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Notes:

Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene

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Increased seed production has been a common goal during the domestication of cereal crops, and early cultivators of barley (*Hordeum vulgare* ssp. *vulgare*) selected a phenotype with a six-rowed spike that stably produced three times the usual grain number. This improved yield established barley as a founder crop for the Near Eastern Neolithic civilization. The barley spike has one central and two lateral spikelets at each rachis node. The wild-type progenitor (*H. vulgare* ssp. *spontaneum*) has a two-rowed phenotype, with additional, strictly rudimentary, lateral rows; this natural adaptation is advantageous for seed dispersal after shattering. Until recently, the origin of the six-rowed phenotype remained unknown. In the present study, we isolated *vrs1* (six-rowed spike 1), the gene responsible for the six-rowed spike in barley, by means of positional cloning. The wild-type *Vrs1* allele (for two-rowed barley) encodes a transcription factor that includes a homeodomain with a closely linked leucine zipper motif. Expression of *Vrs1* was strictly localized in the lateral-spikelet primordia of immature spikes, suggesting that the *VRS1* protein suppresses development of the lateral rows. Loss of function of *Vrs1* resulted in complete conversion of the rudimentary lateral spikelets in two-rowed barley into fully developed fertile spikelets in the six-rowed phenotype. Phylogenetic analysis demonstrated that the six-rowed phenotype originated repeatedly, at different times and in different regions, through independent mutations of *Vrs1*.

domestication | evolution | grass | transcription factor | *vrs1*

Domestication of the major modern cereal crops started $\approx 10,000$ years before the present (yBP) (1–4). Throughout the process of cereal domestication, humans have deliberately selected individuals of wild species to emphasize seed recovery (5–7) and improved seed yield (1, 8). One of the most conspicuous instances of this process that occurred in the Near East was the appearance of six-rowed spikes during the domestication of barley (*Hordeum vulgare* ssp. *vulgare*; Fig. 1A and B). The barley spike is composed of triplets (each with one central and two lateral spikelets) arranged alternately at rachis nodes. All three spikelets of the modern six-rowed barley cultivars are fully fertile and able to develop into grains, but the lateral spikelets of two-rowed barley are reduced in size and are sterile (Fig. 1C–G). The reduced lateral spikelets of this phenotype have greatly reduced stamens and a rudimentary ovary and stigma (Fig. 1H) compared with those of the central spikelets (Fig. 1I). Wild barley (*H. vulgare* ssp. *spontaneum*), the progenitor of cultivated barley (1, 9), is two-rowed, and its arrow-like triple spikelets, a product of disarticulation of the mature inflorescence due to brittleness of the rachis (main axis), are an adaptive specialization that ensures that the seeds will bypass stones and pebbles and reach soil when they fall to the ground (10). This feature is an evolutionary advantage offered by the two-rowed spikes in nature, and spontaneous six-rowed mutants are eliminated naturally and rapidly from wild barley populations because they lack

this adaptation (10). Thus, six-rowed barley occurs primarily as cultivars or weeds (9).

The earliest archaeological specimens of barley seeds were uncovered from preagricultural sites in the Near East dating from 19,000 to 9,000 yBP (1). The specimens showed kernels of a two-rowed barley with a brittle rachis that are essentially identical to present-day wild barley. The earliest domesticated barley (9,500–8,400 yBP) also had two-rowed spikes; cultivation of six-rowed barley started later, with estimated dates ranging from 8,800 to 8,000 yBP (1, 11). Around 7,000–6,000 yBP, when barley was cultivated in the alluvial soils of Mesopotamia and, later, in the soils of Lower Egypt, six-rowed barley soon became dominant, replaced two-rowed barley, and established itself as the most important crop for Near Eastern Neolithic civilizations (1, 11, 12).

