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Leaf primordium size specifies leaf width and vein number among row-type classes in barley

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SUMMARY

Exploring genes with impact on yield-related phenotypes is the preceding step to accomplishing crop improvements while facing a growing world population. A genome-wide association scan on leaf blade area (LA) in a worldwide spring barley collection (*Hordeum vulgare* L.), including 125 two- and 93 six-rowed accessions, identified a gene encoding the homeobox transcription factor, Six-rowed spike 1 (VRS1). VRS1 was previously described as a key domestication gene affecting spike development. Its mutation converts two-rowed (wild-type *VRS1*, only central fertile spikelets) into six-rowed spikes (mutant *vrs1*, fully developed fertile central and lateral spikelets). Phenotypic analyses of mutant and wild-type leaves revealed that mutants had an increased leaf width with more longitudinal veins. The observed significant increase of LA and leaf nitrogen (%) during pre-anthesis development in *vrs1* mutants also implies a link between wider leaf and grain number, which was validated from the association of *vrs1* locus with wider leaf and grain number. Histological and gene expression analyses indicated that VRS1 might influence the size of leaf primordia by affecting cell proliferation of leaf primordial cells. This finding was supported by the transcriptome analysis of mutant and wild-type leaf primordia where in the mutant transcriptional activation of genes related to cell proliferation was detectable. Here we show that VRS1 has an independent role on barley leaf development which might influence the grain number.

Keywords: barley (*Hordeum vulgare* L.), VRS1, homeodomain-leucine zipper class I transcription factors, leaf development, leaf width, vein number.

INTRODUCTION

Feeding the growing population along with reducing agriculture's environmental footprint is a crucial challenge posed to the scientific community in this century, which can be achieved by increasing cropping efficiency and yield (Foley *et al.*, 2011). Barley, the fourth important cereal crop, is considered as the founder crop of Old World Neolithic food production that enabled mankind to move from hunter-gathering to cultivation and agriculture (Harlan and

Zohary, 1966; Zohary *et al.*, 2012; Mascher *et al.*, 2016). Domesticated barley has two principal spike types: two-rowed forms and six-rowed forms governed by a single gene, *Six-rowed spike 1* (*VRS1* ***V****ulgare* ***r****ow-type* ***s****pike 1*; syn. *HvHOX1*). VRS1 negatively affects carpel fertility in the lateral spikelets and generates two-rowed forms; mutations altering the functionality of the protein cause six-rowed forms. It has already been reported that *VRS1* is the result of a recent gene duplication that occurred only in tribe Triticeae (found in Wheat and Rye), and its paralog is known as *HvHOX2*. Although, both genes' tissue-specific expression has been shown, their molecular functions are yet to be described (Komatsuda *et al.*, 2007; Sakuma *et al.*, 2010, 2013).

Tapping the great potential of leaves will give a profound effect on crop improvement since plant leaves are the fundamental energetic unit of terrestrial life generating primary energy from photosynthesis (Blonder *et al.*, 2011). Leaves of dicotyledonous and monocotyledonous plants differ in various aspects, but the initiation of a leaf shares common features and gene networks. Leaf formation is initiated on the flanks of the shoot apical meristem (SAM) as leaf primordia (Gonzalez *et al.*, 2012). It is not clearly understood how lateral-organ primordia are specified in the SAM, but a marker gene, like *DORNRÖSCHEN*, is known to provide identity to lateral-organ founder cells in Arabidopsis (Comelli *et al.*, 2015). The maize *narrow sheath 1* and *narrow sheath 2* (*ns1* and *ns2*) orthologous to rice *NARROW LEAF 2/3* (*NAL2/3*), and homologous to Arabidopsis *PRESSED FLOWER* (*PRS*) are *WUS*-related homeobox 3 (*WOX3*) genes proposed to influence leaf founder-cell recruitment during leaf primordia formation (Scanlon *et al.*, 1996; Matsumoto and Okada, 2001; Nardmann *et al.*, 2004; Cho *et al.*, 2013). The Arabidopsis proteins STRUWWELPETER (SWP) and SAMBA (a negative regulator of anaphase-promoting complex/cyclosome complex) are involved in formation of leaf founder cells by modulating cell proliferation during early development (Hepworth and Lenhard, 2014). Recent studies have shed light on the mechanisms of leaf initiation; the mechanical strain of cell wall and plasma membrane of leaf primordia likely coordinates the PIN-FORMED1 (PIN1) localization and cortical microtubule orientation, which in turn increases auxin accumulation and thereby promotes further growth (Nakayama *et al.*, 2012). Leaf primordia are formed by progression of the cell cycle with a strict temporal regulation of proteins involved in DNA replication and mitosis (Inzé and De Veylder, 2006). Moreover, the cell cycle is influenced by an E3-ubiquitin ligase complex, which degrades the cell cycle dependent kinase inhibitors (Hershko, 2005). Once the leaf is initiated, the leaf grows in three different dimensions namely adaxial-abaxial, proximo-distal, and medio-lateral (Bar and Ori, 2014). Rice genes *NAL7* (Fujino *et al.*, 2008), *NAL9* (Li *et al.*, 2013), *dwarf and narrow leaf 1* (*dnl1*) (Wei *et al.*, 2013), *NAL1* (Jiang *et al.*, 2015) and cellulose synthase-like genes from both rice (*NARROW AND ROLLED LEAF 1*) and maize (Hu *et al.*, 2010; Hunter *et al.*, 2012) are influencing medio-lateral leaf axis development and produce narrow leaves, when they are mutated.

Despite these many narrow/wide leaf phenotypes, very few studies have reported the association of leaf characters with yield relevant traits (Fujita *et al.*, 2013; Takai *et al.*, 2013). Here we combined the power of a Genome-Wide Association Study (GWAS) with molecular analysis and identified the barley homeodomain-leucine zipper class I transcription factor VRS1 that influences the medio-lateral leaf axis formation and plant yield. Phenotypic and histological studies show that the six-rowed domestication allele of *VRS1* (i.e. *vrs1.a*) confers increased leaf width, vein number and leaf nitrogen content. Transcriptome analyses of wild-type and mutant plants suggest that wild-type two-rowed plants have impaired cell proliferation during early leaf primordium development.

RESULTS

VRS1 affects leaf area and grain number

Barley exists in two morphologically distinct spike forms, referred to as 'two-rowed' and 'six-rowed', which also differ in their leaf blade area (LA) (Alqudah and Schnurbusch, 2015). To study the natural genetic variation of LA in spring barley, 218 worldwide accessions (125 two-rowed and 93 six-rowed, Table S1) under greenhouse conditions (Methods S1) at four developmental stages (Zadoks stage, Z): awn primordium (Z31–33), awn tipping (Z49), heading (Z55) and anther extrusion (Z65) (Zadoks *et al.*, 1974; Alqudah and Schnurbusch, 2015) were analysed. A significant difference ($P \leq 0.05$) in the main culm LA between row-types, in which six-rowed forms had significantly larger LA at all developmental stages after awn primordium (AP) (Figure 1a), was detected. High broad-sense heritability (H^2) for the studied trait ($\geq$0.85) indicated that LA is predominantly genetically controlled at the examined developmental stages. GWAS identified clear association signals for LA (on all four developmental stages) at the physical position of SNP markers Barley Oligo Pool Array (BOPA) 2_12_30896 [652031268–652031389 (SNP position: 652031329)] and BOPA2_12_30900 [652031594–652031715 (SNP position: 652031655)] (Mascher *et al.*, 2013). These two SNPs are found to be localized within the physical position of the *VRS1* gene (652031295–652032562) on the long arm of chromosome 2H (Figure 1b), IPK barley BLAST server, Gatersleben (http://webblast.ipk-gatersleben.de/barley/).

