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ORIGINAL PAPER

The genetics of barley low-tillering mutants: *low number of tillers-1* (*lnt1*)

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Abstract Barley (*Hordeum vulgare* L.) carrying recessive mutations at the *Low number of tillers1* (*Lnt1*) gene does not develop secondary tillers and only develops one to four tillers by maturity. Double mutant analysis determined that the *lnt1* mutant was epistatic to five of the six low and high tillering mutants tested. Double mutants of *lnt1* and the low tillering mutant *intermedium-b* (*int-b*) resulted in a uniculm plant, indicating a synergistic interaction and that *Lnt* and *Int-b* function in separate tillering pathways. RNA profiling identified 70 transcripts with either increased or decreased abundance in the *lnt1* mutant compared to wild-type. One gene with reduced transcript levels in the *lnt1* mutant was the *BELL*-like homeodomain transcription factor *JuBel2*. The *JuBel2* allele in the *lnt1.a* mutant contained a frameshift mutation that eliminated most of the predicted polypeptide, indicating that the *Lnt1* gene encodes *JuBel2*. Previous studies with the low-tillering mutant *absent lower laterals* (*als*) showed that the tillering phenotypes and genetic interactions of *als* and *lnt1* with other tillering mutants were very similar. However, the transcriptomes were very different; many transcripts annotated as stress and defense response exhibited increased abundance in the *als* mutant. This difference suggests a functional separation between *Als* and *Lnt1* in the genetic control of tillering.

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Introduction

Plant shoot architecture is based upon the activity of the shoot apical meristem and axillary meristems. The shoot apical meristem produces all above-ground organs including: leaves, nodes, internodes, inflorescence and axillary meristems (Sussex 1989). Vegetative axillary meristems form in the leaf axil of lower leaves at the base of the plant (crown) and produce tillers (branches) in grasses. In barley (*Hordeum vulgare* L.), the predominant above ground vegetative structure are tillers. Primary tillers develop from axillary meristems located in leaf axils of the shoot apical meristem. Tillers developing from axillary meristems in the axils of primary tillers are secondary tillers (Counce et al. 1996). Tiller development is characterized by three general stages: (1) the initiation of an axillary meristem; (2) development of the axillary meristem with leaves (axillary bud); and (3) the subsequent outgrowth of the axillary bud into a tiller (Schmitz and Theres 2005).

Insights into the genetic regulation of branching have come from studies utilizing genes that fall into two general classes (recently reviewed by McSteen 2009; Wang and Li 2008; Doust 2007). One class of genes restricts bud outgrowth and are defined by mutants with increased branching. Examples include the maize *Teosinte branched1* gene (Doebley et al. 1997) and homologs in rice and *Arabidopsis* (Hu et al. 2003; Takeda et al. 2003; Aguilar-Martinez et al. 2007). Other examples include the *Arabidopsis MORE AXILLARY BRANCHING (MAX)* genes (Stirnberg et al. 2002; Sorefan et al. 2003; Booker et al. 2004) and homologs in pea, petunia and rice (Johnson et al. 2006; Sorefan et al. 2003; Snowden et al. 2005; Ishikawa et al. 2005; Zou et al. 2006; Arite et al. 2007). The other class of genes promote axillary meristem development and are defined by mutants with decreased branching. Examples include the *Arabidopsis LATERAL SUPPRESSOR* (*LAS*) gene (Greb et al. 2003) and tomato *Lateral suppressor* and rice *MONOCULM1* (*MOC1*) homologs (Schumacher et al. 1999; Li et al. 2003), and the *Arabidopsis REGULATOR OF AXILLARY MERISTEMS* (*RAX*) genes (Müller et al. 2006).

