covered in its entirety by a 403-kb contig generated by chromosome walking within a barley cv. Morex (*btr2*-type, see Table S3) bacterial artificial chromosome (BAC) library (Figures 1G and S1). A series of single nucleotide polymorphism (SNP) markers that differentiated cvs. KNG and AZ were developed across the contig for the purpose of fine mapping (Table S1).

An enlarged population of 14,058 F_2 individuals, derived from the crosses between cvs. AZ × KNG and OUH602 × cv. KNG (see Experimental Procedures), was then used to locate *btr1* by recombination to within a 1.2-kb interval (Figures 1F and 1G). Annotation of the 1.2-kb sequence identified only a single open reading frame (ORF1), which encodes a 196-residue protein. The 1.2-kb interval featured 18 sites that differed between cvs. KNG and AZ, with six sites located in ORF1. A comparison between the cv. KNG (*btr1*) and cv. AZ (*Btr1*) coding sequences revealed a 1-bp deletion (position +202, relative to the start codon in cv. KNG, see Figure 1H) predicted to induce a frameshift after changing leucine to serine at position 68. While a variable base at position +43 generated a threonine (T) residue at peptide position 15 in cv. AZ as opposed to an alanine (A) in cv. KNG, the wild barley accession OUH602 (*Btr1*) also carried alanine (A) at this position, indicating that this change could not be responsible for the non-brittle rachis phenotype (Figure 1I). The remaining four variable nucleotides were synonymous changes. To confirm the identity of ORF1 and *Btr1*, cv. Golden Promise (*btr1*-type) was transformed with the wild-type OUH602 ORF1 sequence under the control of the OUH602 native promoter (Figure S2A). The resulting primary transgenic plants (T_1) produced brittle spikes (Figures 2A and S2B), reminiscent of those formed by OUH602 (Figure 3A). In the T_2 generation, the transgene co-segregated with the brittle rachis character, confirming that ORF1 complemented the *btr1* allele of cv. Golden Promise (Figures 2A and S2C).

To identify *Btr2*, a set of 12,257 F_2 segregants, derived from crosses of cvs. AZ × KNG and OUH602 × RIL50 (RIL50 line is a *btr2*-type derivative selected from the cross of cvs. AZ × KNG [Komatsuda et al., 2004]; see Experimental Procedures), was analyzed. The *btr2* location was ultimately delimited by recombination to a 4.9-kb interval (Figures 1F and 1G) that contained three short open reading frames, designated ORF2–ORF4, as predicted de novo by applying the gene prediction software FGENESH. Based on sequence comparisons, ORF2 and ORF4 matched scores to hundreds of sequences in many locations across the barley genome (BLASTN and BLASTX to barley genomic sequences at http://webblast.ipk-gatersleben.de/barley/index.php), suggesting that they represent components of as yet un-annotated repetitive elements of the barley genome. The repetitive natures of ORF2 and ORF4 were confirmed by k-mer-statistical analysis against the barley genome (Schmutzer et al., 2014).

In contrast to ORF2 and ORF4, ORF3 represented low copy DNA (see "The Structure of the BTR Protein" section) and encoded a 202 amino acid residue protein. The KNG and AZ sequences covering the genetic interval differed at 69 nucleotide sites. A comparison between the ORF3 sequences present in cvs. AZ (*btr2*) and KNG (*Btr2*) revealed an 11-bp deletion positioned at +254–264 in cv. AZ (Figure 1H), which was predicted to create a frameshift after changing glycine to valine at position 85. The other three polymorphic sites within ORF3 were synonymous (Figure 1J). To test if ORF3 corresponded to *Btr2*, RIL50 was transformed using the construct *pUBI1*::*ORF3*, in which the OUH602 ORF3 sequence was driven by the maize UBIQUITIN1 promoter (Figure S2D). The T_1 plants formed spikes with a brittle rachis at maturity (Figures 2B and S2E; Movie S1), comparable with those of the wild barley OUH602 (Figure 3A), and the transgene co-segregated with the brittle rachis trait among the T_2 progeny (Figures 2B and S2F). Thus, ORF3 complemented *btr2*.

Brittleness Is Associated with Thin Cell Walls

A non-brittle rachis mutant, M96-1 (Figure 3B), was induced by sodium azide treatment of the wild barley accession OUH602 (Figure 3A) and proved to be a *btr1*-type based on allelic testing (Table S3). The M96-1 sequence (GenBank: KR813811) harbors a C-to-T transition at position +466 relative to the OUH602 sequence, which introduces a stop codon and results in a transcript with a truncated ORF1 encoding a 155 amino acid residue protein. In the M96-1 mutant, the dispersal units remain attached to one another at maturity (Figure 3B). In the brittle rachis line OUH602, toluidine blue staining of rachis sections at the anthesis stage revealed that five to six cell layers are expanded above each rachis node (Figure 3C), whereas no such expansion is

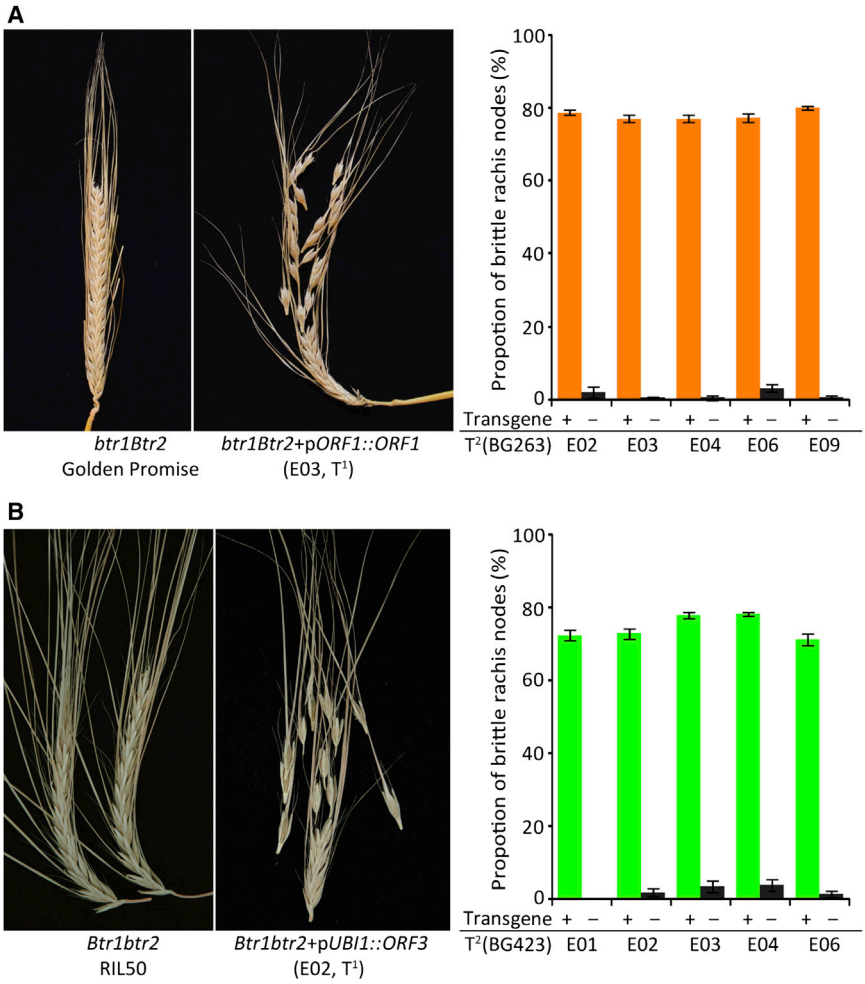


Figure 2. Complementation of *btr1* and *btr2*

(A) Genetic complementation of *btr1* by *ORF1*. The expression of *ORF1* in cv. Golden Promise (*btr1Btr2*) induces the brittle rachis phenotype in the primary transgenic (left) and is correlated with rachis brittleness in the T_2 generation (right).