The allelic variation at *VRS1* also affected grain number per main spike in our GWAS panel (Figure S1a). These observations suggested that the *vrs1* locus is involved in LA regulation along with grain number in barley.

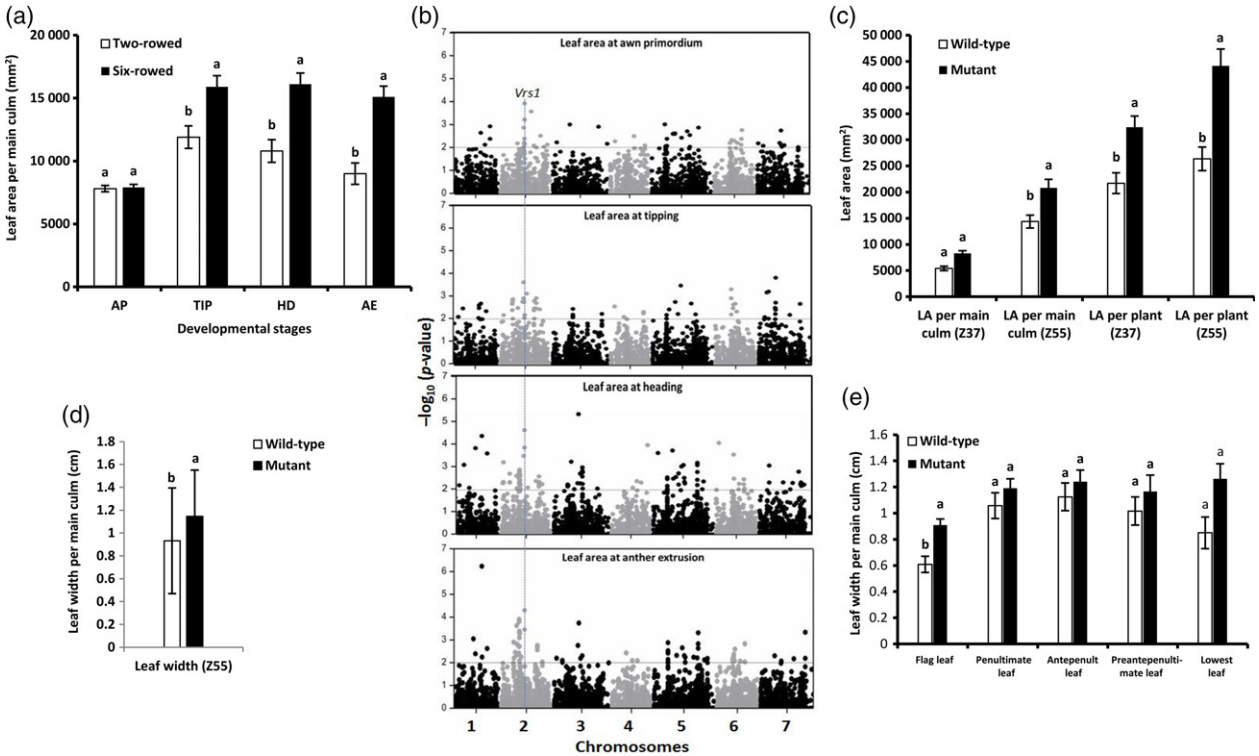

Figure 1. VRS1 regulates leaf blade area (LA) in a worldwide two- and six-rowed barley population.
(a) Phenotypic analysis of LA per main culm for the population at four growth stages (n = 125 and 93 for two- and six-rowed barleys, respectively with three biological replicates per accession).
(b) GWAS for LA shows the significant association of *Vrs1* in Manhattan plot; the vertical blue dotted line indicates the location of *Vrs1* along chromosome 2H, and the gray dotted line denote the threshold significance level, $-\log_{10}$ (P-value of 0.02) (n = 125 and 93 for two- and six-rowed barleys, respectively with three biological replicates per accession).
(c) Phenotypic characterization of LA per main culm and whole plant for four independent *vrs1* mutants and their respective wild-types at Zadoks growth stages Z37 (flag leaf just visible) and Z55 (heading).
(d, e) (d) The average of leaf width for mutants and wild-types at Z55 for main culm and (e) leaf width of individual leaves of main culm. AP: awn primordium, TIP: tipping, HD: heading, AE: anther extrusion. Four independent vrs1 mutants and their respective wild-types represented by three biological replicates per line in Figure 1(c–e). Values marked by different letters differ significantly [$P \leq 0.05$ for (a, c, d, e); $P \leq 0.001$ for (d)] based on Fisher's Least Significant Difference (LSD) test. Error bars are based on mean ± SD.

Leaf width is the critical determinant for increased leaf area in *vrs1* mutants

To further elucidate the role of VRS1 during barley leaf growth and development, LA was measured in four independent *vrs1* mutants (six-rowed) and their wild-types (*VRS1*, two-rowed) at two developmental stages, Z37 (flag leaf just visible) and Z55 (heading) (Zadoks *et al.*, 1974) (Methods S1). The *vrs1* mutants had larger LA (cumulative on main culm and whole plant) than their wild-types at both developmental stages with the maximal difference observed at Z55 (Figure 1c, cumulative; Figure S1b, independent mutants and wild-types). Then, length and width of individual leaves were measured on the main culm at Z55 to investigate which of these parameters mainly influenced the observed difference in LA in the *vrs1* mutant plants. The cumulative leaf width per main culm ($P \leq 0.001$) (Figure 1d) and length and width of the flag leaf were significantly different between wild-type and mutant classes ($P \leq 0.05$; Figure S1c,d, independent mutants and wild-types; Figures S1e and 1e, cumulative). To exclude the effect of leaf and tiller number on the observed difference in LA, leaf number per main culm, total leaf number per plant and tiller number per plant were recorded at both (Z37 and Z55) stages. There was no significant difference ($P \leq 0.05$) between mutant and wild-type for all three characters at Z37. However, at Z55 the mutant plants had significantly lower total leaf number per plant ($P \leq 0.001$) and tiller number per plant ($P \leq 0.01$; Figure S1f). These results implied that VRS1 mostly affects leaf width, which mainly contributed for the LA along with the flag leaf length.

vrs1 mutant had higher leaf nitrogen during anthesis

Pre-anthesis nitrogen storage in vegetative organs like roots and shoots accounts for maximum nitrogen remobilization during grain development in wheat and rice (Hirel *et al.*, 2007). The significant difference for LA observed in

the two- and six-rowed barley population at Z49 (3–5 days before fertilization) might determine their carbon and nitrogen content (Tian *et al.*, 2011). Therefore a pair of wild-type and mutant plants was analysed. Bowman near isogenic line of mutant allele *vrs1.a* [BW-NIL(*vrs1.a*); syn. BW 898] (Druka *et al.*, 2011) was used as a six-rowed representative and cultivar Bowman as two-rowed wild-type. Elemental analysis of carbon and nitrogen contents in the penultimate leaves indicated that the mutant (i.e. wide) leaves contained 1.4-fold more leaf nitrogen (N) (%, $P \leq 0.001$) than wild-type (narrow) leaves (Figure 2a); whereas carbon content (%) was not significantly different (Figure 2b).