The development of a six-rowed spike is controlled by a single allele, *vrs1* (formerly *v* for *vulgare*), that is recessive to the dominant allele responsible for the two-rowed spike (*Vrs1*) (13, 14). Although *Intermedium spike-c.h* (*Int-c.h*), which occurs in six-rowed barley, is involved in enlarging the size of lateral spikelets, the presence of the recessive gene *vrs1* is by itself sufficient to cause two-rowed barley to become six-rowed barley (14). Three additional genes have been identified on different chromosomes through artificially induced mutations; however, none of these genes has been found in known six-rowed cultivars, probably due to the phenotypic disadvantages that occur in these mutants, such as reduced size and reduced fertility of the lateral spikelets on the upper and lower portions of the spikes (14). These observations indicate that *Vrs1* has been the primary target of mutation during the evolution of six-rowed barley. It has been assumed that six-rowed barley developed from domesticated two-rowed barley by means of spontaneous mutation (1, 12), but the origin of six-rowed barley has not been confirmed. In the present study, we isolated the *vrs1* gene and used studies of this gene to reveal the origin of six-rowed barley.

Results

Map-Based Cloning of *Vrs1*. We previously mapped the *vrs1* locus to a 0.90-cM interval between *cMWG699* and *MWG865* (15, 16). In

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Abbreviations: HD-ZIP, homeodomain-leucine zipper; yBP, years before present.

Data deposition: The sequences reported in this paper have been deposited in the EMBL/GenBank/DBJ database (accession nos. AB259782, AB259783, and EF067844).

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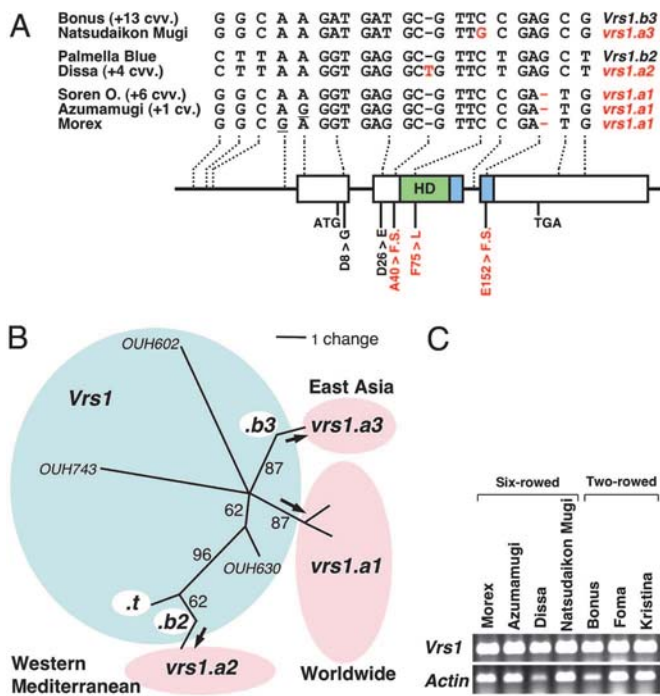


Fig. 4. Molecular events occurred in six-rowed barley. (A) Haplotype analysis of the *Vrs1* region in two- and six-rowed cultivars. (B) Phylogenetic tree of *Vrs1* alleles illustrating the three independent origins of six-rowed barley. Three wild barley lines (“OUH” identifiers) are outgroups. (C) RT-PCR analysis of *Vrs1* expression in immature inflorescences of six-rowed and two-rowed barley.