(B) Genetic complementation of *btr2* by *ORF3*. The expression of *ORF3* in RIL50 (*Btr1btr2*) induces the brittle rachis phenotype in the primary transgenic (left) and is correlated with rachis brittleness in the T_2 generation (right). In (A) and (B), the values shown represent the % of brittle rachis nodes, bars indicate the SE.

See also Figure S2, Table S3, and Movie S1.

visible in M96-1 (Figure 3D). Disarticulation occurs at the so-called "constriction groove" (Ubisch, 1915) immediately below this expanded region (Figures 3C and 1B), although this region is not physically constricted.

The thickness of the cell walls in the wild barley OUH602 separation layer is ~25% of those in the M96-1 equivalent cells (Figures 3E, 3F, S3A, and S3B) with the thicknesses of both primary and secondary walls appearing to be greatly reduced (compare Figures 3E and 3F); the thin cell walls collapse at maturity, resulting in disarticulation across the plane of the cell wall that is marked by a smooth disarticulation scar (Figure 3G). In M96-1, detaching the grains from the rachis node requires the application of considerable force, which results in the formation of a rough, jagged disarticulation scar (Figure 3H). Fluorescence-based immunocytochemical studies using specific antibodies against cell wall polysaccharides revealed no significant differences in levels of heteroxylan, (1,3;1,4)-β-glucan, pectic polysaccharides, or cellulose in the disarticulation zones of both brittle and non-brittle rachis barley lines (Figures S3C–S3N). Furthermore, no reduction in lignin content was apparent in the separation layers (Figure 3M), in contrast to other cereal taxa (e.g., *Oryza*, *Bromus, Agropyron*, *Elymus*), where disarticulation is associated with an apparent reduction in lignin content in the disarticulation layer (Figures 3I–3L), at least using the acridine orange stain. In situ RNA hybridization experiments demonstrated that *Btr2* transcription is localized to within a few cell layers at the rachis node (Figures 3N and 3O), exactly where the five or six layers of cells mentioned previously expand to form the separation layer. This data is consistent with *Btr2* expression playing a key role in the spatial determination of the disarticulation cell layer above the rachis nodes.

The Structure of the BTR Protein

While functionally related, *Btr1* and *Btr2* share no significant similarity with one another at either the nucleic acid or the peptide sequence level, consistent with the hypothesis that they are complementary dominant genes (Takahashi and Hayashi, 1964). BLASTing BTR1 and BTR2 against the National Center for Biotechnology Information nr database revealed no hits within the Conserved Domain Database (CDD) using the default Expect (E)-value of 0.01. Secondary structure prediction by SOSUI (Hirokawa et al., 1998) databases predicted two lipophilic regions in BTR1, suggesting it might be a membrane-bound protein. In contrast, BTR2 was predicted to be a soluble protein. The BTR2 protein showed limited similarity with CAR and PIP motifs present in the IDA protein encoded by the *Arabidopsis thaliana INFLORESCENCE DEFICIENT IN ABSCISSION* gene, but BTR2 and IDA are not considered homologs because the amino acid similarity covers only a short region containing the CAR and PIP motifs. Re-sequencing the *btr1* and *btr2* regions in a large panel of wild and cultivated barleys showed that the two transmembrane domains in BTR1, and the CAR and PIP motifs in BTR2, are fully conserved (Figures S4A and S4B).

BLAST searches of the BTR1 and BTR2 sequences against the current barley genome assembly detected homology with two further hypothetical proteins, that we term BTR1-LIKE and BTR2-LIKE (Figures 4A and 4B). *Btr1-like* and *Btr2-like* genes