VRS1 affects leaf width and vein number

To further understand the effect of VRS1 on leaf development, barley leaves were histologically studied also in Bowman and BW-NIL(*vrs1.a*). The plants were analysed at Z49 once they completed their growth (Methods S1). The *vrs1* mutant [BW-NIL(*vrs1.a*)] formed wider leaves (Figure 2c,e) with more veins (Figure 2d) than wild-type (cv. Bowman). The transverse section of mutant and wild-type mid-rib showed no difference in the appearance of the mid-rib with regards to the ratio of xylem to phloem, its relative positioning, and size or structure (Figure S2a,b). To assess the interveinal space between mutant and wild-type, the ratio of leaf width to vein number was calculated and it was found that wild-type plants had a higher ratio than mutants (Figure S2c). These results implied that mutant plants are not affected in their leaf vascular bundle anatomy; however, interveinal space might be reduced. Furthermore these results show that VRS1 affects LA by increasing leaf width and vein number without extending the interveinal space.

Effect of VRS1 on leaf width is established during leaf primordia development

Genes regulating the medio-lateral development of the leaf can be attributed to their function as early as during development of incipient leaf primordia (Scanlon, 2000) or

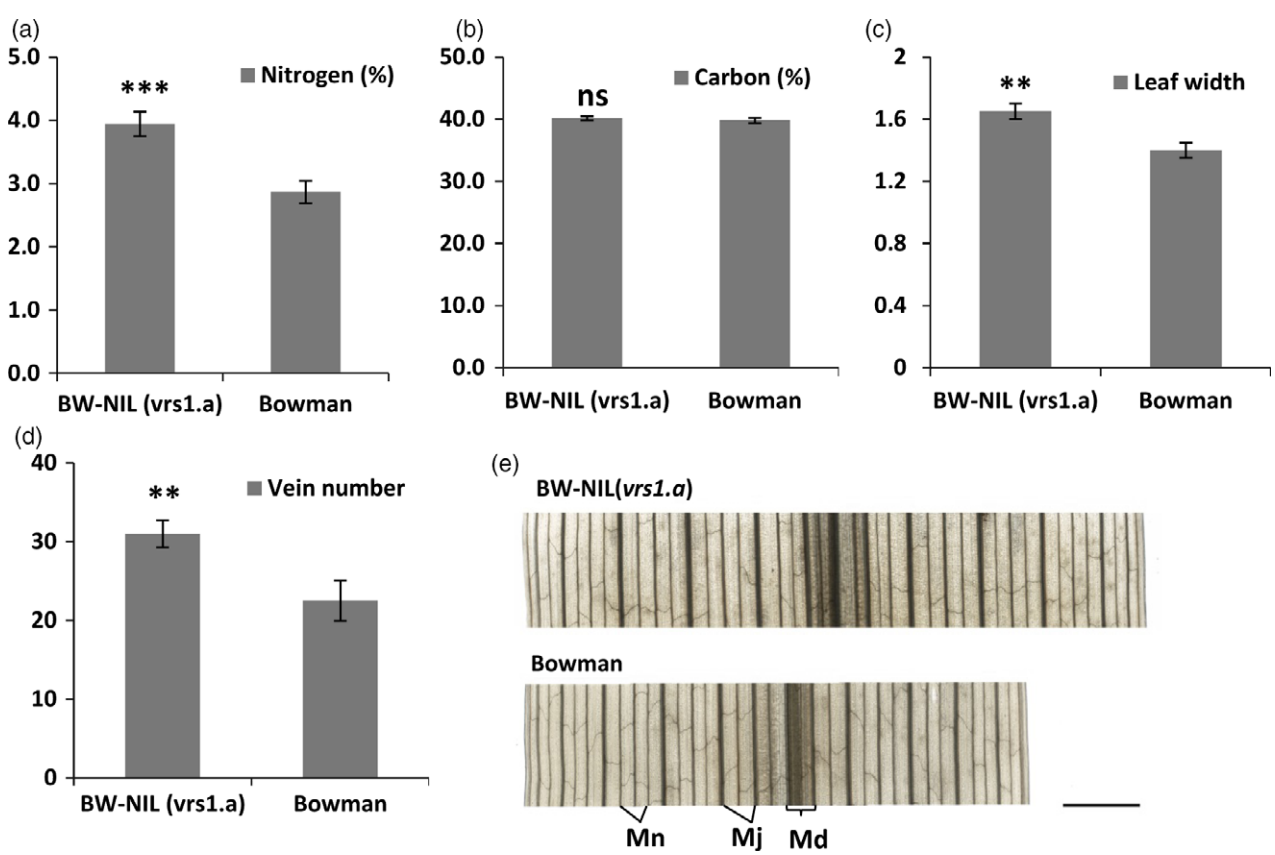

Figure 2. VRS1 regulates leaf nitrogen (%), leaf width and vein number at Z49 (Tipping stage).
(a, b) (a) The elemental analysis for wild-type Bowman and mutant BW-NIL(vrs1.a) penultimate leaves shows, leaf nitrogen (%) is significantly higher (1.4-fold) in mutant than wild-type (b) but not carbon (%).
(c, d) (c) BW-NIL(vrs1.a) has significantly wider leaves and (d) more veins compared to Bowman.
(e) Vein cleared leaves of BW-NIL(vrs1.a) and Bowman. Data in (c, d) were results from the analysis performed in antepenult leaf. Data in (a–d) were subjected to the Student's *t*-test, and values are given as mean ± SEM; $n = 9$ for (a, b) $n = 6$ for (c, d), ***P-value ≤ 0.001, **$P \leq 0.01$. ns: not significantly different at $P < 0.05$; Scale bar 2 mm. Mn: minor vein, Mj: major vein and Md: mid-rib.

during outgrowth of the leaf (Jost *et al.*, 2016). To study the effect of VRS1 during leaf primordia development, serial transverse sections of the shoot apices dissected from embryos of the same wild-type (Bowman) and mutant [BW-NIL(*vrs1.a*)] used above, possessing SAM and young leaves (Figure 3a,b), were made. The area of the SAM was calculated from the tip of the meristem, and for plastochron-1 (P1, the youngest leaf or leaf primordia), both area and width from the base of the leaf were measured (Methods S1). We considered the section as leaf base, when a first visible separation of P1 from SAM was found (Jost *et al.*, 2016). The area of both P1 and SAM was measured by creating the contour maps of each organ's section by using Free-D software (Andrey and Maurin, 2005). The leaf width of P1 was calculated by thinning down the skeletons of P1 as described previously (Klukas *et al.*, 2014). Our analysis revealed that the area of the SAM is not significantly different between wild-type/Bowman and mutant/BW-NIL(*vrs1.a*) (Figure 3c). However, both area and width of the P1 middle region was significantly larger in mutant/BW-NIL(*vrs1.a*) plants (Figure 3d,e). We further verified the observed indifference for the SAM by an independent approach by measuring the diameter of the SAM (Figure S3a,b and Methods S1) in tissue cleared shoot apices (dissected from embryos) of both genotypes, once more confirming no significant differences of the SAM. These results highlight that VRS1's effect on leaf width was already established during leaf primordia development; but that it did not alter the size of the SAM.

