A novel mutation conferring the nonbrittle phenotype of cultivated barley

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Summary

- The nonbrittle rachis, resulting in a seed head which does not shatter at maturity, is one of the key phenotypes that distinguishes domesticated barley from its wild relatives. The phenotype is associated with two loci, Btr1 and Btr2, with all domesticated barleys thought to have either a 1 bp deletion in Btr1 or an 11 bp deletion in Btr2.
- We used a PCR genotyping method with 380 domesticated barley landraces to identify those with the Btr1 deletion and those with the Btr2 deletion.
- We discovered two landraces, from Serbia and Greece, that had neither deletion. Instead these landraces possess a novel point mutation in Btr1, changing a leucine to a proline in the protein product. We confirmed that plants carrying this mutation have the nonbrittle phenotype and identified wild haplotypes from the Gaziantep region of southeast Turkey as the closest wild relatives of these two landraces.
- The presence of a third mutation conferring the nonbrittle phenotype of domesticated barley shows that the origin of this trait is more complex than previously thought, and is consistent with recent models that view the transition to agriculture in southwest Asia as a protracted and multiregional process.

Introduction

Cultivated barley (Hordeum vulgare L. subsp. vulgare), the domesticated form of Hordeum vulgare ssp. spontaneum (C. Koch), was one of the founder crops of agriculture in the Fertile Crescent of southwest Asia (Zohary et al., 2013) and today is the fourth most important in terms of productivity (Ulrich, 2011). Domestication of barley was accompanied by a suite of phenotypic changes brought about by selective pressures resulting from human intervention in propagation of the wild plants (Fuller, 2007). Principal among these changes was an alteration in the architecture of the ears, which results in the mature spikelets (the dispersal propagules containing the grains) remaining attached to the flower head after ripening. This ‘nonbrittle’ phenotype contrasts with the wild ear, which is dehiscent, shattering at maturity and releasing the detached spikelets (Brown et al., 2009).

Genetic studies have associated brittleness with two closely linked loci, brittle-rachis 1 (Btr1) and brittle-rachis 2 (Btr2) (Takahashi & Hayashi, 1964), which have recently been shown to be independent genes located c. 100 kb apart on chromosome 3H (Pourkheirandish et al., 2015). Although the genes and their predicted protein products lack sequence or structural similarity, Btr1 and Btr2 are functionally related, both specifying a thinning of the cell walls in the rachis node, the structure that attaches the spikelet to the ear (Pourkheirandish et al., 2015). As a consequence of this thinning, the mature spikelets of wild plants are able to detach from the ear. The recessive versions of these genes, btr1 and btr2, give rise to rachis nodes with thicker cell walls, which result in a spikelet that can only be detached by threshing. In a recent survey of 240 cultivated barleys, all plants of the btr1 lineage had the same 1 bp deletion in the Btr1 gene, and all btr2 types had the same 11 bp deletion in Btr2 (Pourkheirandish et al., 2015). These results were interpreted as indicating that the btr1 and btr2 types of cultivated barley emerged independently in two different geographical regions, btr1 in the southern Levant and btr2 somewhat later in the northern Levant.

In this paper we report that some nonbrittle barley landraces have neither the 1 bp deletion in Btr1 nor the 11 bp deletion in Btr2 described by Pourkheirandish et al. (2015). Instead, these accessions have a point mutation in a different part of the Btr1 coding sequence. The discovery of this third cultivated lineage has implications for our understanding of the events that resulted in domestication of barley.

Materials and Methods

Barley accessions

We studied 380 barley landraces (Supporting Information Table S1), the set covering all ecological and geographic regions of European barley cultivation, and supplemented with a selection of landraces representing African and Asian regions. The set...
is largely nonoverlapping with the landraces studied by Pourkheirandish et al. (2015).