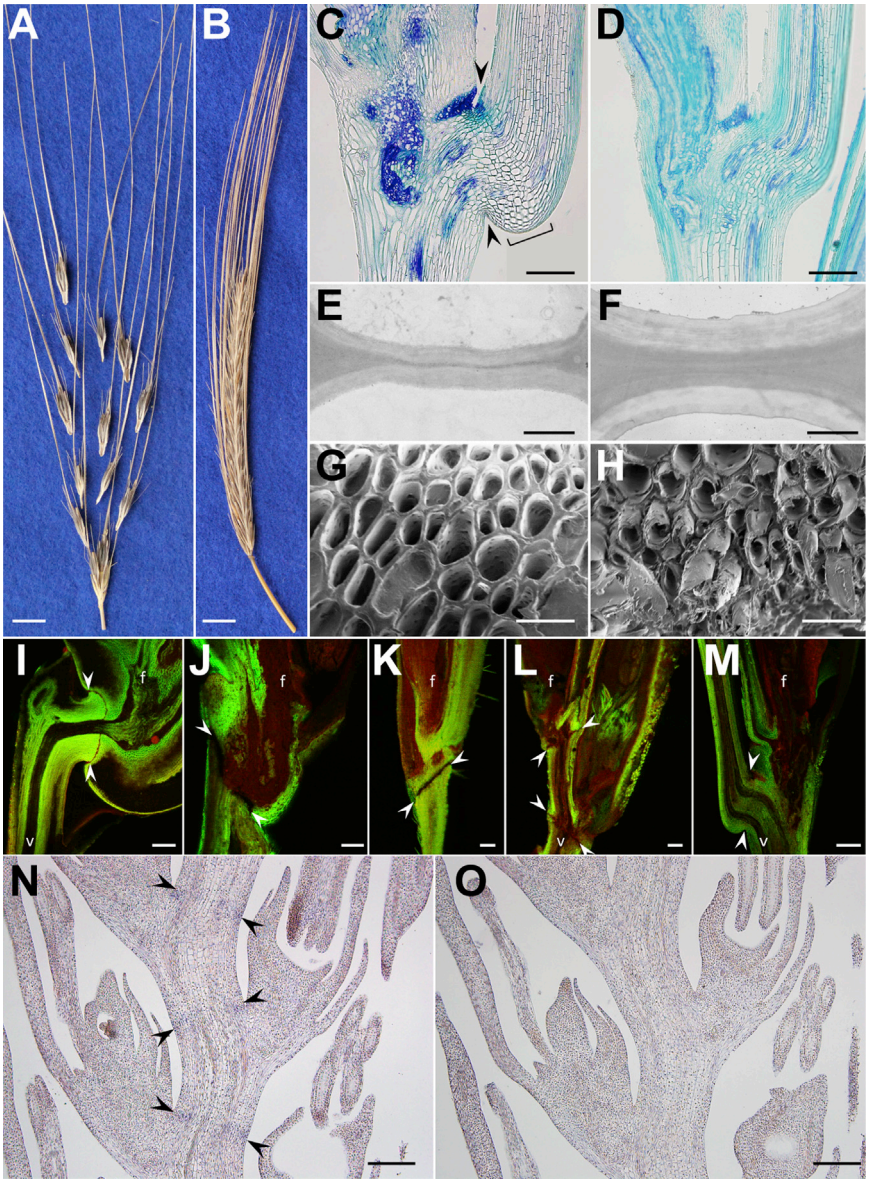


Figure 3. The Brittle Rachis Results from a Reduction in Cell Wall Thickness

(A–H) The rachis of wild barley accession OUH602 (A, C, E, and G) and of the induced non-brittle rachis mutant M96-1 (B, D, F, and H). (A and B) In the mature spike, the rachis is brittle in OUH602 but non-brittle in M96-1. (C and D) Longitudinal sections of the junction between two rachis nodes at anthesis stage, stained with toluidine blue. Arrows indicate the separation layer (or "constriction groove"), while the bracket indicates a layer of expanded cells. (E and F) Cell wall thickness in the separation layer prior to disarticulation, as imaged by transmission electron microscopy. (G and H) Surface of the disarticulation scar as imaged by scanning electron microscopy. In OUH602 (G), disarticulation was spontaneous, but in M96-1 (H), the grain had to be torn away from the spike.

(I–M) Longitudinal sections across the flower and pedicel of *Oryza rufipogon* (I), *Bromus japonicus* (J), *Elymus canimus* (K), *Agropyron cristatum* (L) and OUH602 (M). Acridine orange stains negatively charged materials such as lignin green. Lignin is lost from the abscission zone (arrowed), but not in OUH602.

(N and O) In situ RNA hybridization of *Btr2* in the immature (white anther stage) OUH602 spike. Longitudinal serial sections along the rachis were probed with either an antisense (N) or a sense *Btr2* (O, negative control) sequence. Arrowheads indicate sites of transcription in the abscission zone primordium. f, flower; v, vascular bundle. Scale bars, 1 cm (A and B), 250 μm (C and D), 1 μm (E and F), 25 μm (G and H), 100 μm (I–M), and 200 μm (N and O).

See also Figure S3.

lie in a head-to-head orientation with respect to one another, separated by just 400 bp and their location is only 103 kb away from *Btr2* in the wild barley OUH602 (Figure 1G). Other than those in Figure 1G, we could not detect any further *Btr* and *Btr-like* sequences in cv. Morex, based on the currently available barley genome sequence. This genomic configuration is consistent with *Btr1*/*Btr2* and *Btr1-like*/*Btr2-like* representing the product of a duplication event involving an ancestral gene pair. Dot-plot analysis supports this hypothesis (Figures S1A and S1B). *Btr1* and *Btr2* are separated from one another in Morex by 88 kb, by 111 kb in OUH602, and by 118 kb in cv. Haruna Nijo (Figure 1G). The greater separation between *Btr1* and *Btr2* compared to *Btr1-like* and *Btr2-like* appears to be the result of nested retrotransposon insertions (Figures S1C and S1D). The *Btr* and *Btr-like* genes duplication was detected in the wheat A genome as well (Figure 4A). The *Btr* duplications present in rice are independent from these of barley and wheat (Figure 4A), implying that this local gene duplication event occurred post separation of the Pooideae and Ehrhartoideae lineages some 40–53 million years ago (Aliscioni et al., 2012; International Brachypodium Initiative, 2010) but before the separation of wheat and barley some 8–12 million years ago (Chalupska et al., 2008; Middleton et al., 2014). Despite their sequence similarity, there was no evidence of any transmembrane helices or ligand motifs in either BTR1-LIKE, BTR2-LIKE or any of their homologs in related cereals (Figure 4A). Neither *Btr1-like* nor *Btr2-like* are functional paralogs of *Btr1* or *Btr2* as they are unable to complement *btr1* or *btr2* in barley cultivars. Furthermore, there are very pronounced differences of expression between *Btr1* and *Btr1-like* in spike development stages 1 to 5 and between *Btr2* and *Btr2-like* in stage 4 (Figure 4C). Collectively, these features suggest that *Btr* and *Btr-like* genes diverged functionally after a duplication event that occurred specifically in the Pooideae lineage. This divergence may have occurred during the evolution of the Triticeae as suggested earlier (Sakuma et al., 2011).