VRS1 is expressed during leaf primordia initiation and development

To complement the histological study on leaf primordia formation, the expression of *VRS1* was studied by tagging its native promoter with an *enhanced GFP* (*eGFP*) coding sequence in transgenic barley plants of two-rowed cv. Golden Promise. Five out of 25 independent transgenic events showed GFP expression in the leaf in the T_0 generation. From the five GFP-positive events, T_1 plants of three events were selected, and their GFP expression was confirmed. Fluorescence analyses of 8 days old shoot apices revealed GFP fluorescence in the entire shoot apex including developing leaf primordia (Figure 4a). The detection of *VRS1* transcripts in early shoot apices (11 days old) of BW-NIL(*vrs1.a*) and cv. Bowman using quantitative RT-PCR (qRT-PCR) confirmed our transgenic promoter analysis during early stages of leaf development. No significant difference in transcript abundances of mutant and wild-type plants (Figure 4b) was found, suggesting that only the function of the protein and not transcript levels are responsible for the wide/narrow leaf phenotype. Moreover, *VRS1* was expressed in the leaf epidermal cells, and in the

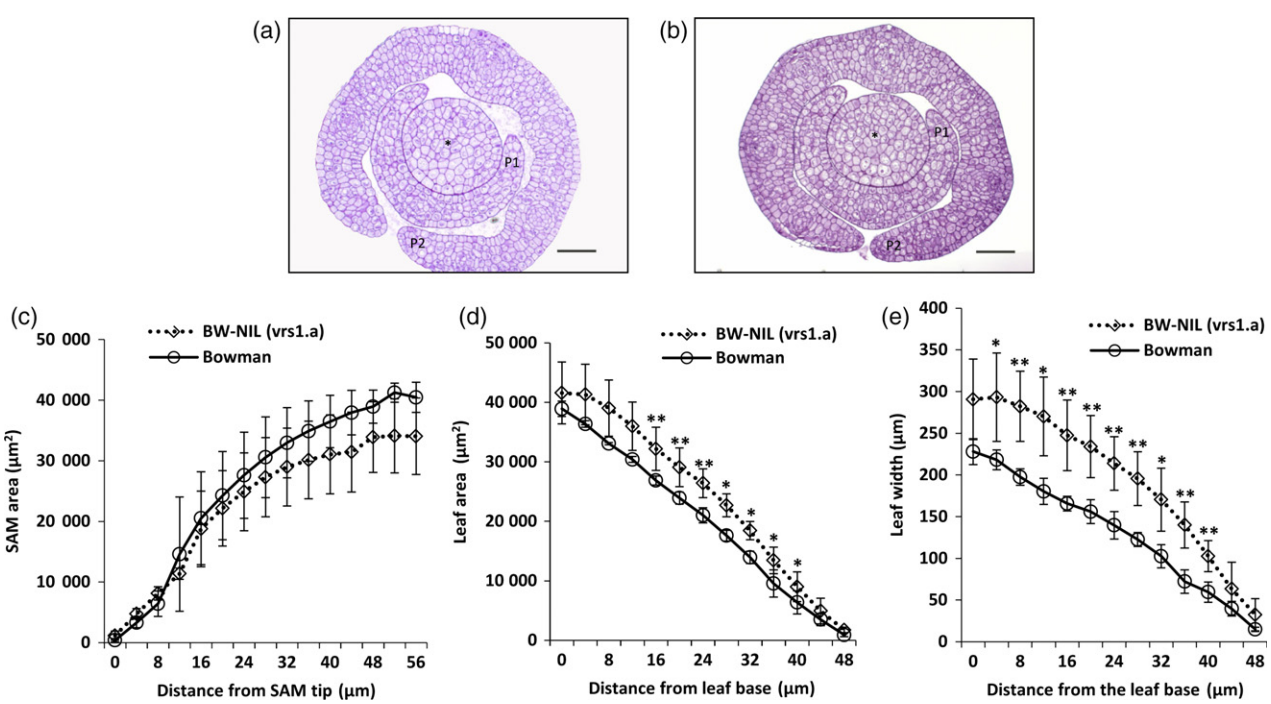

Figure 3. Mutant leaf primordia, plastochron 1 is wider than wild-type.
(a, b) Representative images of the transverse serial sections of the shoot apices dissected from the embryo of BW-NIL(vrs1.a) and Bowman respectively.
(c) Calculated SAM area from the serial sections of mutant and wild-type shows no significant difference from tip to base.
(d, e) Calculated leaf area and width of plastochron 1 are significantly different in mutant especially in the middle region of the P1 leaf. Scale bar 50 µm; Data in (c–e) were subjected to the Student's *t*-test, and values are given as mean ± SD; $n = 4$; $^{**}P$-value ≤ 0.01, $^{*}P$-value ≤ 0.05.

vascular bundle of mature leaves (Figure S4). Also transcripts of *VRS4* (*HvRA2*), an upstream regulator of *VRS1* in spike development (Koppolu *et al.*, 2013) were detected, implying that a similar regulation upon *VRS1* might exist during leaf development (Figure 4b).

Transcriptome analysis of *vrs1* mutant shoot apices suggests a role of VRS1 during early developmental processes of leaf formation

To address the molecular function of the homeodomain transcription factor VRS1 during early leaf development, the transcriptome of 11-day-old shoot apices from mutant and wild-type plants was studied using a custom-made Agilent barley 60k microarray (Koppolu *et al.*, 2013). A Gene Ontology enrichment analysis (GO) on all genes detectable by microarray hybridization indicated a significant over-representation of transcripts/genes associated with developmental processes (Figure S5). Such enhancement was expected for meristematic tissue. The gene entities were filtered with stringent parameters (explained in detail in Experimental Procedures), which yielded 51 differentially expressed transcripts, of which 26 were up- and 25 were down-regulated in the mutant (Figure S6). Transcript levels of selected genes (five up- and three down-regulated) were validated by qPCR (Figure S7). Among the 26 up-regulated genes in the mutant plants, specific functions could be assigned to 12 genes (blast analysis), revealing that transcripts/genes belonging to cell proliferation are preferentially up-regulated consistent with the presence of the wide leaf phenotype. Nine out of 25 down-regulated genes could be linked to apoptosis, jasmonic acid or promotion of flowering, which indirectly

