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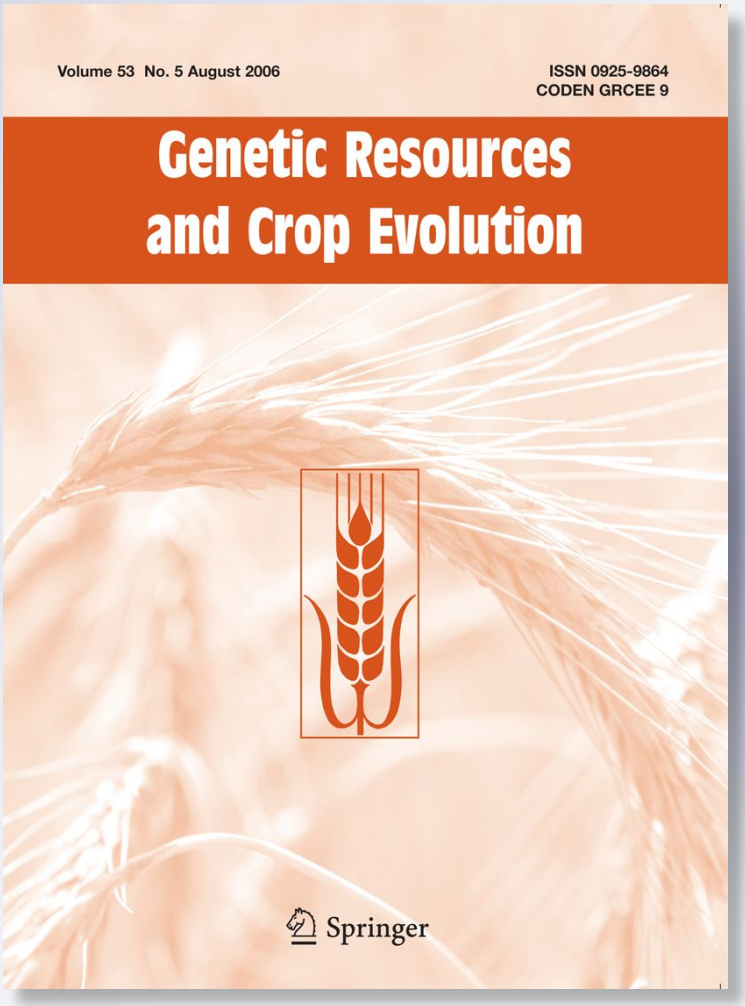

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RESEARCH ARTICLE

Re-sequencing of *vrs1* and *int-c* loci shows that *labile* barleys (*Hordeum vulgare* convar. *labile*) have a six-rowed genetic background

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Abstract *Labile*-barleys (*Hordeum vulgare* convar. *labile* (Schiem.) Mansf.) are found in the highlands of Ethiopia, Eretria and North India-Pakistan districts. They represent a distinct spike form showing row-type alterations even within individual spikes of the same genotypes. Variation at the *six-rowed spike 1* (*vrs1*) locus is sufficient to control barley lateral spikelet fertility, which is also modified by alleles at the *intermedium-c* (*int-c*) locus. This study aimed at re-sequencing these two loci to investigate whether *labile*-barleys have a two-rowed genetic background, resulting in increased lateral spikelet fertility, or show reduced lateral spikelet fertility if they possess a six-rowed genetic background. The *Vrs1* re-sequencing results of 221 supposedly *labile*-barley accessions from Ethiopia revealed 13 accessions with two novel *vrs1.a1* haplotypes. Following the current nomenclature of *vrs1* haplotypes, the new haplotypes were named as haplotypes 66 and 67. Re-sequencing at the *int-c* locus showed that 118 of the *labile*-barleys possessed the previously described *Int-c.a* allele but only one accession was found having a novel *Int-c.a* haplotype in the homozygous state (termed *Int-c.a haplotype1*; *Hap_1*). Interestingly, 101 *labile*-barleys carried the *Int-c.a* allele and *Int-c.a haplotype1* simultaneously, suggesting maintained heterozygosity or recent gene duplication at this locus. Only one accession had a two-rowed haplotype (*Vrs1.b3*, *int-c.b1*) and one accession possessed the *Vrs1.t* (*deficiens*) and *Int-c.a* alleles (six-rowed). These two accessions were considered as misclassified *labile* genotypes and not included in further analysis. Thus, these results confirmed that all of the 219 *labile* accessions studied in this work showed six-rowed alleles at *vrs1* but reduced lateral spikelet fertility. This reduction is most likely caused by the recessive *labile* (*lab*) locus which we are in the process to characterize further.

Keywords *Hordeum vulgare* convar. *labile* · *int-c* · *Labile*-barleys · Lateral spikelet fertility · Novel haplotype · *vrs1*

Helmy M. Youssef and Ravi Koppolu contributed equally to the work.

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Introduction

The inflorescence architecture of barley (*Hordeum vulgare* L.) is unique among Triticeae family members, which also include wheat, rye and triticale. The

barley spike is characterized by a triple spikelet meristem (one central and two lateral spikelets) at each rachis node which bears three one flowered spikelets (von Bothmer et al. 1985). Based on the central and lateral spikelet fertility, barley could be classified into four different groups: (i) two-rowed barley which has fully fertile central spikelets but the two lateral spikelets are sterile and produce only one seed per rachis node, (ii) the *deficiens*-barley includes two-rowed barleys from Ethiopia which have no or extremely reduced lateral spikelets and it produces one seed per rachis node, (iii) six-rowed barley which has three fully fertile spikelets and produces three seeds per rachis node, and (iv) *labile*-barleys which can have the lateral spikelets developed or absent, fertile or sterile even within one spike of the same genotype (Fig. 1).