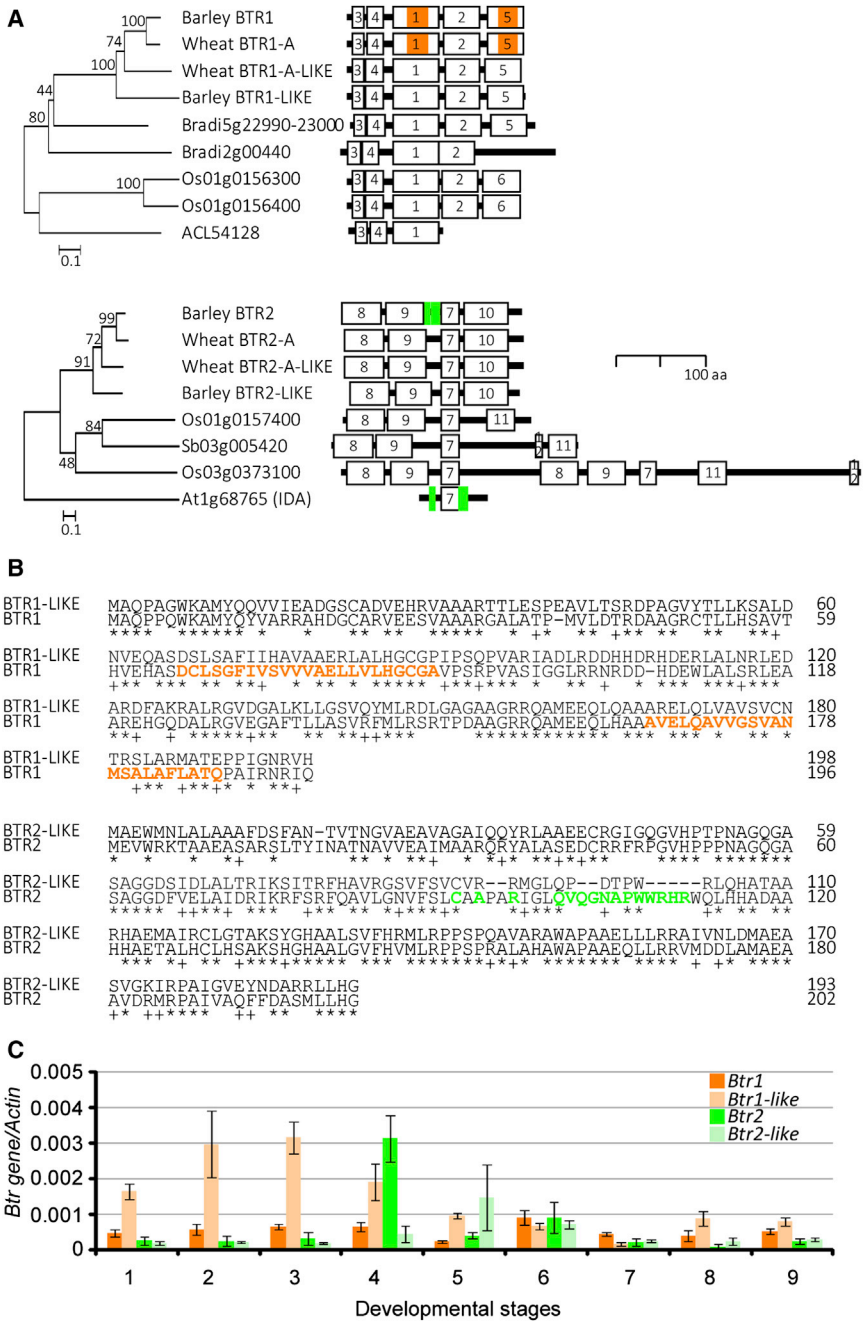


Figure 4. The BTR1 and BTR2 Proteins Determine the Brittle Rachis Trait

(A) Motifs present in BTR1 and BTR2 homologs. A BLASTX search identified one homolog of BTR1 in barley (named BTR1-LIKE) that shares 59% identity, BTR1 and BTR1-LIKE orthologs from wheat A genome (88% and 67% identity), two in *Brachypodium distachyon* (29% and 39% identity), and two in rice (23% and 26% identity). Only the BTR1 of barley and wheat were predicted to form transmembrane helices. For BTR2, one homolog (BTR2-LIKE) was identified in barley (55% identity), BTR2 and BTR2-LIKE orthologs from the wheat A genome (83% and 66% identity), two (12% and 27%) in rice, and one (17%) in sorghum; all of these homologs were also predicted to be soluble. The orange colored boxes in BTR1 indicate predicted transmembrane helices, while the green BTR2 boxes indicate, a CAR and a PIP motif. The peptide sequences of barley BTR1 and barley BTR2 were deduced from full length cDNA sequences of the wild barley OUH602 accession Genbank: KR813338 and KR813339. The peptide sequences of barley BTR1-LIKE and barley BTR2-LIKE-a were predicted from DNA sequences of wild barley OUH602 BAC accession Genbank: KR813335 using FGENESH. The peptide sequence of wheat BTR1-A was predicted from DNA sequences of *Triticum monococcum* subsp. *boeoticum* (KU101-1) sequence accession Genbank: KR813812 and the peptide sequences of wheat BTR1-A-LIKE, wheat BTR2-A, and wheat BTR2-A-LIKE are predicted from *Triticum aestivum* (Chinese Spring) sequence accessions Genbank: KR813813–KR813815 using FGENESH. Sequences prefixed by "Bradi" derive from *Brachypodium distachyon,* by "Os" from rice, by "ACL" from maize, by "Sb" from sorghum, and by "At" from *A. thaliana* were all extracted from public domain databases except for the *Brachypodium* gene "*Bradi5g22990-23000*," which was located between genes *Bradi5g22990* and *Bradi5g23000*, with a sequence position 25139096-25139701 on chromosome 5.

(B) Alignment of peptide sequences encoded by the two pairs of OUH602 *Btr* and *Btr-like* genes. Matching residues are marked by asterisks. Conservative amino acid substitutions are marked by "+" (BLOSUM 62 matrix). The two predicted transmembrane helices in BTR1 are marked in orange. The two BTR2 ligand motifs CAR and PIP (green) were inferred by homology with *A. thaliana* IDA (At1g68765).

(C) Relative transcript abundance of *Btr* and *Btr-like* genes during developmental stages in OUH602 as measured by qRT-PCR. *Actin* was used as the reference gene. 1, lemma primordium stage; 2, stamen primordium stage; 3, awn primordium stage; 4, white anther stage; 5, green anther stage; 6, yellow anther stage; 7, leaf post anthesis; 8, plumule during germination; 9, root during germination. Values represent mean ± SE (n = 3).

See also Figure S4 and Table S2.

The Immediate Wild Ancestors of *btr* Alleles and Their Geographical Distribution

A series of test hybrids was generated to reveal the genotype of 274 barley cultivars in a world core collection (Table S5). The *btr1* tester was cv. KNG and the *btr2* tester was RIL50. Scoring for brittle rachis in the hybrids revealed that 130 of the cultivars were *btr1* and 123 were *Btr1* (Figure 5A) and the remaining 21 cultivars could not be phenotyped because no F_1 plants were obtained. The *btr1*-type cultivars are widely distributed but are found at higher frequency in European and Middle Eastern barleys compared with those from East Asia (Figure 5B). Re-sequencing the 2.4-kb stretch of DNA spanning the locus (Table S2) revealed that all of the *btr1* lines carried the 1-bp deletion present in cv. KNG, while none of the *Btr1* carriers carried

this mutation (Table S5). While this further supports the hypothesis that the 1-bp deletion is responsible for the non-brittle rachis phenotype, it also indicates that the *btr1* allele is monophyletic (Figure 5C).

The equivalent test for *btr2* revealed that 124 of the cultivars carried *btr2* and 129 *Btr2* (Figure 5A). The remaining 21 cultivars could not be phenotyped because no F_1 plants were obtained. The *btr2*-type cultivars are distributed mainly in East Asia but also in North Africa (Figure 5B). Re-sequencing the key 4.9-kb interval (Table S2) showed that all of the *btr2* lines carried the 11-bp deletion (Table S5). No *Btr2* lines carried this deletion. We conclude that the 11-bp deletion is responsible for the non-brittle rachis in *btr2* lines and that this *btr2* allele is also monophyletic (Figure 5D).

An examination of sequence diversity at both loci, covering cultivated barley and a geo-referenced set of wild barley accessions representative of the geographical range of the species (Tables S5, S6, and S7), was based on a concatenated *Btr1*/*Btr2* sequence template. A total of 232 haplotypes was represented among 343 wild barley accessions and 53 among 267 domesticated cultivars (Table S4). In wild barley, nucleotide diversity (as measured by the parameters π and θ, see Experimental Procedures) declines steeply within the two coding regions, indicating that both coding sequences have remained relatively conserved in wild populations (Figure S5A). In cultivated barleys, the reduced level of diversity throughout the sequence almost certainly reflects the outcome of domestication (Figure S5B). Separation of the cultivated barleys into the *btr1*- and *btr2*-type subsets revealed a marked reduction in genetic diversity throughout the region (Figures S5C and S5D). These data indicate that only two alleles at each of the two loci have been retained after domestication, constituting a major genetic bottleneck. Network analysis revealed that, despite the large number of haplotypes, all of the cultivars belong to either the *btr1* or *btr2* lineage (Figures 5C and 5D), indicative of two independent origins and thus two domestication events.

Molecular dating analysis was conducted using a Bayesian inference method, with an uncorrelated relaxed clock for each *btr* locus separately. Both the complete sequence alignment and an alignment with the coding sequence were used to obtain estimated dates of divergence. These produced virtually identical results indicating that the *btr1*-type allele diverged $\sim$50,000 $\pm$ 10,000 years ago and the *btr2*-type allele diverged $\sim$40,000 $\pm$ 10,000 years ago.