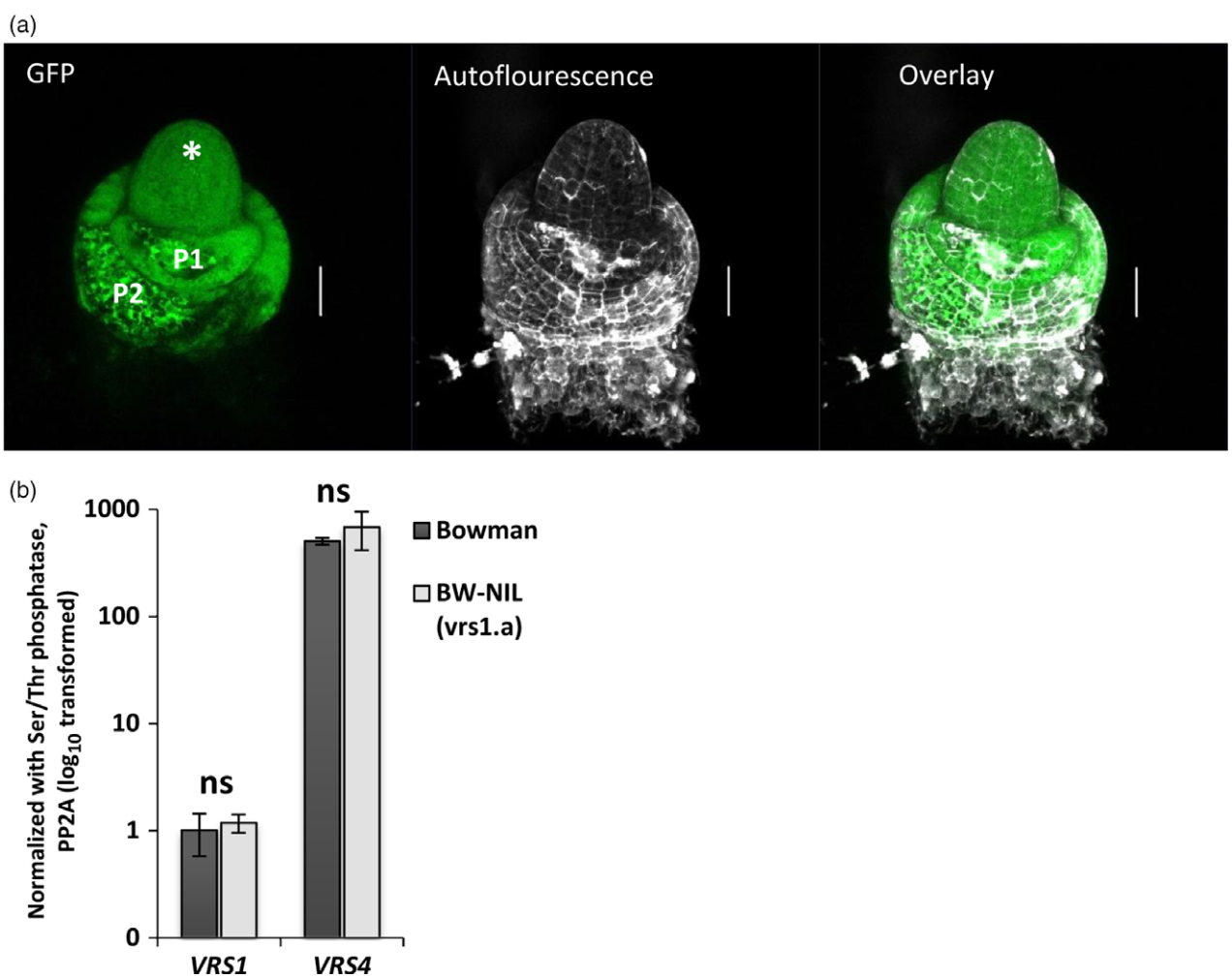

Figure 4. VRS1 expresses during early leaf development specifically during leaf primordia initiation and development.
(a) *VRS1* promoter activity in SAM and developing leaf primordia demonstrated by the expression of GFP in *ProVRS1::GFP* transgenic plants. Panel-1 shows the GFP expression as green color, Panel-2 shows the autofluorescence of apex cell walls and Panel-3 is the combined image of the above two panels. SAM is indicated by asterisk, P1 and P2 are developing leaf primordia; scale bar 50 μm.
(b) *VRS1* and *VRS4* transcript abundance as assessed by qRT-PCR in Bowman and BW-NIL(vrs1.a) shoot apices. Abundances were $\log_{10}$ transformed and compared using the Student's *t*-test. Values are given in the form of mean ± SEM (n = 3); ns: non-significant.

points towards down- regulation of cell differentiation processes in the mutant. In both up- and down-regulated gene sets, a number of kinases were found, which might imply that several signalling cascades involved in cell proliferation and differentiation were also differentially regulated in mutant and wild-type.

Phylogenetic analysis of HD-ZIP class I proteins and their expression pattern

We performed a phylogenetic analysis by using the web-based tool 'SALAD' (http://salad.dna.affrc.go.jp/CGViewer/en/) for barley, rice, maize and Arabidopsis HD-ZIP class I proteins (Figure 5a). The SALAD dendrogram is different from conventional phylogenetic trees since it provides a phylogenic tree along with putative motifs, which are evolutionary conserved. This feature helps to identify motifs with relevance to the functionality of a protein or cluster. The barley VRS1 and its paralog HOX2 formed a sub-family (Figure 5b) with their putative orthologs from rice and maize and the homologous Arabidopsis δ class proteins (ATHB21, -40 and -53) (Henriksson *et al.*, 2005) based on the conserved motif 4 (Figure S8a). The motif 16 (Figure S8b) separates the monocot proteins (barley, rice, and maize) from dicot proteins (Arabidopsis) (Figure 5b). The monocot-specific cluster is further divided into two different branches. The GT1 clade (41) is defined by the presence of motif 17 (Figure S8c) and consists of GRMZM2G005624 (GRASSY TILLERS1, GT1 from maize), LOC_Os03g10210 (OsHOX12 from rice) and full-length clone AK365295 (putative HvHOX12 from barley). VRS1 and HOX2 are grouped together in the VRS1 clade based on the presence of motifs 21 (Figure S8d) and 59 (Figure S8e) with LOC_Os07g39320 (OsHOX14 from rice) and GRMZM2G062244 (HOX107 from maize). Among the motifs (4, 16, 17, 21, & 59) found in the VRS1 clade, motif 21 was identified as putative 'AHA' (Aromatic Hydrophobic Acidic amino acids) motif required for the transcriptional activity of HD-ZIP class I proteins (Capella *et al.*, 2014). This