Double mutant studies in *Arabidopsis* (Müller et al. 2006), rice (Komatsu et al. 2003; Oikawa and Kyozuka 2009), and barley (Dabbert et al. 2009) suggest that the genetic pathways that regulate branching are complex. In rice, Komatsu et al. (2003) found that mutations at the *LAX PANICLE1* (*LAX1*) gene or the *SMALL PANICLE* (*SPA*) gene alone did not affect tillering. However, tillering was reduced in the *lax1; spa* double mutant, arguing that these two genes function in separate genetic pathways. Recently, *SPA* was identified as an allele of *MOC1* (Oikawa and Kyozuka 2009). Similarly, double mutant studies of the low tillering barley mutants *absent lower laterals1* and *intermedium spike-b* resulted in plants with fewer tillers than either single mutant, indicating that these genes function in separate pathways that promote tillering (Dabbert et al. 2009). Moreover, double mutants of the low branching *Arabidopsis rax1* and *las* mutants also resulted in decreased branching (Müller et al. 2006). These studies indicate that the genetic regulation of branching is controlled by two or more pathways.

Low- and high-tillering mutants have been described in barley (Babb and Muehlbauer 2003; Dabbert et al. 2009). These tillering mutants fall into the two general classes defined by Bennett and Leyser (2006). One class of genes promote axillary meristem development and are defined by mutations that result in a low-tillering phenotype. In barley, these mutations include: *low number of tillers1* (*lnt1*), *absent lower laterals1* (*als1*), *intermedium-b* (*int-b*), *uniculm2* (*cul2*), *uniculm4* (*cul4*), and *uzu*. Conversely, the other class of genes repress axillary meristem development and are defined by mutations that result in a high-tillering phenotype. In barley these mutations include: *densonidosum6* (*den6*), *granum-a* (*gra-a*), *intermedium spike-m* (*int-m*), and *many noded dwarf1* (*mnd1*).

The barley tillering mutants includes genes that appear to be required at different stages and result in altered gene expression. The *Cul2* gene product is required for all vegetative axillary meristem development; no tillers form in *cul2* mutants (Babb and Muehlbauer 2003). However, only primary tillers develop in *als1* mutant plants and the mature plant exhibits between one to four tillers. Thus, the *Als1* gene product is required for secondary tiller development (Dabbert et al. 2009). In *als1 mutant* plants, axillary buds were not observed developing in the axils of primary tillers, indicating that tiller development was inhibited before axillary bud development (Dabbert et al. 2009). Transcriptome analysis using the Barley1 GeneChip identified over 100 genes that exhibited either increased or decreased transcript levels in *als1* mutant plants compared to wild-type (Dabbert et al. 2009). Approximately 40% of the transcripts exhibiting increased accumulation in the *als1* mutant were annotated as stress and defense response, indicating that tillering is genetically related to the stress response. The low tillering phenotype of the *lnt1* mutant closely resembles *als1*. Given the similarity of the tillering phenotypes of the *als1* and *lnt1* mutants, we were interested in determining whether or not these similarities would extend further.

To gain an understanding of the genetic control of axillary meristem and axillary bud development in barley we examined the *lnt1* mutant. The objectives of this study were to (1) identify the block in tiller development in the *lnt1* mutant; (2) to identify genetic pathways regulating tillering; (3) to examine the genetic similarities between *lnt1* and *als1* mutants; and (4) to identify candidate genes for the *Lnt1* gene.

Materials and methods

Genetic materials

The *lnt1.a* allele is a single gene recessive mutation that was identified as a spontaneous mutation (cv. Mitake) in a bulk population from a cross between Chikurin Ibaraki 2 and Miho Hadaka (Nonaka 1973). We used a near-isogenic line developed through eight backcrosses of the Mitake-derived *lnt1.a* allele into Bowman. A second *lnt1* allele, *int-l.81*, was also used (Franckowiak and Lundqvist 1997). The *int-l.81* allele has been backcrossed into Bowman six times. Other barley tillering mutants used in this study contain single gene recessive mutations that have been backcrossed into the Bowman genetic background a minimum of five times (Babb and Muehlbauer 2003; Lundqvist et al. 1997). Table 1 lists the genetic stocks used.