Labile-barley (*Hordeum vulgare* L. convar. *labile* (Schiem.) Mansf.), previously known as '*irregular*' barley, was found among barleys originating from the

Fig. 1 Barley spikes showing different row-types; (**a**) two-rowed barley, (**b**) six-rowed barley, (**c**) *deficiens*, (**d**) spikelets from *top* to *bottom*: two-rowed, six-rowed and *deficiens* and (**e**) different spike forms of *labile*-barleys showing increased lateral fertility from *left* to *right* (Awns were clipped off for clarity)

highlands of Ethiopia, Eritrea (Åberg and Wiebe 1945) and also the North India-Pakistan districts (Takeda and Saito 1988). The *labile* row-type has been considered as a distinct spike character especially among Ethiopian barleys (Bjørnstad and Abay 2010) and 'irregular spike'-forms have been growing in most of the barley cropping areas throughout Northern Ethiopia (Abay and Bjørnstad 2009; Hadado et al. 2009). Recent phenotypic and molecular evidence linked the occurrences of *labile*-barleys mainly to higher altitudes above 2,800 m a.s.l. (subpopulation T6; Hadado et al. 2010). However, among all the naturally occurring row-type variants in barley, the *labile*-barleys are genetically least described probably owing to their high phenotypic plasticity, which caused difficulties while classifying them (Mansfeld 1950). The *labile*-phenotype can vary from spike to spike even within a single plant; for example one spike can be completely reduced to a *deficiens* phenotype with other spikes expressing various degrees of lateral spikelet fertility within an individual plant. There is a continuous variation in the number of fertile lateral spikelets from genotype to genotype (Djalali et al. 1970). It has been suggested that two genetic factors are necessary for the manifestation of the *labile* character: (i) the recessive allele for the six-rowed phenotype (*vrs1.a*), and (ii) the recessive allele at the *lab* gene for the *labile* character (Djalali 1970). The two-rowed allele at *Vrs1* appeared to be epistatic to the *lab* gene and the *lab* gene contributes to variable expression in lateral spikelets (Djalali et al. 1970). The F_1 and F_2 plants arising from crosses between *labile* genotypes were also found to show the *labile* character, indicating the complete penetrance of the *labile* phenotype. However, lateral spikelets of F_1 plants from *labile* and two-rowed barley crosses were completely developed but sterile. F_2 individuals segregated for two-rowed, six-rowed and *labile* phenotypes (Djalali 1970).

Understanding the developmental genetics of the barley inflorescence such as spikelet initiation, abortion and fertility has started to emerge relatively recently. Komatsuda et al. (2007) provided the first step in the elucidation of lateral spikelet fertility in barley and showed that loss-of-function of the wild-type *Vrs1* gene (responsible for two-rowed phenotype) resulted in complete fertility of lateral spikelets displaying the six-rowed phenotype. The *Vrs1* gene belongs to the HD-ZIP I class of homeobox transcription factors. Lack of the VRS1 protein in lateral spikelet primordia enabled complete fertility, suggesting that VRS1 protein suppresses the development of lateral spikelets in barley. Previous and recent studies revealed that different alleles at the *vrs1* locus are responsible for the size and fertility of lateral spikelets, for example barleys classified as convar. *hexastichon* (L.) Alef. possess the *vrs1.a* allele, that of convar. *deficiens* (Steud.) Mansf. carry the *Vrs1.t* allele, convar. *distichon* (L.) Alef. have the *Vrs1.b* allele and some barleys belonging to the convar. *intermedium* display the *int-d* allele (Komatsuda et al. 2007; Lundqvist and Lundqvist 1989).

Lundqvist and Lundqvist (1988) showed that the phenotypic effect of *Vrs1.b* can be influenced by ten independent *intermedium* (*int*) genes distributed all over the barley genome. In addition to this, natural quantitative variation in the size and fertility of the lateral spikelets has also been observed, particularly in progenies of two-rowed by six-rowed crosses (Lundqvist and Lundqvist 1989). Genetic studies indicated that this quantitative variation is largely due to the effect of alleles at the *int-c* locus. Alleles at the *int-c* locus either complement or repress the fertility of lateral spikelets based on the allelic status at the *Vrs1* gene. For example in six-rowed barley, the loss-of-function *vrs1.a* allele is generally complemented by the *Int-c.a* allele, and in two-rowed barley the *Vrs1.b* allele is complemented by the *int-c.b* allele (Lundqvist et al. 1997). Recently Ramsay et al. (2011) identified *int-c* as an orthologue of the maize (*Zea mays* L.) domestication gene, *Teosinte branched 1* (*ZmTB1*). *ZmTB1* is mainly involved in the control of axillary organ growth and also in female inflorescence development in maize (Doebley et al. 1997) rather than inflorescence architecture. It was observed that the induced mutant allele *int-c.5* significantly increases the tiller number during the juvenile stages (Ramsay et al. 2011). However, it is presumed that tillering mediated by *Int-c.a* in six-rowed cultivars is under the masking effect of the reduction in tiller number associated with six-rowed alleles at the *Vrs1* gene (Kirby and Riggs 1978). Apart from the naturally occurring six-rowed mutants *vrs1* and *int-c*, there are three induced mutants *vrs2*, *vrs3*, *vrs4* which can individually convert two-rowed to six-rowed barley (Druka et al. 2011).