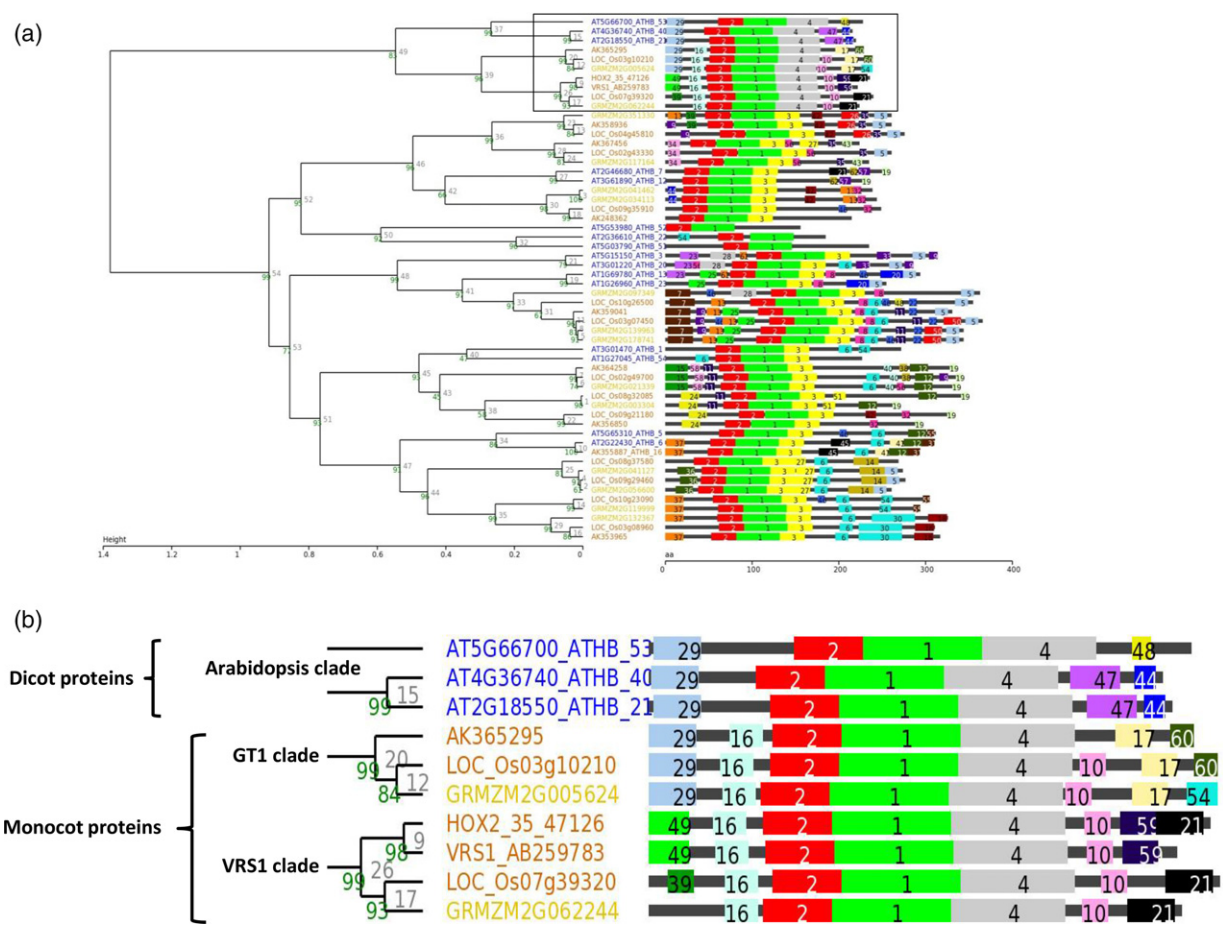

Figure 5. Phylogenetic analysis of class I HD-ZIP proteins in Arabidopsis, rice, maize and barley identified the important homologs and putative orthologs of VRS1.
(a) Evolutionarily conserved motif based dendrogram generated by SALAD for HD-ZIP I proteins shows the clustering of VRS1 and its homologs.
(b) Magnification of VRS1 cluster shows the three different sub-clusters namely Arabidopsis clade, GT1 clade and VRS1 clade based on the presence of motifs 4, 16, 17 and 21. The values in green color are bootstrap probability, and gray colors are approximate unbiased *P*-value.

motif is present in all members of the VRS1 clade except VRS1 itself. It indicates that HOX2 is the putative ortholog of rice LOC_Os07g39320 (OsHOX14) and maize GRMZM2G062244 (HOX107) (Sakuma *et al.*, 2010). Taking the expression pattern of *VRS1* homologs (Table S2) from the GT1 and VRS1 clade into account, the results suggest that VRS1, like all other clade members, is also involved in the development of leaves and leaf-derived structures (bracts).

DISCUSSION

VRS1 affects leaf width and establishes narrow leaves in two-rowed barley

Barley is highly suitable for the identification of causative gene loci underlying natural phenotypic trait variation using GWAS (Ramsay *et al.*, 2011; Comadran *et al.*, 2012; Alqudah *et al.*, 2014, 2016). Our GWAS analysis for LA in the spring barley panel identified two SNPs physically localized inside the *VRS1* gene which highlights the importance of this gene in the natural variation of LA between two- and six-rowed types. The significant reduction of LA in two-rowed plants (wild-type) implied that the functional *VRS1* allele is negatively associated with LA. The influence of total leaf number and tiller number on LA was excluded, as they were not significantly different at stage Z37 between six-rowed (mutant) and two-rowed (wild-type), or higher at Z55 in two-rowed types (Figure S1f). This suggests that the observed variation in LA is mainly attributable to either leaf width or length. It was then clarified from the independent phenotyping of *vrs1* mutants and corresponding wild-types (showing wide and narrow leaves, respectively, Figures 1d,e and 2) that leaf width is the major contributor for the observed difference on LA between row-types. The LA difference from awn primordium to heading stage (Figure 1a) indicates the importance of the *VRS1* gene for barley development, since this period of growth is characterized as 'the critical period of spikelet survival' (Alqudah and Schnurbusch, 2014). Moreover, the larger LA at tipping (around anthesis) and heading stages (post-anthesis) of six-rowed barley may increase its photosynthetic and storage capacity, which themselves can improve spikelet formation, grain filling and subsequently grain yield. Lowered leaf mass area (LMA, i.e. weight per unit leaf area) has been shown to be associated with increased net photosynthetic capacity (A_{max}) and leaf nitrogen (N) (Reich *et al.*, 1998). Interestingly, Alqudah and Schnurbusch (2015) found that six-rowed barley (wide leaf) also had significantly lower LMA than two-rowed types under different growth conditions, implying that six-rowed forms may have higher leaf nitrogen (N) than two-rowed. The observed significant increase of leaf nitrogen (%) during the important pre-anthesis phase in six-rowed barleys supported this. Overall, the increased LA could very well be linked with improved grain yield by reducing absolute spikelet abortion in six-rowed barley (relative spikelet abortion in two-rowed is approximately 30% and in six-rowed 42%), even though they produce three times more spikelets than two-rowed barley (Alqudah and Schnurbusch, 2014). The association of *VRS1* with grain number per spike in the GWAS panel supports the link between LA and grain number. The many positive effects of mutant *vrs1* (six-rowed) on larger LA, higher leaf nitrogen (%) and increased grain number per spike might have impacted negatively on tiller production (Figure S1c), which rather suggests a trade-off relationship (negative pleiotropic effect) with tiller number (Kirby and Riggs, 1978; Alqudah *et al.*, 2016).

The co-expression of *VRS4* (*HvRA2*) (in this study) also suggests its involvement during leaf development. It is also reported that *VRS2* influences leaf area, leaf and tiller number (Youssef *et al.*, 2017). Similarly, *INTERMEDIUM SPIKE-C* (*INT-C*, *VRS5*) has been reported to be involved in tillering at the juvenile stage (Ramsay *et al.*, 2011). All these reports strongly suggest that six-rowed spike genes (*VRS*) may also have an independent role in early vegetative development apart from their major role in spike architecture.