vice versa, because the wild barley lines (OUH602, OUH630, and OUH743) were outgroups. The *vrs1.a2* allele has an insertion of one nucleotide in exon 2, which results in a frame shift of the deduced amino acid sequence (Fig. 4A). The *vrs1.a3* allele has a substitution of one nucleotide in which the phenylalanine (F) at amino acid position 75 becomes leucine (L) (Fig. 4A). The F is highly conserved in the DNA-binding domains of plants, animals, and yeasts, as shown by BLASTP analysis against the database of conserved domains (20). Three *hex-v* mutants (*hex-v.12*, *hex-v.13*, and *hex-v.14*) also revealed the replacement of F by another nucleotide at position 75, supporting the importance of this amino acid in the DNA-binding domain. The *vrs1.a3* allele occurred in one six-rowed cultivar (“Natsudaikon Mugi”) in East Asia. The most common allele, *vrs1.a1*, which is distributed around the world (10 cultivars), has a deletion of one nucleotide in exon 3, which results in a different frame shift (Fig. 4A). The progenitor of the *vrs1.a1* allele remains to be identified (Fig. 4B). It was noteworthy that the *vrs1.a1* allele has been differentiated into three subhaplotypes by point mutations in the 5′ noncoding region (Fig. 4A). Two- and six-rowed barleys showed equal levels of transcription of *Vrs1* (Fig. 4C), thus single-base-pair mutations in *vrs1* appear to be responsible for the functional changes observed in all three natural six-rowed alleles.

Discussion

The evolution of barley was not a single event but has been an ongoing process of continuous specialization, as suggested by the concept of diffuse centers of differentiation (12). All of the present barley forms have resulted from specialization as a result of cumulative mutations, and tracing the origin of six-rowed barley would require us to find the least-specialized form of barley either conceptually (based on genetic analysis) or archaeologically. One of the main arguments in the debate over the evolution of barley relates to genetic changes between the brittle and nonbrittle rachis and between the two- and six-rowed spikes. We previously inferred

the phylogeny of rachis brittleness (21) and of the number of rows of spikelets (22) based on molecular markers that are tightly linked to the *btr1/btr2* and *vrs1* genes. The genealogy based on each domestication gene (here, a domestication gene is one that motivates humans to domesticate a plant) allows adequate inferences about the origins of cultivated barley. In the present study, cloning of the *Vrs1* gene substantiated our proposed phylogeny for the number of rows of spikelets. Wild and cultivated barley are interfertile (9, 23), and mutual introgression of genes appears to be substantial between the two forms (24). Therefore, random markers do not appear to represent the two- and six-rowed types of barley, and evolutionary studies based on DNA markers that are inherited independently of the domestication genes might provide different insights into the specialization of barley (25, 26). In the present paper, we focus exclusively on the origin of six-rowed barley. In a more strict sense, we focus on the *Vrs1* gene rather than the whole genome of barley to elucidate the origin of six-rowed barley, even though our data might have some implications for evolution of the species.

An HD-ZIP I Homeobox Gene Determines the Number of Rows of Spikelets in Barley. Our study revealed that *Vrs1* encodes a member of the HD-ZIP class of transcription factors. Analysis of induced mutants clearly confirmed the identity of *Vrs1* and demonstrated its biological function. Although complementation analysis by transformation is another option (27), our data, based on as many as 57 independent mutant lines, together with the specific gene expression patterns, confirm that *Vrs1* encodes an HD-ZIP I protein. Although HD is universal, the HD-ZIP is unique to the plant kingdom. HD-ZIP protein forms dimers via ZIP and correctly binds by means of HD to dyad-symmetrical recognition sequences of DNA based on the strict spatial relationship between HD and ZIP (28, 29), and the DNA-binding activity of HD-ZIP was demonstrated (30). HD-ZIP proteins have been grouped into four families (I–IV) (31), and the barley VRS1 protein belongs to family I (data not shown). The HD-ZIP III and IV genes are functionally well characterized, being involved in (i) development of the apical meristem, (ii) vascular development, and (iii) establishment of cell fates in epidermal (32). The biological function of the HD-ZIP I and II genes has not yet been clarified, although involvement in plant growth and development was suggested (32). Our study shows a conspicuous association between an HD-ZIP I gene and a plant developmental process. The spatial and temporal specificity of *Vrs1* gene expression suggests that VRS1 is a transcription factor involved in the development (suppression) of lateral spikelets in two-rowed barley. Most domestication genes have been found to encode transcription factors (33), and our results agree with these observations.