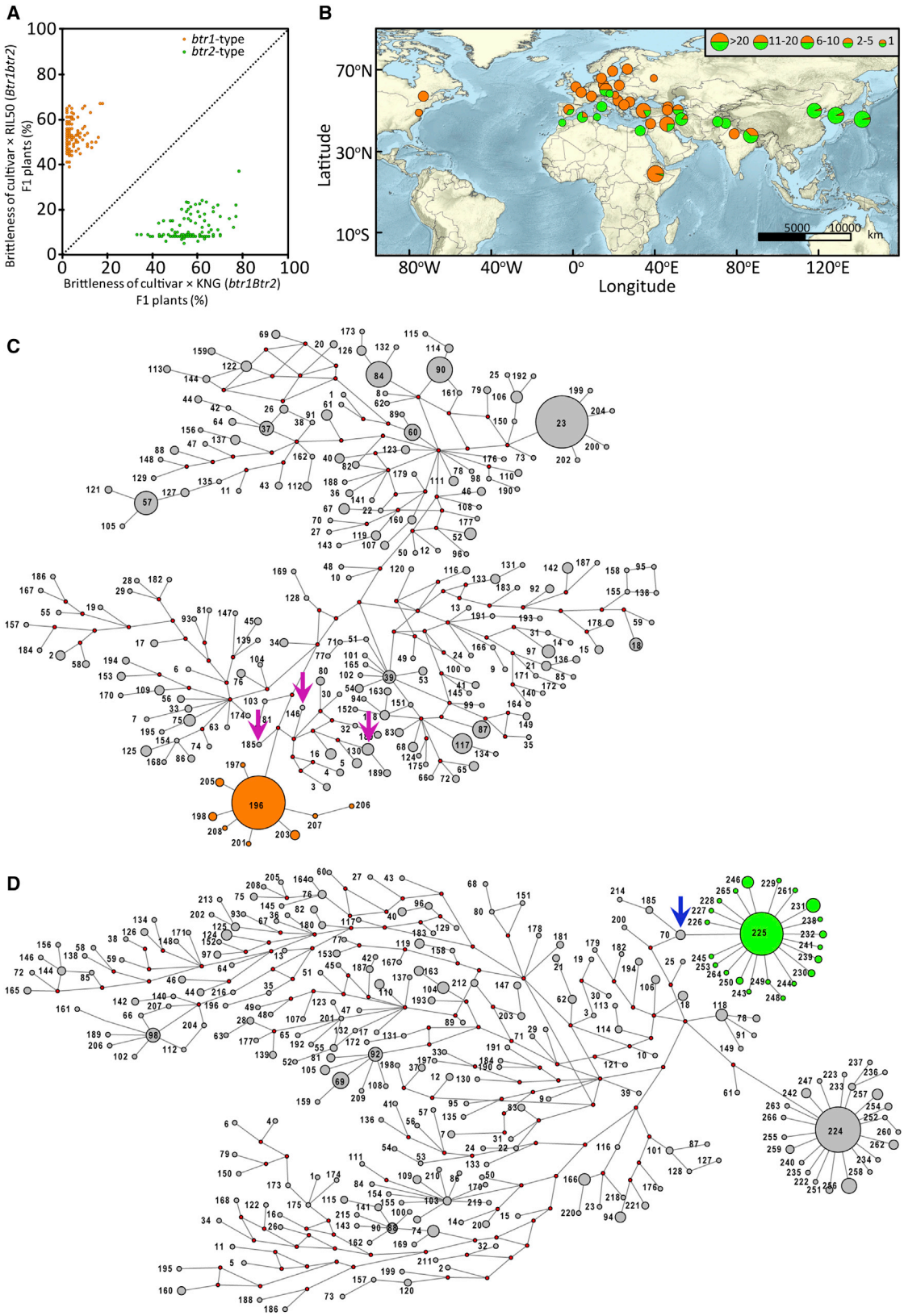
Evolutionary theory predicts that the immediate wild ancestors of cultivated barley should carry the same haplotypes as the cultivated ones, except for the mutations responsible for the loss of function of the key wild-type gene. We therefore explored whether the sequences of *btr1* and *btr2* loci in the wild germplasm could shed light on the domestication history of the species. The *btr1*-type cultivar subset featured nine haplotypes, of which the most common was B1Hap196 (indicated as "196" in the network of Figure 5C). None of wild barleys carried an immediate ancestral sequence of the *btr1*-type cultivars, and the haplotype most closely related to B1Hap196 was recovered in accessions originating from the southern Levant and Central Asia. With respect to the southern Levant, the relevant accessions were "2076" (ICWB181463) from Jordan (carrying B1Hap185 indicated as "185") and FT643 from Israel (carrying haplotype B1Hap146 indicated as "146") (Figures 5C and S6A). In Central Asia, the ancestral haplotype most similar to the *btr1*-type (B1Hap130, indicated as "130") is found in six accessions originating from Turkmenistan, Uzbekistan, and Afghanistan (Figures 5C and S6A; Tables S6 and S7). The next closest, both from Israel, were FT064 (B1Hap030) and FT075 (B1Hap032).

The *btr2*-type cultivar subset featured 22 haplotypes, with B2Hap225 by far the most common (indicated as "225" in the network of Figure 5D). For *btr2*-type cultivated barleys, we found four wild barley accessions with sequences (B2Hap070 indicated as "070" in Figure 5D) at the *btr2* locus that were identical to those present in the *btr2*-type cultivars, except for the 11-bp deletion (Figure 5D). Furthermore, these four wild barley accessions carried the B1Hap023 haplotype for *Btr1* that is identical to that in the *btr2*-type cultivars (Figure 5C), in accordance with the notion that B2Hap070 is the immediate ancestor of *btr2*-type. All four derive from western Syria/Southeast Turkey (Figure 6). The most closely related wild haplotype to B2Hap070 is B2Hap185, differing by just two nucleotides within 4.9-kb, while B2Hap214 differs by a further single nucleotide (Figure 5D; Tables S6 and S7). Two wild barley accessions having B2Hap185 were collected from Syria and a wild barley accession with B2Hap214 originated from Lebanon. The similar geographical origin of the accessions carrying B2Hap070, B2Hap185, and B2Hap214 suggests that they form a single evolutionary clade and implies that the progenitor of cultivated *btr2*-type cultivars grew in the northern part of Syria and Southeast Turkey (Figure 6).

To test this assertion, we used genotyping-by-sequencing (Poland et al., 2012) to obtain genome-wide genotypic data for 243 diverse cultivated and wild accessions of barley and assessed the genetic distance between them by principal component analysis (Figure 7). The first principal component clearly separated domesticated from wild barley (Figure 7B), while the second and third principal components reflected the geographic origins of accessions in the Fertile Crescent (Figures 7A and 7C). The closest wild barleys to *btr1*-type can be divided into two groups that are located at distinct positions in the plots; one from the southern Levant (FT643) and another genetically homogeneous group from Central Asia (FT566, FT567, and FT568). Three immediate ancestors of *btr2*-type (FT262, FT590, and FT670) from the northern Levant were closely related to each other genetically, but distinct from FT643 (Figure 7C). As expected, cultivated accessions were closely related to each other and distinct from wild species.