Two-rowed barely has impaired cell proliferation during early leaf development

Early leaf development is largely attributable to cell division (Scarpella *et al.*, 2010); as *VRS1* expresses during leaf primordia initiation and development, it may affect cell division. The wider P1 of *vrs1* mutant (six-rowed) plants during early leaf development strongly suggested that two-rowed leaf primordia has impaired cell proliferation. An impact of cell expansion on leaf width can be excluded as the interveinal distance determined by the leaf width/vein number ratio was found to be reduced in the mutant (Figure S2c) (Smillie *et al.*, 2012; Hur *et al.*, 2015). *AtHB6*, an HD-Zip I gene from Arabidopsis, was also found to be expressed specifically in leaf primordia and involved in cell proliferation (Soderman *et al.*, 1999). It was previously shown in Arabidopsis and rice that proteins regulating cell proliferation during lateral-organ formation also influence the number of veins (Kang *et al.*, 2007; Qi *et al.*, 2008; Scarpella *et al.*, 2010). These findings support our hypothesis that VRS1 acts during the early leaf development and contributes to leaf width and vein number. The reduction of maximum thickness in mutant P1 (Figure S3c) reiterates that the wider and thinner leaves of mutants are already defined during early leaf primordia development. The potential role of VRS1 in early leaf development was further supported by the identification of differentially expressed genes related to active cell division. Over-representation of genes like Werner syndrome ATP-dependent helicase related to helicase activity for more DNA replication (Knoll and Puchta, 2011), UDP-glycosyl-transferase

related to enhanced biosynthesis of polysaccharides for cell wall formation (Lao *et al.*, 2014) and *MICROTUBULE ORGANIZATION1* involved in arrangement of microtubules, which exerts a mechanical strain for the proper cell division planes and cellular growth (Kawamura *et al.*, 2006; Smolarkiewicz and Dhonukshe, 2013) were detected. Similarly, the enhanced expression of kinases like *CRINKLY 4 RELATED 3* (*CCR3*) and *GEMINIVIRUS REP INTERACTING KINASE 1* (*GRIK1-like*), involved in formative cell division signalling (Becraft *et al.*, 2001; Baena-Gonzalez *et al.*, 2007; De Smet *et al.*, 2008), *CLAVATA 3/ESR* (*CLE*)*-25* involved in the initiation of lateral-organ primordia (Jun *et al.*, 2007), and the putative maize *BRANCH ANGLE DEFECTIVE 1* (*BAD1*), that possibly promotes cell proliferation in lateral organs (Bai *et al.*, 2012), were found in the shoot apex of *vrs1* mutant plants. The phylogenetic analysis of *VRS1* homologs from rice and maize with similar expression profiles, suggests their involvement in basic developmental processes (Table S2). It is known that homeobox genes (which contain the highly similar 60-aminoacid homeodomain) are evolutionarily conserved transcription factors that regulate basic developmental processes such as regional specification, patterning, and differentiation in animals, fungi and plants (Duverger and Morasso, 2008).

In conclusion, until now, only WOX3 was shown to be involved in the recruitment of leaf founder cells during early leaf formation (Scanlon, 2000). An attempt to explore the function of BROAD LEAF 1 in barley showed that it is not affecting leaf primordia development but rather leaf outgrowth (Jost *et al.*, 2016). Results presented here show that leaf primordium size correlates with leaf width and vein number in *vrs1* mutants. Moreover, the concurrent spatiotemporal expression pattern, wide leaf with more veins and alterations in down-stream gene expression support the hypothesis that VRS1 negatively contributes to the early development of leaf primordium in wild-type plants, i.e. two-rowed types. It can be speculated that VRS1 might suppress the cell proliferation of leaf founder cells that form the leaf primordia. The detected expression of *VRS1* in the SAM without phenotypic alterations between row-types implies that: (i) VRS1 only exerts subtle effects, or (ii) participation of some yet unknown development promoting factors in the SAM. Clonal analysis of *vrs1* mutant and wild-type plants during early leaf development could shed more light in the underlying mechanics of VRS1 during leaf formation. Our phylogenetic analysis suggests the importance of characterizing putative orthologs of *VRS1* in rice, maize and wheat, which might have a profound impact on crop improvement by modifying canopy architecture. Additionally, the significant association of *VRS1* with grain number variation and increased leaf nitrogen (%) during pre-anthesis development corroborates the essential link between LA and grain number and how it influences the yield of barley plants.

EXPERIMENTAL PROCEDURES

Plant cultivation

Plants were grown on the substrate containing four parts of autoclaved compost, two parts of 'Rotes Substrat' (Klasmann-Deilmann GmbH, Geeste, Germany), 1.6 parts of sand and 0.8 parts of peat. Grains were planted in either 54 or 96 well plastic trays and germinated in climate chamber or temperature controlled greenhouse for 4 weeks at 11°C day and 7°C night with 10 h light. After 4 weeks of growth, seedlings were transferred to pots (diameter 16 cm^2) and allowed to mature in the greenhouse. Further growth conditions were divided into four phases: first phase at 14°C day and 9°C night with 12 h light for 4 weeks, second phase at 16°C day and 9°C night with 14 h light for 2 weeks, third phase at 20°C day and 12°C night with 16 h light for 2 weeks and final fourth phase at 20°C day and 14°C night with 16 h light until harvest. Plants were fertilized with 'Plantacote plus' (AGLUKON GmbH, Duesseldorf, Germany) (15 g/pot) during vegetative phase and with liquid fertilizer 'Hakaphos Rot' (AGLUKON GmbH, Germany) (once a week, 2–4%) from the start of spike development. For the association panel, 30 seedlings of each of the 218 accessions (125 two-rowed and 93 six-rowed) were germinated for 10 days under a 16-h photoperiod, 20°C/16°C, vernalized for 28 days (10-h photoperiod, 4°C), acclimated for 7 days (16 h photoperiod, 14°C/12°C), and finally transplanted into 0.5 L pots and grown in a greenhouse.

Genotyping and association analysis

The genotypic data required for the GWAS was obtained from a 9K Illumina SNP chip (Comadran *et al.*, 2012) (Illumina's GoldenGate Technology, Illumina, San Diego, CA, USA). Only SNPs having a minor allele frequency of the association panel of at least 5% were included (4320 SNPs). The genetic marker positions anchored by physical map positions for SNP markers based on Barke × Morex RILs population sequencing (POPSEQ) populations were used (Mascher *et al.*, 2013). The mixed-linear model (Yu *et al.*, 2006) was chosen and Eigen analysis was employed to correct for population structure (Price *et al.*, 2006). The significance level of the association *P*-value was determined by calculating the level of the false discovery rate (FDR) at ≥0.05%. To correct for multiple testing, FDR was calculated using GenStat v. 16 (GenStat, 2014) to exclude false-positive associations. More details about the approach of GWAS analysis were described previously (Alqudah *et al.*, 2014).

Transgenesis

The *VRS1* (AB259782.1, GI: 119943316) coding sequence was taken as a BLAST query to identify their promoter sequences. The promoter was amplified from the genomic DNA of cv. Bonus (two-rowed barley), along with the 5′-UTR in the form of 991 bp for *VRS1* promoter, which was inserted into the pCR4.0 TOPO TA plasmid (ThermoFisher SCIENTIFIC, Carlsbad, CA, USA) to produce pCR4.0 TOPO-*VRS1* promoter. The insert was validated by sequencing and then amplified from pCR4.0 TOPO-*VRS1* promoter using primers engineered to produce a *BamH*I restriction site at one end and a *Pst*I site at the other end. Amplicons were then inserted into p*NOS*-AB-M (DNA Cloning Service, Hamburg, Germany) to produce the constructs *pNOS-ProVRS1*. The *eGFP* coding sequence was amplified from pFF_{19}eGFP (DNA Cloning Service) using *Pst*I and *Eco*RI restriction site containing primers, and the amplicon was then inserted into *pNOS* vectors to produce the expression units *pNOS-ProVRS1*::*eGFP*, which was transferred

into p6U (DNA Cloning Service) using the *Sfi*I sites, and from then into barley cv. Golden Promise *via Agrobacterium*-mediated gene transfer (Hensel *et al.*, 2009). Transgenesis was verified by both a PCR and a Southern hybridization assay. The primer sequences employed are given in Table S3.