Genotyping and sequencing the Btr loci

We designed rapid genotyping systems capable of distinguishing the dominant and recessive versions of the Btr1 and Btr2 genes, assuming the underlying mutations giving rise to the recessive alleles were as previously reported (Pourkheirandish et al., 2015). To detect the 1 bp deletion characteristic of btr1, we used a PCR in which the 3’-end of the reverse primer spanned the 1 bp deletion site and produced a 205 bp product from the wild-type Btr1 allele and no product from the recessive btr1 allele. The Btr2 genotyping system amplified a 154 bp product from the wild-type allele, and a 143 bp product from btr2. Both PCRs were carried out in 12.5 μl reactions comprising 2 ng μl⁻¹ DNA, 9 μl Q5 reaction buffer, 1 μl Q5 high GC enhancer, 0.5 μM each primer, 0.2 mM each dNTP, 0.02 U μl⁻¹ Q5 high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) with an initial denaturation of 30 s at 98°C, followed by 35 cycles of 10 s at 98°C, 20 s at 66°C, 10 s at 72°C, and a final extension of 2 min at 72°C. Primer sequences are given in Table S2(a).

The status of those landraces that were genotyped as Btr1 Btr2 according to this screen was checked by sequencing of the Btr1 and Btr2 loci. The Btr1 gene was amplified as a 2425 bp fragment and the Btr2 gene as a 4942 bp fragment, using the primers described in Table S2(a) and the PCR conditions described earlier, except that for Btr1 the annealing step was performed at 62°C and the synthesis step was carried out for 1 min, and for Btr2 annealing was at 60°C and synthesis for 2 min. Amplicons were purified (MinElute PCR Purification Kit; Qiagen) and sequenced (ABI 3730 DNA analyzer; ABI, Foster City, CA, USA) using various primers (Table S2b). Novel sequences have been deposited in GenBank, accession numbers KX722223–KX722226.

Sequence data analysis

Median joining networks (Bandelt et al., 1999) were prepared by aligning the sequences that we obtained with GenBank accessions KR813340–KR813547 (Btr1/btr1) and KR813548–KR813810 (Btr2/btr2) (Pourkheirandish et al., 2015). Alignment gaps were treated as missing data and all polymorphic sites were used in network construction. In these networks, edge lengths are proportional to the number of substitutions, but the node sizes do not reflect haplotype frequencies. Protein Variation Effect Analyzer (PROVEAN) v.1.1 (Choi & Chan, 2015) and the transmembrane orientation of the BTR1 protein predicted by SOSUI

Fig. 1 Structure of the novel btr1 allele in domesticated barley. (a) Alignment between the amino acid sequences of the wild-type brittle allele (Btr1), the previously reported nonbrittle allele (here called btr1A), and the novel nonbrittle allele that we report (btr1B). The Btr1 and btr1A sequences are from Hordeum spontaneum OUH602 and Hordeum vulgare cv KNG, respectively (Pourkheirandish et al., 2015). The BTR1 protein has two hydrophobic regions indicated by green shading. Changes giving rise to the two btr1 alleles are highlighted in yellow. (b) Transmembrane orientation of the BTR1 protein predicted by SOSUI (Hirokawa et al., 1998). The hydrophobic regions and the Leu–Pro substitution in the btr1B product are indicated using the same colours as in (a).
(Hirokawa et al., 1998) were used to assess the impact of amino acid changes.

Results

The genotyping screen identified 300 of the 380 barley accessions as btr1 Btr2 and 78 as Btr1 btr2, each of these accessions having the canonical 1 or 11 bp deletion described by Pourkheirandish et al. (2015). The remaining two landraces were typed as Btr1 Btr2. These two landraces were PI 374426, collected in 1971 at Rása, Serbia (described as awned, six-rowed, spring type, hulled), and HOR 683, collected in 1942 from the Peloponnese, Greece (spring type). Sequencing of the Btr1 and Btr2 loci in these accessions confirmed that neither possessed the 1 or 11 bp deletions associated with btr1 and btr2, respectively. Instead, both accessions displayed a single T→C transition converting a leucine to proline at position 111 in the Btr1 protein product (Fig. 1a). Henceforth, we refer to this novel mutation as the btr1b allele, in contrast to btr1a, which displays the canonical 1 bp deletion.

Btr1 codes for a transmembrane protein similar to signal transduction receptors. The amino acid substitution specified by the btr1b allele is predicted to lie within the cytoplasmic component of the protein (Fig. 1b). PROVEAN assigned a score of −5.0 (deleterious) with 95% probability, reflecting the different structural and chemical properties of proline compared with leucine (Wu, 2013). It is therefore reasonable to hypothesize that the substitution will have an impact on the function of the protein.