DISCUSSION

Here, we have identified two *btr* genes and causal mutations that are responsible for the evolution of the non-brittle rachis phenotype that was central in the process of barley domestication. By comparing non-brittle to brittle haplotypes observed in a geo-referenced collection of wild barley's our data highlight two distinct geographical regions where early farmers must have independently selected for mutations of the brittle rachis



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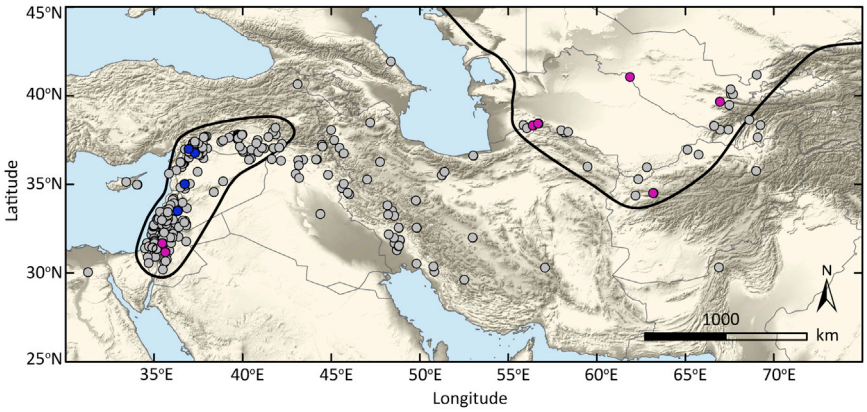

Figure 6. Cultivated Barley Originated from South and North Levant

Sites of domestication. The GIS-based map of the Fertile Crescent indicates the collection sites of the wild barley accessions harboring the proposed ancestral *btr1* (in purple, located in the southern portion of the Levant and Central Asia) and *btr2* (in blue, located in the northern portion of the Levant) alleles. The other wild barley analyzed are indicated with gray dots. Black lines indicate the Levant (left) and Central Asia (right).

See also Tables S6 and S7.

phenotype of wild barleys. Our data similarly provide an insight into the evolutionary history of the original brittle rachis genes.

The *Btr1* and *Btr2* genes are closely linked on chromosome 3H of barley and the nucleotide deletions that define the *btr1* and *btr2* alleles are consistent with the known dominance of the brittle over non-brittle phenotype (Johnson and Åberg, 1943; Schiemann, 1921; Takahashi and Hayashi, 1964; Ubisch, 1915). The two genes are separated from one another by ~100 kb, ensuring tight genetic linkage (Takahashi and Hayashi, 1964). However, recombination between these genes was detected in a large F_2 population, allowing the identification of individuals with either the *Btr1Btr2* genotype (two recombinant lines called AK-12691 and AK-34321), which formed a brittle rachis spike, and the *btr1btr2* genotype (a recombinant line called AK-11128) with a non-brittle rachis. As *btr1btr2* plants grow normally in the field, the lack of any cultivars of this genotype can be simply attributed to the tight linkage between the two genes.

The Evolution of Brittle Rachis

The brittle-rachis character is specific to species within the Triticeae tribe (family Poaceae), because only these species produce a spike as their unit of inflorescence. An alternative dispersal mechanism, referred to as "shattering," is widespread among Poaceae species, occurring in about half of the members of the Triticeae tribe (Sakuma et al., 2011). In this more ancient process, grains break off from the rachilla, a secondary axis in the grass inflorescence. That disarticulation at the rachis in barley and other members of the Triticeae is distinctive may relate to the more recent evolution of the spike compared to the panicle (Judd et al., 1999). The apparent reduction in lignin content in species with a rachilla type disarticulation layer could not be detected in the brittle rachis of wild-type barley. The disarticulation system that evolved in barley can also be distinguished from that occurring in grain or seed abscission in rice (Zhou et al., 2012) and *Arabidopsis thaliana* (Butenko et al., 2003), and from leaf abscission (Beck, 2010; Wang et al., 2010). In the latter cases, the removal of de-esterified homogalacturonan in the middle lamella weakens cellular cohesion and results in disarticulation (Bowling and Vaughn, 2011). The brittle rachis disarticulation system therefore appears to be unique to the Triticeae. In wild barley, it is associated with reduced cell wall thickness along the separation layer. This reduction in cell wall thickness is similar to that seen in barley *brittle culm* mutants, which are deficient in certain types of cellulose (Kimura et al., 1999) and that may be caused by lesions in *CesA* cellulose synthase genes (Burton et al., 2010). However, no obvious differences in the concentrations of cellulose, lignin or other cell wall polysaccharides could be detected in the separation zones of brittle and non-brittle barley rachis lines, suggesting that brittle rachis-related genes affect cell wall thickness per se but not wall composition. Given that *Btr1* and *Btr2* genes are detected in barley and wheat but neither in rice nor *Brachypodium*, it is tempting to speculate that the evolution of *Btr1* and *Btr2* was a significant driver in the conversion of the rachilla-type disarticulation system to rachis-type found in the Triticeae.

The biological functions of BTR1 and BTR2 proteins are unknown, although given their functional complementarity one might predict that they work together to produce the relatively

Figure 5. Cultivated Barley Arose Twice Independently

(A) The genotype of 274 accessions obtained by test crossing with cv. KNG (x axis) and RIL50 (y axis). Two genotypes were identified: *btr1btr1Btr2Btr2* (shown in orange) and *Btr1Btr1btr2btr2* (green).

(B) Geographical distribution and frequencies of 240 cultivated barley (120 *btr1*-type in orange and 120 *btr2*-type in green). Genotyping of 240 accessions by two test crosses and sequencing of *btr1* and *btr2* loci confirmed in this study. Cultivars were assigned to the capital of their source country.

(C) MJ network for the *Btr1/btr1* sequence (GenBank: KR813340–KR813547), based on the re-sequencing of 505 wild and 270 cultivated barley (145 *btr1*-type, 125 *btr2*-type) accessions. The multiple sequence alignment covered 2,914 nt and revealed 208 haplotypes. *Btr1* and *btr1* carriers are shown in gray and orange, respectively. Circle sizes correspond to the frequency of individual haplotypes. Closest wild haplotypes to *btr1*-type are indicated with purple arrows. Haplotype 1 corresponds to B1Hap001.

(D) MJ network for the *Btr2/btr2* sequence (GenBank: KR813548–KR813810), based on the re-sequencing of 357 wild barley and 267 cultivated barley (142 *btr1*-type, 125 *btr2*-type) accessions. The multiple sequence alignment covered 5,410 nt and revealed 263 haplotypes. *Btr2* and *btr2* carriers are shown in gray and green, respectively. Immediate wild ancestral haplotype to *btr2*-type are pointed with blue arrow. Haplotype 1 corresponds to B2Hap001.

See also Figures S5 and S6 and Tables S4 and S5.