GFP assay

Shoot apices of presumed transgenic barley plants were collected from 10 to 12 days old T_1 or T_2 seedlings. Transverse and longitudinal leaf sections were cut by a razor blade, and GFP fluorescence was detected in these sections by the LSM 510 META or the LSM 780 confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), following their excitation by a 488 nm laser line in combination with a 490–530 nm bandpass filter. The chlorophyll in the samples was detected by excitation with a 633 nm laser line in combination with 650 nm long pass filter and cell wall autofluorescence with 405 nm laser line in combination with 410–480 nm bandpass filter. In all samples authenticity of the GFP signal was analysed by photospectrometric unmixing using the Lambda detector.

Elemental analysis of carbon and nitrogen

Carbon and nitrogen analysis was carried out with an elemental analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany), which can also measure Hydrogen and Sulphur. The instrument was switched on about 3–5 h before the analysis, and the measurement was performed with carbon nitrogen (CN) mode. About 3–5 mg of ground and the frozen dried sample was weighed in a tin (Symbol Sn) boat, folded and placed in the autosampler. The boats containing the samples are combusted in the chamber with excess oxygen kept at 950°C, where it is mineralized by the catalysts. The various gases (CO_2, H_2O und NOx) formed from the combustion passes through a Sodium Hydroxide column packed with copper granules held at 550°C (reduction tube), where the remaining oxygen is bound, and nitric/nitrous oxides are reduced to N_2. All gases are removed at respective traps except the analytically important CO_2 and N_2, which are subsequently detected by the thermal conductivity detector. High-purity helium (Quality 5.0) is used both as a carrier and reference gas. Blank values are obtained from empty tin boats, and calibration is done by elemental analysis of standard substances (Acetanilide contains 10.36% N_2 and 71.09% C) supplied by the instrument's manufacturer.

qRT-PCR and microarray hybridization

RNA was extracted from the shoot apices of 11 days old cv. Bowman and BW-NIL (*vrs1.a*) seedlings using a plant mini RNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, and its quality and quantity assessed with a Nano drop device (Peqlab, Erlangen, Germany). A 500-ng aliquot was taken as the template for the synthesis of the cDNA first strand, primed by oligo(dT), using a RevertAid cDNA kit (ThermoFisher SCIENTIFIC, Waltham, MA, USA). Each genotype was represented by three independent sets of 80–90 shoot apices. The subsequent qRT-PCR was based on the Power SYBR® Green PCR Master Mix (ThermoFisher SCIENTIFIC) and conducted in an Applied Biosystems 7900HT Fast Real-Time PCR system (ThermoFisher SCIENTIFIC) following the manufacturer's protocol. Relative transcript abundances were obtained using $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) and were normalized against the abundance of *serine/threonine phosphatase PP2A* transcript. The primer sequences employed are given in Table S3. The presence of a unique PCR product was verified by dissociation analysis and each qRT-PCR is repeated at least three times. Each biological replicate was represented by three technical replicates. For the microarray procedure, the same RNA samples extracted from three biological replicates was used and the quality of the RNA was verified with a Bioanalyzer 2100 device (Agilent Technologies, Santa Clara, CA, USA). The RNA was labeled through the application of a Low input QuickAmp Labeling kit (Agilent Technologies) and hybridized, following the manufacturer's protocol, to a custom-synthesized 60 k Barley Microarray (Agilent Technologies) (Koppolu *et al.*, 2013). The resulting data were analysed using GeneSpring 13.0 GX software (Agilent Technologies). After quantile normalization and baseline transformation to the median of all samples, the probesets (genes) were filtered by Coefficient of Variation <50%, followed by Moderated T-Test and Bonferroni-Holm multiple testing corrections; finally, probesets passed the *P*-value cut-off ≤0.05 with the fold change of ≥2.0 are taken for interpreting the data. Data sets are available at e!DAL (Arend *et al.*, 2014) (https://doi.org/10.5447/ipk/2016/10, https://doi.org/10.5447/ipk/2016/10). GO terms of the Harvest35 assembly were annotated via UniProt and significantly up- and down-regulated genes were tested for enrichment using topGO (Adrian Alexa, 2010). Significance of enrichment was tested using the classic Fishers Exact test as implemented in topGO.

Phylogenetic and BLAST analysis

The HD-ZIPI protein sequences of Arabidopsis and rice were taken from TAIR and TIGR databases respectively, and their sequence IDs were from the earlier publications (Henriksson *et al.*, 2005; Agalou *et al.*, 2008). Barley and maize homolog proteins were collected from 'IPK Barley BLAST server' (http://webblast.ipk-gatersleben.de/barley/viroblast.php) and 'Gramene database' (http://www.gramene.org/) respectively. Phylogenetic analysis was done from the SALAD database, which performs the clustering based on the presence and the similarity of the evolutionary conserved motifs, extracted by using the MEME software (http://meme.sdsc.edu/meme/intro.html). The putative function of the differentially abundant transcripts and VRS1 orthologous proteins was derived from the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE =BlastHome), gramene and BARLEX (http://apex.ipk-gatersleben.de/apex/f?p=284:10) (Colmsee *et al.*, 2015) databases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

V.T., A.M.A, R.K., T.S. and M.K. analysed the data. M.K., T.S., V.T., A.M.A and N.S. conceptualized the research and wrote the manuscript; A.M.A performed the GWAS analysis. A.M.A and R.K. were responsible for the phenotyping.

A.G. provided the genotypic information used for GWAS. G.H. and J.K. created the transgenic barley plants, which were analysed by V.T. Microscopic analyses were performed by V.T. and T.R. GO analysis was performed by A.B.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. VRS1 affects grain number in two- and six-rowed population; phenotyping of leaf length, leaf number per main culm and whole plant along with tiller number in Z37 and Z55.

Figure S2. VRS1 might not affect vascular anatomy, however it likely influences vein number.

Figure S3. The width of SAM is not significantly different between mutant and wild-type and mutant has thinner leaf (Plastochron 1) compared to wild-type.

Figure S4. *VRS1* promoter activity in epidermis and vascular bundles of matured leaves.

Figure S5. Gene ontology enrichment analysis of all expressed genes detected by 60k microarray hybridization.

Figure S6. Heat map of the significantly differentiated genes

Figure S7. Confirmation of the expression of selected genes from microarray analysis by qRT-PCR.

Figure S8. Alignment of amino acid sequences for the identified motifs from HD-ZIP I phylogenetic analysis.

Table S1. Description and phenotypic analysis results of leaf area across the association panel of 218 spring barley plants.

Table S2. Expression of *VRS1* and its homologs in different plant organs.

Table S3. Primer sequences used for qRT-PCR and construction of the *ProVRS1*::*eGFP* transgene.

Methods S1. Phenotyping.

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