Until today, almost all cultivated six-rowed barleys are known to carry the recessive (loss-of-function)

vrs1.a allele (Komatsuda et al. 2007) and the alternative *Int-c.a* allele (Ramsay et al. 2011), enabling complete lateral spikelet fertility. In contrast, two-rowed barleys, carrying the functional *Vrs1.b* allele (Komatsuda et al. 2007), and *int-c.b* allele (Ramsay et al. 2011), develop always infertile lateral spikelets, and therefore, produce only one fertile central spikelet. Previous genetic studies including *labile*-barleys found contradicting results, suggesting that *labile*-barleys were either derived from two-rowed (Breitenfeld 1957) or six-rowed barleys (Nötzel 1952). In an attempt to reveal the haplotype structure at the *vrs1* locus in *labile*-barleys, a set of 14 *labile* accessions had been analyzed and all carried the *vrs1.a* allele, (Saisho et al. 2009). However, the sample size used in the Japanese study was very limited and may not provide a representative and sufficient coverage of the available haplotypes. In order to get a better understanding in which genotypic background the *labile* character is most reliably detectable we initiated the present study to determine the genotypic status of 221 Ethiopian barleys at the two known row-type genes, *Vrs1* and *Int-c*.

Materials and methods

Plant materials

221 Ethiopian barley accessions categorized as *labile* and maintained at the IPK Genebank, Gatersleben, Germany, were selected for the present study (Supplementary table 1). Two two-rowed (Barke and Ametyst) and two six-rowed (Morex and Streptoe) spring barley cultivars were grown alongside with all *labile* accessions as controls.

Growing conditions and spike phenotyping

The expression of the *labile* character was found to be influenced by day length. Djalali (1970) found that, a period of short day (12 h light and 12 h dark) treatment for 20–30 days after seed germination resulted in reduced lateral fertility. It was also noted that the expression of the *labile* character is more pronounced under continuous short days. To account for this observation, *labile* accessions and controls were grown under 12 h/12 h (day/night) light conditions and a temperature of ~14°C during the day and ~12°C during the night. After anthesis plants were scored for visible phenotypes such as Filled Lateral Spikelets (FLS) with developed kernels, Unfilled Lateral Spikelets (ULS) with enlarged lemma and palea with awns, but without developed kernels, Developed Lateral Spikelets (DLS) comprising both FLS and ULS, Potential Spikelets (PS = Number of rachis nodes/spike (in case of expected two-rowed or *deficiens* accessions) and number of rachis nodes/spike* 3 (in case of expected six-rowed accessions)), Potential Lateral Spikelets (PLS = number of rachis nodes/spike* 2 (in case of expected six-rowed accessions) and zero (in case of expected two-rowed or *deficiens* accessions)), Unfilled Central Spikelets (UCS) and Developed Spikelets (DS) which comprise of filled, unfilled central spikelets and developed lateral spikelets; all calculations were performed on the total number of heads averaging 5 heads on a single plant.

Genomic DNA isolation

Leaf samples were collected from single plants (at 3–5 leaf stage) of each accession for DNA extraction. The total genomic DNA was extracted using the Doyle and Doyle (1990) method. DNA quality and quantity were checked on 0.8% agarose gels and the concentration was adjusted to ~20 ng/µl for PCR.

PCR amplification, sequencing and sequence analysis

Three primer pairs were designed to cover the 2,062 bp fragment at the *vrs1* locus in order to obtain sequence data for the whole gene by Sanger sequencing. The three primer pairs include *Vrs1-1*F (5′-TATCTAGAGGAACTCGATGAACTTGAG-3′), *Vrs1-1*R (5′- GTACCATTGGCCGCGAA-3′) covering promoter and 5′ untranslated region (5′ UTR), *Vrs1-2*F (5′-ACACCAACAGGCAACAGAACAACCTA-3′), *Vrs1-2*R (5′-GGACGCACATCATCAGGTCATCGT-3′), covering exon1, exon2, exon3 and *Vrs1-3*F (5′-CAAACATATGGCCAGCTGCT-3′), *Vrs1-3*R (5′-TGATCTTCAAGAGAGCTGCCA-3′) covering the 3′ UTR. For the *HvTB1* gene a single primer pair was designed to amplify a 1,074 bp fragment. The primer pair for this locus include *HvTB1*F (5′-TCCTTTCTATGATTCCCCAAGCCCC-3′) and *HvTB1*R (5′-CCACTCCACCGAGCTC

CC-3′). PCR amplifications with individual primer pairs for *Vrs1* and *Int-c* were performed in all 221 barley accessions.

PCR amplifications were carried out in a 25 µl reaction volume containing 20 ng of DNA, 2.5 µl of PCR buffer (10X) (Qiagen, Hilden, Germany), 5 mM dNTPs, 5 µl of Q-solution (Qiagen, Hilden, Germany), 5 pM primers, and 1U of *Taq* polymerase (Qiagen, Hilden, Germany). The PCRs were conducted using a thermal cycler (SensoQuest Thermal Cycler, USA) and the touchdown PCR amplification profile has an initial denaturation step for 3 min at 94°C followed first by 8 cycles of 94°C for 40 s, 61°C for 40 s (for *vrs1-1* and *vrs1-3*) or 65°C (for *Vrs1-2* and *HvTB1*) and 72°C for 2 min, with 1°C decrement in temperature per each cycle, then followed by 45 cycles of 94°C for 40 s with constant annealing temperatures (55°C- for *vrs1-1* and *vrs1-3* or 60°C for *Vrs1-2* and *HvTB1*) for 40 s and 72°C for 2 min, followed by a final extension for 10 min at 72°C. The PCR products were tested on 1.2% agarose gels to check the amplification.