To confirm that the btr1b allele confers the nonbrittle phenotype, and that accessions carrying this allele are not mislabeled wild barley accessions, we germinated seeds of PI 374426 and grew plants to maturity. The resulting seed heads had a typical domesticated six-rowed phenotype (Fig. 2). The mature rachis was tough and remained intact after the spikelets had been

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Fig. 2 Comparison of the seed head of barley landrace PI 374426, possessing the btr1b allele, with a wild barley seed head. (a) Seed head of PI 374426, displaying a nonbrittle, six-row phenotype. (b) After forcibly removing spikelets (right) from the PI 374426 ear, the rachis remains intact (left). (c) The mature seed head of wild barley accession PI 662202. (d) With PI 662202, the mature rachis disarticulates into segments that remain attached to the spikelets. It is impossible to remove spikelets without breaking the rachis. Bars, 2 cm.
forcibly removed from the ear. DNA was then extracted from seeds from two spikelets to confirm the presence of the \textit{btr1b} allele. By contrast, with the wild accession PI 662202, which was genotyped as \textit{Btr1 Btr2}, the mature rachis disarticulated into segments that remained attached to the spikelets, and it was impossible to remove spikelets without breaking the rachis.

Median joining networks were constructed to identify the relationships between the \textit{btr1b} and \textit{Btr2} haplotypes of PI 374426 and HOR 683 and the \textit{Btr1} and \textit{Btr2} haplotypes of wild barley published by Pourkheirandish et al. (2015). In the \textit{Btr1/btr1} network, the two \textit{btr1b} haplotypes were located together at a position distant from the \textit{btr1a} sequences, and were most closely related (one to four substitutions) to the \textit{Btr1} haplotypes of four brittle wild barleys (IPK IDs FT266, FT624, FT730, FT747), all of which were collected from the Gaziantep region of southeast Turkey (Fig. S1a). The \textit{Btr2/btr2} network gave a similar result, the \textit{Btr2} haplotype of PI 374426 and HOR 683 showing the closest relationship with the \textit{Btr2} haplotypes of the same four wild Gaziantep accessions (Fig. S1b).

**Discussion**

The assumption that there are two lineages of cultivated barley, originally called the ‘Oriental’ and ‘Occidental’ families, dates back 60 yr to the pioneering genetic studies of Takahashi (1955). Recently, genomic work has suggested that all cultivated accessions fall into one or other lineage, characterized by a 1 bp deletion or 'brittle rachis' mutation in the \textit{Btr1} or \textit{btr2} genes (Poets et al., 2015; Civán et al., 2013; Willcox, 2013; Poets et al., 2015). However, we note that those wild accessions with \textit{Btr2} haplotypes closest to \textit{btr2} come from northern Syria and the Gaziantep region of Turkey (Pourkheirandish et al., 2015). It is therefore possible that the domesticated lineages with the \textit{btr2} and \textit{btr1b} alleles derive from the same wild population source.

Hypotheses regarding the origins of agriculture in the Fertile Crescent of southwest Asia have undergone a dramatic shift in recent years. The earlier interpretation of the transition from hunter-gathering to agriculture as a rapid ‘revolutionary’ event, with each of the founder crops of agriculture emerging from one or, at most, two discrete geographical locations (Diamond, 2002), is now being replaced by a more sophisticated model in which the transition to agriculture was a protracted and multiregional process, with the domesticated germplasm of each crop derived from a variety of wild populations (Civán et al., 2013; Willcox, 2013; Poets et al., 2015). The presence of additional brittle rachis mutations in cultivated barley is consistent with this model and emphasizes the geographically dispersed nature of the anthropological and microevolutionary events that resulted in the emergence of agriculture in southwest Asia.

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**Author contributions**

P.C. carried out the laboratory work and data analysis. P.C. and T.A.B. jointly conceived the project and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Median-joining networks showing the relationships between the cultivated accessions containing btr1b alleles and wild barley accessions.

Table S1 Barley landraces.

Table S2 Primers: PCR primers and sequencing primers.

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