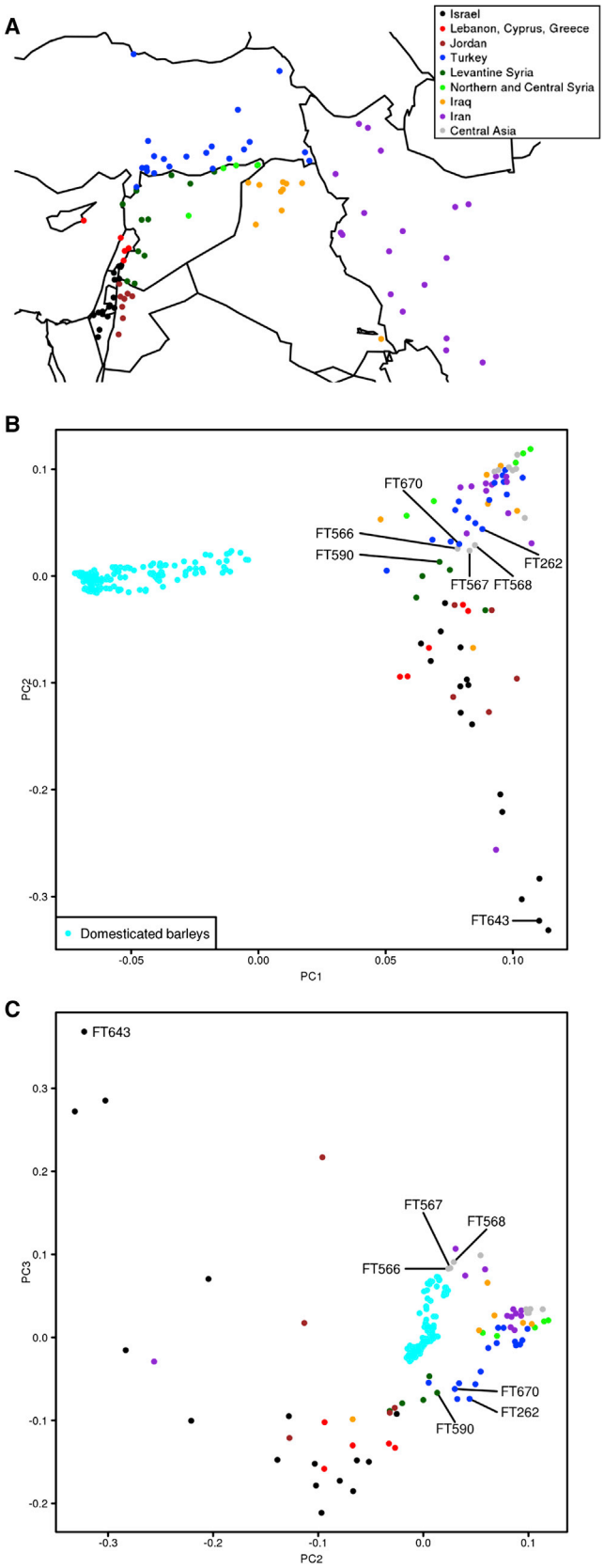

Figure 7. Genome-wide Diversity in Wild and Domesticated Barley

(A) Geographic origins of 81 wild barley accessions from the Fertile Crescent. (B and C) Principal component analysis based on 17,758 bi-allelic SNPs obtained by genotyping-by-sequencing. The first principal component (PC1) separates wild and domesticated germplasm (B). The placement of accession in the 2D space spanned by PC2 and PC3 (C) corresponds to their geographic origins in the Fertile Crescent. Domesticated accessions are shown in cyan in (B) and (C). Otherwise, colors correspond to countries of origins as described in the legend. Accession numbers with haplotypes close to *btr1*-type or *btr2*-type are indicated inside (B) and (C).

thin cell walls in the separation zones of the rachis. The close genetic linkage of pairs of functionally related genes has been observed in a number cases (Hurst and Lercher, 2005; Makino and McLysaght, 2008). For example, genes encoding a receptor protein kinase (*SRK*) and a cysteine-rich protein (*SCR*) are separated from each other by just 3 kb in a number of Brassicaceae genomes, and their products act as a receptor-ligand pair within the self-incompatibility response (Kachroo et al., 2002). We hypothesize that BTR1 and BTR2 as a potential receptor-ligand pair that, inter alia, mediates cell wall thickness. It must be emphasized that the supporting evidence for the receptor-ligand model is restricted to prediction tools and further studies are needed to test this hypothesis.

Geographical Origin of *btr* Alleles

Our data strongly suggest that *Btr1* and *Btr2* are domestication genes and that the barley non-brittle rachis phenotype evolved on two occasions. This contrasts with a previous proposal that non-brittle rachis had a single origin (Badr et al., 2000) but is consistent with the proposition that barley was domesticated more than once (Azhaguvel and Komatsuda, 2007; Takahashi and Hayashi, 1964). Our data allow us to identify the regions where the two crucial mutation events occurred, or at least where farmers started selecting for the non-brittle phenotype. The wild barley accessions most closely related to *btr1*-type cultivars were recovered from the southern Levant region and from Central Asia. The most genetically diverse extant stands of wild barley, along with the location of the most ancient archaeological finds, map to the southern portion of the Levant (Jakob et al., 2014; Zohary et al., 2013). One of the earliest finds of non-brittle barley was at Tell Aswad in modern day Syria, a site that dates from some 10,500 years ago (Tanno and Willcox, 2012). The southern Levant was a refugium for wild barley during the last glacial maximum (Jakob et al., 2014). Wild barley populations in Central Asia are not as genetically diverse as those in the Levant (Jakob et al., 2014) and archaeological finds are rare. A likely scenario is that these Central Asian accessions were derived from material spread by humans from the Levant. Overall, the genetic data suggest that the origin of *btr1*-type cultivated barleys in the southern Levant was a discrete domestication event. In an alternative scenario, the *btr1* allele could have been generated as a result of an intra-locus recombination within the coding sequence following an outcross involving wild barley (Figure S6B).

The immediate ancestors of *btr2*-type cultivated barley were found in two wild accessions from Syria and two from Turkey. The probability of a reverse mutation, which would require the insertion of an 11-bp segment into a non-functional *btr2* locus

to restore its function, is extremely low. Thus, these four lines cannot be secondary or feral populations that escaped from cultivation. A more plausible scenario is that they harbor the haplotype of the immediate ancestor of *btr2*-type cultivated lines. We therefore propose that the first *btr2*-type barley, a natural primary non-brittle rachis mutant, was selected in the northern Levant. While archeological records are incomplete, non-brittle barley grains recovered from the Syrian steppe at Tell Abu Hureyra and Tell Halula (80–120 km east of Aleppo, Syria) have been dated to 9,800 to 9,000 years ago (Hillman et al., 1989), while finds from Salat Cami (Southeast Turkey) date to 8,300 years ago (Tanno and Willcox, 2012). Pre-domestication sites, around which wild materials were probably cultivated (Weiss et al., 2006), have been identified across northern Syria and Southeast Turkey (Tanno and Willcox, 2006; Weiss et al., 2006; Zohary et al., 2013). The evidence therefore supports the emergence of *btr2*-type later than *btr1*-type non-brittle rachis. Genotyping by sequencing data support our findings that the closest wild barley accessions to *btr1*-type and *btr2*-type are highly diverged. The distribution of cultivated barleys is consistent with a recent study using transcriptome data (Dai et al., 2014) and is similar to the "Occidental" and "Oriental" cline of *btr1* and *btr2* types presented 60 years ago (Takahashi, 1955). Two "transition zones" were found, where major frequency changes between *btr1*- and *btr2*-types occur: the region between Iran and Afghanistan and the Levant and the southern part of the Mediterranean Sea. Besides these two transition zones *btr1*-types were found to predominate in India and Ethiopia. While possible that *btr1*- and *btr2*-type barleys may be better adapted to different eco-climatic zones—an alternative scenario is that their current distribution is a direct result of human migration.