For direct-sequencing of PCR products, the PCR products were first purified using MinElute 96UF PCR purification kit (Qiagen, Hilden, Germany), then sequenced using BigDye Terminator v3.1 cycle sequencing Kits (Applied Biosystems, USA). DNA sequence analysis, quality score assignments and the construction of contigs were achieved using Sequencher 4.7 DNA sequence assembly software. Multiple sequence alignments were carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The *Vrs1.b2*, *Vrs1.b3*, *Vrs1.t*, *vrs1.a1*, *vrs1.a2* and *vrs1.a3* alleles (Saisho et al. 2009) for the *vrs1* locus and *Int-c.a*, *int-c.b1* and *int-c.b2* alleles (Ramsay et al. 2011) for the *int-c* locus were considered as the reference alleles.

Results

Genotypic status of the *vrs1* locus in *labile*-barleys

A total of 221 Ethiopian barley accessions classified as *labile* (Supplementary table 1) were sequenced at the *vrs1* locus. The sequence analysis revealed that 217 accessions had the *vrs1.a1* allele which is responsible for the six-rowed phenotype (Table 1). Meanwhile, two accessions (HOR3529 and HOR3587) carried the *vrs1.a3* allele, one accession possessed the two-rowed allele *Vrs1.b3* (HOR5281) and one accession (HOR5471) had the *deficiens* allele *Vrs1.t*. Since *Vrs1* seems to be epistatic to the *labile* locus, these two accessions possessing two-rowed alleles (HOR5281 & HOR5471) can be considered as misclassified *labile* genotypes, and hence, were omitted from further analyses, thus reducing the effective number of *labile* accessions to 219. Interestingly, 13 of the 217 accessions carrying the *vrs1.a1* allele showed two novel haplotypes either in the promoter region or in the highly conserved HD domain. Among the 13 accessions nine of them (HOR6178, HOR6179, HOR6180, HOR6279, HOR7729, HOR7734, HOR9405, HOR 10421 and HOR10490) had a unique single nucleotide polymorphism (SNP) within the promoter region at 230 base pair (bp) (Fig. 2). The sequence carrying an SNP at 230 bp in different accessions has been named as *haplotype 66* of *vrs1.a1* (*Hap_66*) in continuation to the previously named haplotypes of *Vrs1* (Saisho et al. 2009). The remaining four of the 13 accessions, HOR5172, HOR6400, HOR6420 and HOR6440, showed an SNP at bp position 987 (Fig. 2) within the conserved HD domain that resulted in an amino acid substitution from alanine to aspartic acid (supplementary Figure 1). Thus, the novel haplotype identified by the amino acid substitution in the HD domain has been named as *haplotype 67* of *vrs1.a1* (*Hap_67*) (Fig. 2). Sequence data of these two novel haplotypes (*Hap_66 and Hap_67*) were submitted to the National Center for Biotechnology Information (NCBI) and is available under the following accession numbers JF904736 and JF904737, respectively. In

Table 1 *Vrs1* and *Int-c* alleles and haplotypes in all studied 221 Ethiopian barley accessions

	Int-c alleles/haplotypes				
	Int-c.a	*Int-c.a/ Hap_1*	*Int-c.a/ Hap_2*	*int-c.b1*	Total
Vrs1 alleles					
vrs1.a1	115	1	101		217
vrs1.a3	2	–	–	–	2
*Vrs1.b3**	–	–	–	1	1
*Vrs1.t**	1	–	–	–	1
Total	118	1	101	1	221

* Two-rowed and *deficience* barleys were misclassified as *labile*-barleys (confirmed by re-sequencing of *vrs1*)

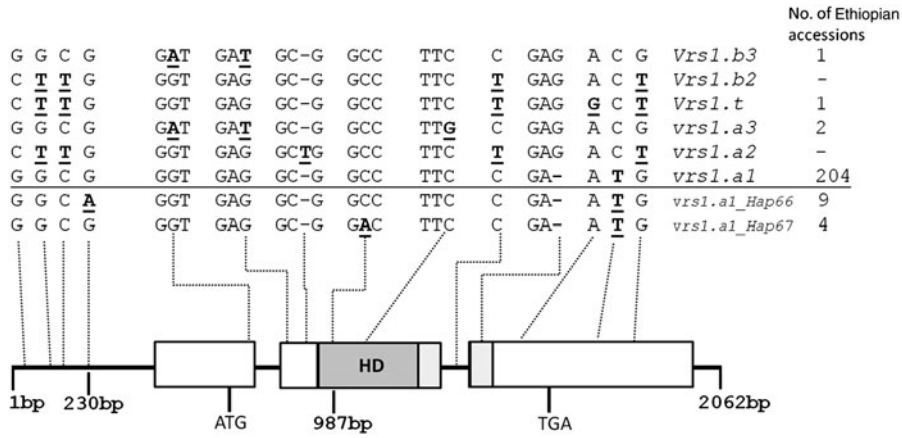

Fig. 2 Alleles and haplotypes at the *vrs1* locus in two- and six-rowed cultivars as well as *labile*-barley accessions. *Vrs1.b3* (two-rowed allele) was identified in one accession, *Vrs1.t.* (*deficiens* allele) in one accession, *vrs1.a3* (six-rowed allele) in two accessions, *vrs1.a1* (six-rowed allele) in 217 accessions were identified. Three overlapping fragments were aligned to cover the *vrs1* locus, spanning in total 2,062 bp

summary, and in accordance with Saisho et al. (2009), all the *labile* accessions (100%) showed six-rowed alleles at the *vrs1* locus.