During the domestication of barley and wheat, rudimentary spikelets or flowers of wild species have been restored to their full functionality to increase seed number (1, 8). In the present study, we demonstrated that loss of function of *Vrs1* is sufficient to convert the rudimentary spikelets of two-rowed barley into the fertile spikelets of six-rowed barley. The mutations found in six-rowed cultivars were commonly represented by single-nucleotide polymorphisms, but even more drastic changes such as complete deletion of *Vrs1* were also found in several induced mutants (Figs. 3 and 4). This loss of function agrees with the facts that six-rowed spikes are genetically recessive and that two-rowed spikes are controlled by the wild-type allele, which occurs naturally in wild barley. The creation of six-rowed spikes is similar to the gigantism that occurs during domestication because both types of change can be caused by loss of function of genes (34). The dominant nature of *Vrs1* and the potential DNA-binding activity of HD-ZIP I proteins suggest that VRS1 is a repressor protein that binds to the DNA of genes that regulate the development of lateral spikelets. Further investigation of the subcellular localization of VRS1 proteins will be necessary to test this hypothesis.

Multiple Origins for Six-Rowed Barley Implied by the Mutant *Vrs1*/HD-ZIP I Gene. The number of rows of spikelets is a key characteristic in inferring the origin of cultivated barley, and it has been debated whether the progenitor of cultivated barley was six-rowed (35), two-rowed (12, 36), or both (37, 38). Recently, the two-rowed progenitor hypothesis was supported by (i) archaeological specimens showing preagricultural utilization of wild barley and the existence of domesticated two-rowed remains that were older than six-rowed barley remains, (ii) the dominance of the two-rowed phenotype, and (iii) the rather weedy nature of brittle six-rowed barley in Israel and Tibet (9–12).

Our phylogenetic tree (Fig. 4B) shows two lineages of domesticated two-rowed barley that diverged considerably from each other: *Vrs1.b3* stood alone, and the other lineage further differentiated into *Vrs1.b2* and *Vrs1.t* (Fig. 4B). Owing to its prevalence in the western Mediterranean, the *Vrs1.b2* allele is likely to be an immediate progenitor of the *vrs1.a2* six-rowed allele, which predominates in the western Mediterranean. This is an indication that one of the six-rowed alleles originated in this region. Similarly, it is clear that the *vrs1.a3* six-rowed allele derived directly from the *Vrs1.b3* two-rowed allele by means of a point mutation (Fig. 4A). Indeed, the Korean *vrs1.a3* cultivar has great similarities to old European two-rowed cultivars, such as a spring growth habit, nonbrittle rachis due to the recessive *bt1* gene (38), lax spikes, a long stem, and the K haplotype of *cMWG699*, which is specific to two-rowed barley (22). It is interesting to speculate that the *vrs1.a3* six-rowed cultivar might have originated from European two-rowed barley introduced into Eastern Asia. Our results suggest that, at least for the *vrs1.a2* and *vrs1.a3* alleles, six-rowed barley was derived from cultivated two-rowed barley. Because the number of lines of wild barley was limited in the present study, further collection of wild barley will be necessary to find a potential wild ancestor for each allele.

We were unable to resolve the origin of the most common *vrs1.a1* six-rowed allele in two-rowed barley (Fig. 4B). A notable feature of the *vrs1.a1* allele is that it has three subhaplotypes (Fig. 4A). If we assume the minimum number of changes, the *vrs1.a1* allele must have originated from a hypothetical *Vrs1.b1* allele in a single, unknown, two-rowed ancestor, and the first *vrs1.a1* allele (which today exists in cv. 'Soren Oomugi 19329'; Fig. 4A) has differentiated into two additional haplotypes by means of background point mutations in the 5' noncoding region. The *vrs1.a1* cultivars correspond to the major Type I cultivars classified by use of a marker tightly linked to *vrs1*, and the dominant Type I cultivars are distributed throughout the world (22). Because of its predominance and higher haplotype diversity, we hypothesize that the *vrs1.a1* allele may represent the most ancient six-rowed allele (dating to 7,000–6,000 yBP), which was widely present in Neolithic agriculture in the Near East.