Genetic analysis

To initiate construction of double mutants, the *lnt1.a* mutant was crossed to six other tillering mutants (Table 1). The F_1 plants were self pollinated to generate F_2 families segregating for both single mutants and the double mutant. Phenotypes from the F_2 families were examined in 90 to 150 individuals. Single mutants could be distinguished from double mutants by a combination of traits including tiller number, plant height, and spike morphology. Double mutant phenotypes were confirmed by screening 10–20 F_3 families that were segregating for one of the mutants and homozygous for the other, and by examining F_3 families derived from F_2 plants exhibiting the putative double mutant phenotype. All double mutant analysis was conducted either on field or greenhouse grown plants. In the greenhouse, four plants were grown in a 6 in. pot in potting soil (Metromix 200, Scott-Sierra Hort. Prod. Co., Marysville, OH). The greenhouse was set at 22°C for 16 h in the light followed by 18°C for 8 h in the dark. Plants were fertilized with Osmocote 14/14/14 (Marysville, OH). Phenotypes from the double mutant families were compared with the corresponding single mutants. Interactions between mutations were classified as epistatic, synergistic, or additive (Babb and Muehlbauer 2003). Interactions were classified as epistatic when the double-mutant phenotype was the same as one of the parents. When the double-mutant phenotype was more severe than either parent, the interaction was classified as synergistic. When the two mutants appeared to act independently giving a phenotype with characteristics of both mutants, the interaction was classified as additive.

Morphological analysis

The effect of the *lnt1* mutant on tillering was followed from 1-week-old seedlings to adult plants. Plants were grown in the greenhouse following the same protocol used for the double mutant analysis. Tissue sections containing the shoot apex from 1- and 2-week-old seedlings were prepared for light microscopy as previously described (Babb and Muehlbauer 2003). Later stages of tiller development were followed by dissecting the crown, first, and second nodes from plants at 3, 4, 5, and 6 weeks after planting. The number of tillers and axillary buds were recorded from a minimum of four plants. Tiller numbers from adult plants were counted in greenhouse grown plants and under field conditions.

Transcriptome analysis

A randomized design with three replications was used for Bowman and Bowman-*lnt1.a* to obtain tissue samples from

Table 1 Genetic stocks

Genetic stocks[a]	Tillering habit[b]	Chromosome location	Backcrosses to Bowman	Source
lnt1.a	Low	3HL	7	GSHO 1984[c]
int-l.81	Low	3HL	6	GSHO 1961
int-b.3	Low	5HL	6	Franckowiak[d]
uzu1.a	Low	3HL	7	GSHO 1963
den6, mnd6.6	High	5HL	7	GSHO 2235
gra-a.1	High	3HL	7	GSHO 1980
int-m.85	High	Unknown	5	Franckowiak
mnd1.a	High	Unknown	9	GSHO 2038
Bowman	Wild-type	NA	NA	Franckowiak
Morex	Wild-type	NA	NA	Rasmusson[e]
Steptoe	Wild-type	NA	NA	Rasmusson
Chikurn-Ibaraki2	Wild-type	NA	NA	CIho7370
Miho Hadaka	Wild-type	NA	NA	PI182617
Mitake	Low	NA	NA	GSHO 833

NA not applicable

[a] Mutant alleles are backcrossed into Bowman

[b] Tiller habit as compared to wild-type

[c] GSHO numbers, CIho7370 and PI182617 were obtained from the USDA-ARS National Small Grain Germplasm Research Facility, Aberdeen, ID

[d] Gift from J. Franckowiak, Department of Plant Sciences, North Dakota State University, Fargo, ND

[e] Gift from D. Rasmusson, Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN

four tissue types for the transcriptome analysis. Seed was surface sterilized and grown as described in PLEXdb (http://www.plexdb.org), accession number BB47. The four tissues sampled were: seedling tissue at growth stage "first leaf just emerging through the coleoptile" (GRO:007059); crown tissue was sampled at the "first leaves unfolded" growth stage (GRO:0007060); immature inflorescence tissue was sampled at the "third node detectable" growth stage (GRO:0007084); embryos were sampled 16–20 days after anthesis from the "expanded cotyledon stage" (PO:0001078) (Dabbert et al. 2009). For each replication/genotype/tissue combination ten plants were sampled for RNA extraction. RNA extraction and labeling were performed as described previously (Boddu et al. 2006).