Genotypic status of the *int-c* locus in *labile* barleys

Sequencing of the *int-c* locus in 221 Ethiopian barley accessions (supplementary Table 1) revealed that 220 of them carried dominant, functional *Int-c* alleles and only one accession possessed the recessive two-rowed *int-c.b1* allele (HOR5281). Of the 220 accessions having dominant *Int-c* alleles, 118 showed the previously described and lateral fertility promoting *Int-c.a* allele (Table 1). Apart from this, two interesting sequence variations at this locus were observed in the remaining accessions; one of them included a non-synonymous nucleotide change at position 437 bp from the start codon within the TCP domain of *HvTB1* that lead to an amino acid substitution from aspartic acid to valine (supplementary Figure 2). This kind of sequence variation within the previously described dominant allele of *Int-c.a* was observed in one *labile* accession, HOR1643, and hence has been denoted as a novel *Int-c.a haplotype1* (*Hap_1*) (Fig. 3). The GenBank accession number of the novel *Int-c.a haplotype1* is JF904738. The second sequence variant showed a highly consistent, putatively heterozygous position at the *Int-c.a* allele, where the characteristic SNP of the *Int-c.a haplotype1* (*Hap_1*) (SNP "T" at 437 bp instead of "A" in *Int-c.a1*) occurred simultaneously. This feature was observed in 101 *labile* accessions and has been denoted as *Int-c.a haplotype2* (*Int-c.a/Int-c.a haplotype1*) (*Hap_2*). Sequence analysis of the *labile* accessions also revealed that all of them (100%) carried the lateral fertility promoting alleles at the *int-c* locus.

vrs1 and *int-c* allele/haplotype combinations in *labile* accessions

In order to have a better understanding of the row-type status in *labile*-barleys, the haplotypes at *vrs1* and *int-c* loci were compared with that of normal two-rowed (*Vrs1.b*, *int-c.b*) and six-rowed (*vrs1.a*, *Int-c.a*) haplotypes. As shown in Table 1, of the 221 barley accessions studied, 115 accessions had the *vrs1.a1*, *Int-c.a* haplotypes (six-rowed alleles), 101 accessions carried the *vrs1.a1*, *Hap_2* haplotypes (six-rowed alleles) and one accession (HOR1643) showed the *vrs1.a1*, *Hap_1* haplotypes (six-rowed alleles). Apart from this, two accessions (HOR3529 and HOR3587) displayed the *vrs1.a3*, *Int-c.a* combination (six-rowed alleles). Among the remaining two accessions, interestingly, one accession (HOR5281) showed both two-rowed alleles, i.e. *Vrs1.b3*, *int-c.b*, and the other accession (HOR5471) showed a *deficiens* allele background *Vrs1.t*, *Int-c.a* at *vrs1* and *int-c* loci. These two accessions have been considered as misclassified *labile* genotypes and were omitted from further analyses. In summary, all 219 *labile* accessions

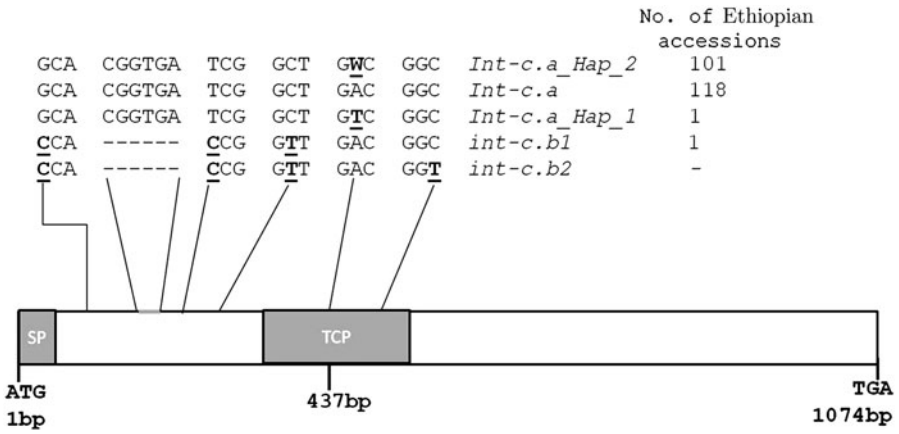

Fig. 3 Allele and haplotype analysis of the *int-c* locus in two- and six-rowed cultivars as well as *labile*-barley accessions. *Int-c.a1* (promoting lateral spikelets) in 118 accessions, *Int-c.a2* (promoting lateral spikelets) in one accession, *Int-c.a1/.a2* (both promoting lateral spikelets) in 101 accessions, and i*nt-c.b1* (impairing lateral spikelets) in one accession were identified. Following the International Union of Pure and Applied Chemistry (IUPAC) notation for degenerate base symbols the "W" at bp position 437 represents the two nucleotides A and T. Re-sequencing comprised one PCR fragment of 1,074 bp in length

(100%) revealed a six-rowed genotypic background (*vrs1.a*, *Int-c.a*) at the two loci. Thus, data generated in this study can provide concrete evidence that the *labile* accessions possess a six-rowed rather than two-rowed genotypic background.