In summary, it appears that the non-brittle rachis evolved from wild barley as a result of anthropogenic selection for mutations in two adjacent complementary dominant genes, the products of which are suggestive of a signal transducing receptor and its protein ligand that likely act in concert to control cell wall thickening in the disarticulation zone of the rachis node. However, the underlying mechanism for this process remains unclear. Four separate strands of evidence indicate that the *btr1*- and *btr2*-type barleys emerged independently in both time and location. The first centers on the observation that the most closely related sequence haplotypes in the wild species are closer to current cultivated *btr2*-type haplotypes than to *btr1*-type haplotypes; the second is that the archaeological record supports the pre-domestication cultivation of wild barley in the southern Levant occurring earlier than cultivation in the northern Levant; third is molecular dating of *Btr* genes; and forth is that wild barley survived the last ice age in the southern Levant. Based on this evidence, we conclude that *btr1*-type barleys emerged in the southern Levant prior to the appearance of *btr2*-types in the northern Levant.

EXPERIMENTAL PROCEDURES

Cloning of the *btr1* and *btr2*

Cultivar (cv.) "Kanto Nakate Gold" (KNG, JP 15436) and cv. "Azumamugi" (AZ, JP 17209) were obtained from NIAS, Tsukuba, Japan. Wild barley (*H. vulgare* subsp. *spontaneum*) accession OUH602 was obtained from the Institute of Plant Science and Resources, Okayama University, Kurashiki, Okayama, Japan. Three mapping populations were developed—from crosses between cvs. AZ × KNG (10,084 F_2 plants, segregating at both *btr1* and *btr2*), cv. KNG × OUH602 (3,974 F_2 plants segregating only at *btr1*), and RIL50 × OUH602 (2,173 F_2 plants segregating only at *btr2*). Ten hybrid plants produced by crossing each putative mutant plant with either cv. KNG (*btr1Btr2*) or RIL50 (*Btr1btr2*) were sown in the field and the proportion of brittle rachis nodes present calculated. The physical location of *btr1* and *btr2* was derived by constructing a localized BAC contig based on cv. Morex, cv. Haruna Nijo and a wild barley line OUH602. See the Supplemental Experimental Procedures.

Complementation by Transformation

The segment included the native *ORF1* promoter as part of a 3.2-kb sequence upstream of the start codon, 591 bp of the *ORF1* coding sequence and 1.1 kb of downstream sequence following the stop codon. The *ORF1* transgene (candidate *Btr1* allele of OUH602) was moved into immature embryos of cv. Golden Promise (*btr1Btr2*) to test for complementation (Table S3). The *ORF3* fragment, flanked by the maize *UBIQUITIN1* (*UBI1*) promoter plus the first intron of *UBI1* and the *A. tumefaciens NOS* terminator, was cut out by *Stu*I and introduced into a *Stu*I-linearized p6U binary vector to form the construct p*UBI1*::*ORF3*. The *ORF3* transgene (candidate *Btr2* allele of OUH602) was moved into RIL50 (*Btr1btr2*). See the Supplemental Experimental Procedures.

qRT-PCR Analysis

Immature spikes were developmentally staged and three replicate bulked samples (50 mg fresh weight) were taken for the purpose of RNA extraction. See the Supplemental Experimental Procedures.

RNA In Situ Hybridization Analysis

See the Supplemental Experimental Procedures.

TILLING

See the Supplemental Experimental Procedures.

Mutagenesis of Wild barley

OUH602 grains were treated with sodium azide and M_2 plants were raised in the field in a screen for non-brittle rachis mutants. See the Supplemental Experimental Procedures.

Cell Wall Morphology and Composition

Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and histochemical staining of sections to examine cell wall morphology and lignin content were performed using standard techniques. The presence and relative abundance of particular wall polysaccharides was investigated using fluorescence immunocytochemical procedures. See the Supplemental Experimental Procedures.

Prediction of the BTR Protein Structure

Sequence annotation, prediction of secondary structure, phylogenetic tree, and peptide motif analysis were performed using standard techniques. See the Supplemental Experimental Procedures.

Geographical Origin of *btr* Alleles

A panel of 274 cultivars representative of global barley diversity was obtained from Kazuyoshi Takeda, Institute of Plant Science and Resources (IPSR), Okayama University, Kurashiki, Japan (Table S5). A set of 150 accessions of subsp. *spontaneum* was obtained from Jan Valkoun, ICARDA, Syria (Table S6); finally, a composite collection of 379 entries of wild barleys was freshly collected from natural populations by B.K. (Table S7). Standard techniques for sequence analysis and molecular dating were outlined in the Supplemental Experimental Procedures.

Genotyping-by-Sequencing of Wild and Cultivated Barleys

Genotyping-by-sequencing (GBS) libraries were constructed for 243 barley accessions as described previously (Wendler et al., 2014). Further information about sequenced accessions is available under the following Digital Object Identifier (DOI): http://dx.doi.org/10.5447/IPK/2015/2. See the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession numbers for the analysis of the DNA sequences of the *btr1* and *btr2* alleles present in OUH602, cv. Morex, and cv. Haruna Nijo reported in this paper are GenBank: KR813335–KR813337.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, seven tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.07.002.

AUTHOR CONTRIBUTIONS

M.P., T.K., M.S., N. Senthil, G.C., P.A., S.K.N., and Y.N. carried out the fine mapping. M.P., T.K., and M.S. assessed rachis type. M.P. and T.K. constructed physical contigs, which were sequenced by H.K. and T.M. M.P., C.P.M., and T.W. annotated the contig sequences. C.P.M. analyzed molecular dating. M.P., G.H., T.K., and J.K. were responsible for creating transgenic material. M.P. and S.S. analyzed transcription. M.P., S.S., and A.T. performed RNA in situ hybridization. F.Y. performed TEM analysis. G.B.F. performed the cell wall analyses by immunofluorescent microscopy. K.S. produced the *btr1* mutant. S.G. and M.P. analyzed TILLING mutants of *btr2*. M.P., H.K., B.K., S.D., R.S., and N. Stein carried out haplotype analysis. B.K. and R.S. analyzed population genetic data and studied recombination rates and linkage disequilibrium. G.W. provided and analyzed the archaeobotanical data. A.W. created the GIS-based topographic maps. A.H. performed genotyping-by-sequencing. M.M. analyzed the data. M.P., B.K., and T.K. designed the experiments. M.P., B.K., R.S., G.W., G.B.F., R.W., and T.K. wrote the manuscript. All authors have reviewed and commented on the manuscript.

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