Our study demonstrates three independent processes for the origins of six-rowed barley (Fig. 4B). (This result does not necessarily mean that barley has a polyphyletic origin as a species, a distinction that is often misunderstood.) We previously hypothesized two independent origins of six-rowed barley, one of which gave rise to the six-rowed barley that has spread around the world and the other of which gave rise to the six-rowed barley endemic to the western Mediterranean (22). Our present study supports the concept of multiple origins of six-rowed barley. We believe these origins are straightforward because we used the DNA sequence encoding the *Vrs1* gene itself. Creation of the three alleles (*vrs1.a1*, *vrs1.a2*, and *vrs1.a3*) cannot be deduced through intragenic recombination by mutual hybridization of six-rowed barleys possessing different alleles (Fig. 4A), which suggests that their origins were independent. Divergence of these alleles cannot be explained by simple mutation. Thus, six-rowed barley must have originated repeatedly from cultivated two-rowed or wild barley by means of a loss-of-function mutation of an HD-ZIP I-class homeobox gene. Six-rowed barley originated

independently at different times and in different regions (Fig. 4B), probably as a result of conscious selection by delighted farmers who greatly appreciated the improved seed yield.

Implications for the Evolution of Barley. The DNA sequence of *Vrs1* may trace the origin and migration of two-rowed barley. Both two- and six-rowed barleys emerged in Greece between 8,000 and 6,000 yBP, but six-rowed barley dominated in the Balkans and Central Europe (5,000–2,000 yBP) and in Southern Europe and North Africa (7,000–4,000 yBP) (1). After its disappearance from ancient Mesopotamia and Egypt, two-rowed barley does not reappear in the archaeological record in these regions until 1,100 yBP (11). Cultivation of two-rowed barley is virtually unknown in Central and Northern Europe until 1,000 yBP, and it has been assumed that two-rowed barley was introduced into Europe at 900–800 yBP by Crusaders from the Near East, as suggested by Fischbeck (39). In the present study, 14 two-rowed cultivars carrying the *Vrs1.b3* allele did not show any polymorphism in the DNA sequence (Fig. 4A). This indicates that the origin of the *Vrs1.b3* allele is relatively recent and that the allele has expanded rapidly throughout the world and become dominant, which partially supports Fischbeck's hypothesis (39). The interpretation that *Vrs1.b3* is a recent allele agrees with the fact that only one *vrs1.a3* six-rowed cultivar (derived from *Vrs1.b3*) has been detected thus far (Fig. 4A). This hypothesis does not necessarily contradict the hypothesis of Helbaek (11) that two-rowed barley was cultivated earlier than six-rowed barley, because the DNA sequences of archaeological specimens remain unknown. Moreover, the type of lateral spikelet in the ancient domesticated two-rowed cultivars (*deficiens*, *Vrs1.t*; *distichon*, *Vrs1.b*) is not clear. We favor the hypothesis that the common progenitor of *Vrs1.t* and *Vrs1.b2* occurred earlier than that of *Vrs1.b3*, based on the genetic distances seen in the allele coalescence and polymorphism results for the Ethiopian and western Mediterranean cultivars (Fig. 4A). Detailed genetic analysis of archaeological barley specimens would help to answer these questions.

The inflorescence architecture in the Poaceae is a continuous story of reduction from a more original "panicle" of spikelets (as seen in rice and oats) to a "spike" of spikelets (40), resulting in three sessile spikelets per node in barley and a single sessile spikelet per node in wheat and rye. In wild *Hordeum* species, the three spikelets and their slender awns form a light dispersal unit that permits both anemochory and zoochory (9). In two-rowed barley, strict temporal and spatial regulation of *Vrs1* expression leads to reduction and sterility of the lateral spikelets. We speculate that either strong alleles or differential expression or regulation of *Vrs1* orthologs could lead to complete repression of lateral spikelet formation at inflorescence nodes of other species in the Poaceae, as is found in wheat and rye. A Poaceae-wide assessment of the variability and regulation of *Vrs1* orthologs would be an exciting and productive way to improve our understanding of plant development and the evolution of grass species.