Haplotype combination/phenotype relationships

The phenotypic data (Table 2) clearly showed that, the *vrs1.a3*, *Int-c.a1* haplotype combination in *labile* accessions resulted in 99.5% of DS, however, it should be noted that about 10% of the DS were UCS. At the same time, the other six-rowed haplotypes resulted in 41.8% (*vrs1.a1*, *Hap_2*) to 51.6% (*vrs1.a1*, *Hap_1*) of DS with a decrease in the number of UCS (1.0–8.5). Regarding the lateral spikelet development in different haplotypes, almost all lateral spikelets were developed (99.3% of DLS/PLS) in accessions having *vrs1.a3*, *Int-c.a* haplotype (six-rowed haplotype), 54.5% of them include ULS. Meanwhile, the other expected six-rowed haplotypes ranged from 13.7 (*vrs1.a1*, *Int-c.a_Hap2*) to 27.4% (*vrs1.a1*, *Int-c.a_Hap_1*) DLS. On the other hand, 101 *labile* accessions had the *vrs1.a1*, *Int-c.a1/.a2* haplotype and showed 13.7% DLS compared to 14.3% DLS in the typical six-rowed haplotype *vrs1.a1*, *Int-c.a1* which was found in 115 accessions. There was no significant phenotypic effect of the putative heterozygous *Int-c.a* allele (*Int-c.a/Hap_2*) on the number of DLS compared to the homozygous *Int-c.a1* allele (*P*-value = 0.69), suggesting that the altered protein sequence within the TCP domain of *Int-c.a/Hap_2* might not have any deleterious or beneficial phenotypic effect on lateral spikelet fertility.

Discussion

Lateral spikelet fertility forms an important basis for grouping barleys into two-rowed (unfertile lateral spikelets) and six-rowed (fertile lateral spikelets) forms. The *labile*-barleys show an intermediate phenotype between two-rowed and six-rowed condition, where the lateral spikelets are occasionally developed but in other cases development is aborted. Variation in lateral spikelet fertility within the spike, and from spike to spike on a single plant, and even from plant to plant within one progeny in *labile*-barley poses an interesting question on its row-type genotypic background (Djalali et al. 1970). So, studying the genetic mechanism(s) underlying lateral spikelet fertility may similarly contribute to a better general understanding of spikelet development in barley or related grass species. As a first step towards this goal, the current study has been initiated to reveal the allele/haplotype structure of *labile*-barleys at two of the known row-type genes *Vrs1* and *Int-c*.

Table 2 *Vrs1* and *Int-c* allele/haplotype combinations and their corresponding spike phenotypes of 219 *labile* accessions after anthesis. Spikelet numbers were calculated from all heads per plant

Allele/haplotype combinations	Number of accessions	Expected row-type	All spikelets				Lateral spikelets				
			PS	DS	UCS	%DS/PS	PLS	DLS	ULS	FLS	% DLS/PLS
vrs1.a1, *Int-c.a*	115	6-r	201.7 ± 69	84.6 ± 31	6.5 ± 7	42	134.5 ± 46	19.3 ± 16	7.2 ± 7	12.1 ± 13	14.3
vrs1.a1, *Int-c.a/Hap_2*	101	6-r	185.0 ± 72	77.4 ± 31	8.5 ± 8	41.8	123.4 ± 48	16.9 ± 17	5.2 ± 4	12.2 ± 15	13.7
vrs1.a1, *Int-c.a_Hap_1*	1	6-r	126	65	1	51.6	84	23	1	22	27.4
vrs1.a3, *Int-c.a*	2	6-r	201.0 ± 81	200.0 ± 76	20.0 ± 22	99.5	134.0 ± 53	133.0 ± 49	54.5 ± 62	78.5 ± 12	99.3
vrs1.a, *Int-c.a* (six-rowed controls)	–	6-r	238.8 ± 6.6	239.6 ± 8.3	0.4 ± 0.5	100.3	160.0 ± 5.7	159.6 ± 5.5	11.0 ± 7.8	148.6 ± 12.5	99.8
Vrs1.b, *int-c.b* (two-rowed controls)	–	2-r	111.7 ± 10.4	111.7 ± 10.4	0.5 ± 0.5	100.0	0	0	0	0	0

PS Potential spikelets (=rachis nodes number * 3 (in case of six-rowed haplotype) and * 1 (in case of two-rowed or *deficiens*)); as total of all spikes per plant

DS Developed spikelets, *FLS* filled lateral spikelets, *ULS* unfilled lateral spikelets, *UCS* unfilled central spikelets, *DLS* developed lateral spikelets, *PLS* potential lateral spikelets

Sequence analysis of 221 Ethiopian barleys at the *vrs1* locus revealed four previously reported alleles and two new haplotypes at *vrs1.a1*. The Four previously reported alleles include *vrs1.a1*, *vrs1.a3* (six-rowed alleles), *Vrs1.b3* (two-rowed allele) and *Vrs1.t* (*deficiens* allele), were identified in an earlier study by Komatsuda et al. (2007). All of the 219 *labile* accessions in the present study showed six-rowed alleles at *vrs1* (*vrs1.a1*- 99.08%, *vrs1.a3* – 0.92%) and only two misclassified accessions had either of the two-rowed alleles *Vrs1.b3* (HOR5281) or *Vrs1.t* (HOR5471). The *vrs1.a1* and *vrs1.a2* alleles identified by Komatsuda et al. (2007) are due to a premature stop codon resulting in a truncated and non-functional protein; whereas *vrs1.a3* has an amino acid substitution from phenylalanine to lycine in the conserved HD domain. The *vrs1.a1* allele is found in six-rowed barleys from all over the world, whereas *vrs1.a2* and *vrs1.a3* are specific to the regions of Western Mediterranean and East Asia, respectively (Komatsuda et al. 2007). The novel *vrs1.a1 haplotype 67* (*Hap_67*) identified in the present study is caused by an amino acid substitution (aspartic acid to valine) within the conserved HD domain and has been observed in four of the *labile* accessions, whereas the other novel *vrs1.a1* haplotype (*Hap_66*) was due to an SNP in the promoter region. The newly found haplotypes of *vrs1.a1* could be specific to the highlands of Ethiopia and Eretria, which *labile*-barleys originated from; however, such hypothesis can be tentative since the number of accessions carrying these alleles are very limited (*Hap_66*- nine accessions and *Hap_67*- four accessions). The low number of accessions carrying these two haplotypes may suggest that these evolutionary events were rather recent. Further studies with a larger set of *labile* and non-*labile* accessions may provide a deeper insight into the rise of the two novel haplotypes. The *Vrs1.t* allele, specific to *deficiens* barleys from Ethiopia, was found in one of the accessions and is most likely due to a misclassification as *labile*-barley. To confirm this, the corresponding accession (HOR5471) was re-grown under long day and short day conditions and the similar *deficiens* phenotype was observed again. The recessive *vrs1.a2* (Komatsuda et al. 2007) and *vrs1.a4* allele, identified by Cuesta-Marcos et al. (2010), were not observed in the present set of *labile* accessions. The *vrs1.a2* allele was identified as being specific to accessions originating from the Western Mediterranean region

(Komatsuda et al. 2007); whereas in case of *vrs1.a4*, of the 102 accessions used in Cuesta-Marcos study four accessions showed the *vrs1.a4* allele and all four accessions originated from Northern America (three from Virginia and one from Washington), suggesting that the *vrs1.a4* allele could be unique to the Northern American region.

By re-sequencing of the *int-c* locus in an association panel of two-rowed and six-rowed barleys (190 lines) Ramsay et al. (2011) identified that all two-rowed barleys used in their study carried the recessive *int-c* allele and, conversely, almost all six-rowed barleys had the alternative *Int-c.a* allele, irrespective of their particular *vrs1.a* (six-rowed) allele. Another study reported that the specific dominant *Int-c.a* allele is necessary for the commercial six-rowed phenotype, but dominant or recessive *int-c.b* alleles are found in cultivated two-rowed germplasm (Cuesta-Marcos et al. 2010). In our study, re-sequencing of *int-c* in *labile* revealed that all (100%) the *labile* accessions carried *Int-c.a* allele (*Int-c.a*- 118 accessions, *Int-c.a_Hap1*- one accession and *Int-c.a/Int-c.ahaplotype1*- 101 accessions) which further confirms that *labile*-barleys have a six-rowed genetic background; only a single accession showed the two-rowed allele *int-c.b1* at the *int-c* locus along with dominant *Vrs1* allele (misclassified *labile*). The newly identified *Int-c.a* haplotype (*Hap_1*) is due to an amino acid substitution in the conserved TCP domain, it occurred in only one of the accessions in the homozygous state and it is too early to infer that it is specific to *labile*-barleys at this stage. In fact, across 101 accessions the presences of the *Int-c.a* allele and *Hap_1* were simultaneously detected, suggesting that heterozygosity had been maintained at the *int-c* locus. Why heterozygosity appeared to be maintained at this particular locus remains puzzling, given that barley is a highly self-fertilizing species. Hence, another possibility might be that the *int-c* locus has been duplicated in the 101 accessions and that both *int-c* genes differ by only one SNP; however, future work is required to clarify these hypotheses. From our phenotypic analyses it became evident that the apparent heterozygosity in 101 accessions did not significantly alter lateral spikelet fertility compared with the 118 homozygous *Int-c.a* accessions.

The haplotype combinations observed at the *vrs1* and *int-c* loci indicated that all the *labile*-barleys showed preference of six-rowed alleles over two-rowed alleles, i.e. 100% of the accessions have one or the other of the aforementioned six-rowed allele combination. The phenotypic data obtained from the *labile* accessions and its comparison to the observed allele/haplotypes combinations (Table 2) showed that, in spite of the *vrs1.a* and *Int-c.a* (genotypically six-rowed alleles) being present in the majority of the analyzed accessions, the observed phenotypic data did not support the expected six-rowed phenotype in *labile*. The *labile*-barley spike phenotype displays a variable number of fertile lateral spikelets at each rachis node (0–2 seeds/rachis node; the present study, but also Djalali et al. 1970; Takeda and Saito 1988). Except for the *vrs1.a3*, *Int-c.a* haplotype combination, which was found in two accessions that gave 99.5% DS, all six-rowed haplotypes, irrespective of their particular allele combinations, showed a maximum of 51.6% of DS. In the *vrs1.a3*, *Int-c.a* haplotype (two accessions) 99.3% of the lateral spikelets were fertile, but in the other six-rowed haplotypes (217 accessions) only from 13.7 to 27.4% of the lateral spikelets were developed. The *Vrs1.b3*, *int-c.b1* haplotype combination (two-rowed phenotype) showed an unexpected increase in the number of DS, and subsequently increased developed and fertile lateral spikelet number. This phenotype could potentially be associated with a naturally derived mutation at any of the three other *six-rowed spike* loci such as *vrs2*, *vrs3* or *vrs4* in this accession.