Materials and Methods

Plant Materials. Barley cultivars representing geographical distribution and three wild lines used in this study are maintained in the laboratory of T.K. (see SI Table 4). Mutants consisting of 39 *hex-v* and 16 *Int-d* lines were obtained from the Nordic GenBank (Alnarp, Sweden). Two more *hex-v*-type mutants derived from Japanese two-rowed cultivars New Golden (41) and Misato Golden (42) by gamma irradiation were obtained from T. Makino, National Institute of Crop Science (Tsukuba, Japan).

Genetic Mapping. Three fine-mapping populations of 6,269 gametes were analyzed previously (15, 16). An additional 1,781 F₂ plants of *vrs1.a1* × *Vrs1.b3* were screened with *cMWG699* and *BC12063*, and recombinants were analyzed further (see SI Table

5). ESTs contigs BC12348 (AJ468022 and CB881790) and BC12063 (BU973565 and BU987455) were converted to sequence-tagged site markers. Other markers were generated from BAC DNA sequences.

BAC Contig and Sequencing. A BAC library of cv. Morex (43) was obtained from Clemson University Genomic Institute (Clemson, SC) and has been screened by PCR (27). BAC contig was made by chromosome walking (see *SI Methods*). Shotgun sequencing of the BACs was carried out by the standard method (44). Assembled sequence contigs were correctly ordered, oriented with information of the cloning sites of the vector and the end-sequences of the bridge subclones, and annotated (see *SI Methods*). Contig gaps were filled by full-sequencing the bridge subclones either by primer walking or by GPS-1 transposon sequencing (New England Biolabs, Ipswich, MA).

RNA Analysis. Immature inflorescences were developmentally staged by measuring under a microscope. RNAs were extracted from leaves or immature inflorescences excised from 2-month-old plants by using TRIzol (Invitrogen, Carlsbad, CA). First-strand cDNA was generated by using SuperScript II (Invitrogen). RT-PCR and full-length cDNA sequencing were carried out with gene-specific primers (see *SI Methods*).

In Situ Hybridization Analysis. A 315-bp RT-PCR fragment including only the 3'-UTR of *Vrs1* was amplified by using primers M111L15F84380 (5'-CAT ACT TAA CGC ACG CCT AGA GATC-3') and M111L15R84670 (5'-TAG CTG CTG CCG CCG CCA AAT CCTC-3') and cloned into pCR4-TOPO vector (Invitrogen); after digestion by EcoRI, the insert was subcloned

into pBluescript II KS (+) vector (Stratagene, La Jolla, CA). Two clones with different insert orientations were linearized by using NotI and were used as templates to generate antisense and sense probes by using T3 RNA polymerase. Hybridization with a digoxigenin-labeled RNA probe, and immunological detection, were conducted. *In situ* hybridization was conducted according to the methods of Kouchi and Hata (45), with thorough modifications for improved reactions (see *SI Methods*).

Haplotype Analysis. Genomic DNAs (2.1-kb) including *Vrs1* were amplified from wild barley, cultivars, and mutants by PCR using the gene-specific primer pair M111L15F84204 (5'-GAA AGA TGA TTG CCA ACT ACC-3') and M111L15R86329 (5'-GTC ATA ACT CGG CAA ACA TAG-3'), and their DNA sequences were determined by using internal primers designed by taking advantage of the BAC sequence (see *SI Methods*).

Phylogenetic Analysis. A phylogenetic tree was constructed by the neighbor-joining method (46) using PAUP 4.0b10 (47). The DNA sequences of single haplotypes or subhaplotypes represented the cultivars sharing identical sequences. Phylogenetically informative insertions/deletions (indels) were included for analysis. All substitutions and indels were weighted equally. Bootstrap analysis with 1,000 replicates was performed.

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