From previous work it was assumed that the *labile* locus interacts with the recessive six-rowed *vrs1* locus to produce irregular spikes with variable abortion of lateral spikelets (Lundqvist and Franckowiak 2003). Djalali et al. (1970) reported a recessive mode of gene action for *labile* in F_1 and F_2 plants when they crossed *labile*-barley with two-rowed barleys. Currently we are in the process to better understand the *labile* genetics and its corresponding phenotype by developing crosses between six-rowed and *labile*-barleys. Future work is required to elucidate the molecular basis for the apparently random spikelet fertility in *labile*-barleys.

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References

Abay F, Bjørnstad A (2009) Specific adaptation of barley varieties in different locations in Ethiopia. Euphytica 167: 181–195

Åberg E, Wiebe GA (1945) Ash content of barley awns and kernels as influenced by location, season and variety. J Agronomy 37:583–586

Bjørnstad A, Abay F (2010) Multivariate patterns of diversity in Ethiopian barleys. Crop Sci 50:1579–1586

Breitenfeld C (1957) Genetische Untersuchungen zur Phylogenie und Systematik der *intermedium*- und *labile*-Gersten. Z Pflanzenzüchtung 38:275–312

Cuesta-Marcos A, Szucs P, Close TJ, Filichkin T, Muehlbauer GJ, Smith KP, Hayes PM (2010) Genome-wide SNPs and re-sequencing of growth habit and inflorescence genes in barley: implications for association mapping in germplasm arrays varying in size and structure. BMC Genomics 11:707

Djalali M (1970) Investigations on expressivity and penetrance of *labile* character of barley (*Hordeum vulgare* L.). Z Pflanzenzüchtung 63:274–322

Djalali M, Hoffman W, Plarre W (1970) Genetics and variability of the *labile*-gene in barley under different environmental conditions. In: Nilan RA (ed) Barley genetics, vol II. Proceedings, second international barley genetics symposium, Pullman, 1969. Washington State University Press, Pullman, Washington, pp 201–207

Doebley J, Stec A, Hubbard L (1997) The evolution of apical dominance in maize. Nature 386:485–488

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Druka A, Franckowiak J, Lundqvist U, Bonar N, Alexander J, Houston K, Radovic S, Shahinnia F, Vendramin V, Morgante M, Stein N, Waugh R (2011) Genetic dissection of barley morphology and development. Plant Physiol 155:617–627

Hadado TT, Rau D, Bitocchi E, Papa R (2009) Genetic diversity of barley (*Hordeum vulgare* L.) landraces from the central highlands of Ethiopia: comparison between the Belg and Meher growing seasons using morphological traits. Genet Resour Crop Evol 56:1131–1148

Hadado TT, Rau D, Bitocchi E, Papa R (2010) Adaptation and diversity along an altitudinal gradient in Ethiopian barley (*Hordeum vulgare* L.) landraces revealed by molecular analysis. BMC Plant Biol 10:121

Kirby EJM, Riggs TJ (1978) Developmental consequences of two-row and six-row ear type in spring barley: 2. Shoot apex, leaf and tiller development. J Agric Sci Camb 91: 207–2016

Komatsuda T, Pourkheirandish M, He CF, Azhaguvel P, Kanamori H, Perovic D, Stein N, Graner A, Wicker T, Tagiri A, Lundqvist U, Fujimura T, Matsuoka M, Matsumoto T, Yano M (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proc Natl Acad Sci USA 104:1424–1429

Lundqvist U, Franckowiak JD (2003) Diversity of barley mutants. In: von Bothmer R, van Hintum T, Knüpffer H, Sato K (eds) Diversity in barley (*Hordeum vulgare*). Elsevier Science BV, Amsterdam, pp 77–96

Lundqvist U, Lundqvist A (1988) Induced *intermedium* mutants in barley: origin, morphology and inheritance. Hereditas 108:13–26

Lundqvist U, Lundqvist A (1989) The co-operation between *intermedium* genes and the six-row gene *hex-v* in a six-row variety of barley. Hereditas 110:227–233

Lundqvist U, Franckowiak JD, Konishi T (1997) New and revised descriptions of barley genes. Barley Genet Newsl 26:22–516

Mansfeld R (1950) Das morphologische System der Saatgerste, *Hordeum vulgare* L. s. l. Züchter 20:8–24

Nötzel H (1952) Genetische Untersuchungen an röntgeninduzierten Gerstenmutanten. Kühn-Arch 66:72–132

Ramsay L, Comadran J, Druka A, Marshall DF, Thomas WTB, Macaulay M, MacKenzie K, Simpson C, Fuller J, Bonar N, Hayes PM, Lundqvist U, Franckowiak JD, Close TJ, Muehlbauer GJ, Waugh R (2011) *INTERMEDIUM-C*, a modifier of lateral spikelet fertility in barley, is an ortholog of the maize domestication gene *TEOSINTE BRANCHED 1*. Nat Genet 43:169–172

Saisho D, Pourkheirandish M, Kanamori H, Matsumoto T, Komatsuda T (2009) Allelic variation of row type gene *Vrs1* in barley and implication of the functional divergence. Breed Sci 59:621–628

Takeda K, Saito W (1988) Inheritance of the percentage of missing lateral florets in '*Irregular*' barley. Japan J Breed 38:72–80 (in Japanese with English summary p 80)

von Bothmer R, Jacobsen N, Jorgensen RB (1985) Two new American species of *Hordeum* (Poaceae). Willdenowia 15:85–90