Transcript accumulation in wild-type and *lnt1.a* tissues were measured using the Affymetrix Barley1 GeneChip® (Close et al. 2004). Hybridizations and data generation were performed at the Biomedical Image Processing Facility at the University of Minnesota (Minneapolis, Minnesota) using Affymetrix procedures (http://www.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf). The Genedata Expressionist Pro v3.1 software (Genedata, San Francisco, CA) was used to analyze data as described previously (Boddu et al. 2006). Correlation coefficients were calculated to be >0.92 between all replications. Transcript accumulation differences between *lnt1.a* and wild-type were calculated for each tissue type were identified using ANOVA ($p < 0.001$). A false discovery rate (FDR) of <2% was imposed and the transcripts were subject to a 2.0-fold difference in transcript abundance. Annotations for differentially accumulated transcripts were obtained using the BLASTX function in HarvEST (http://harvest.ucr.edu). Selected transcripts containing annotations all contained an e-score $\leq 10^{-17}$. Genes were classified into the following functional categories: biomacromolecule recycling, cell wall/membrane associated metabolism, energy metabolism, growth and development, general metabolism, regulatory, stress response, signal transduction, transport, miscellaneous, and unknown. Map positions of select gene transcripts were obtained from HarvEST (http://www.harvest-web.org/hweb/bin/wc.dll?hwebProcess~hmain~&versid=4). GeneChip data were deposited at PLEXdb (http://www.plexdb. org) with accession number BB47.

Genetics of *lnt1*

An F_2 mapping population was created by crossing Bowman-*lnt1.a* with Morex. The F_2 Bowman-*lnt1.a*/Morex population contained 161 individuals. Individuals were phenotyped for tiller number in the F_2 generation. Genotypes of F_2 plants exhibiting a wild-type tillering phenotype were determined in the F_3 generation by planting 15–20 seeds from each family. SSR marker genotyping of the F_2 population was conducted as previously described in Dabbert et al. (2009). Linkage analysis was performed using the Kosambi mapping function in Map Manager QTX (Manly et al. 2001).

A polymorphic *Cvi*AII restriction endonuclease site was used to map the *JuBel2* sequence. Primers 14L and 14R were used to amplify *JuBel2* sequences (Supplemental Table 1). PCR conditions used were 94°C: 5 min, 35 cycles of 94°C: 30 s, 58°C: 30 s, 72°C: 45 s, and then 72°C for 7 min. PCR products were then digested with *Cvi*AII. This cleaved amplified polymorphic sequence (CAPS) marker was used to genotype individuals in the Bowman-*lnt1.a*/Morex population.

Southern blot analyses were performed as described previously (Babb and Muehlbauer 2003).

Mutant analysis and *JuBel2* sequencing

JuBel2 sequences were amplified using PCR from the Bowman, Steptoe, Chikurin-Ibaraki2, Miho Hadaka, Mitake and Bowman-*lnt1.a*. PCR primers were developed using the published *JuBel2* sequence (Müller et al. 2001) and the Primer3 primer design program (Rozen and Skaletsky 2000). Primer sequences are provided in Supplemental Table 1. Products were amplified using HotStarTaq from Qiagen Inc. (Valencia, CA) with a touchdown program (Chin et al. 1996). Amplicons were purified using the Qiagen MinElute PCR purification kit (Qiagen Inc., Valencia, CA), and then sequenced by the University of Minnesota BioMedical Genomics Center. *JuBel2* sequences from Bowman, Steptoe, Bowman-*lnt1.a*, Chikurin-Ibaraki 2, and Miho Hadaka were deposited in GenBank, accession numbers GQ853057, GQ853058, GQ853059, GU722209, and GU722210, respectively.

Results

Morphology of *lnt1* mutants

Recessive mutations of *Lnt1* affect tiller and inflorescence development. Wild-type barley plants generally exhibit 10–30 tillers depending on space allocation and resource availability (Fig. 1a). Plants carrying the *lnt1.a* allele typically develop two or three tillers, although occasional plants may have no tillers or up to four tillers (Fig. 1d). This reduction in tillers appeared to be due to the lack of secondary tillers in *lnt1.a* plants. Figure 1c shows a primary and a secondary tiller from a wild-type Bowman plant. Primary tillers were present in *lnt1.a* plants, but not secondary tillers (Fig. 1f). Spikes on *lnt1.a* plants were largely normal, except for an occasional lack of spikelets in the central or upper regions of spikes (Fig. 1e).

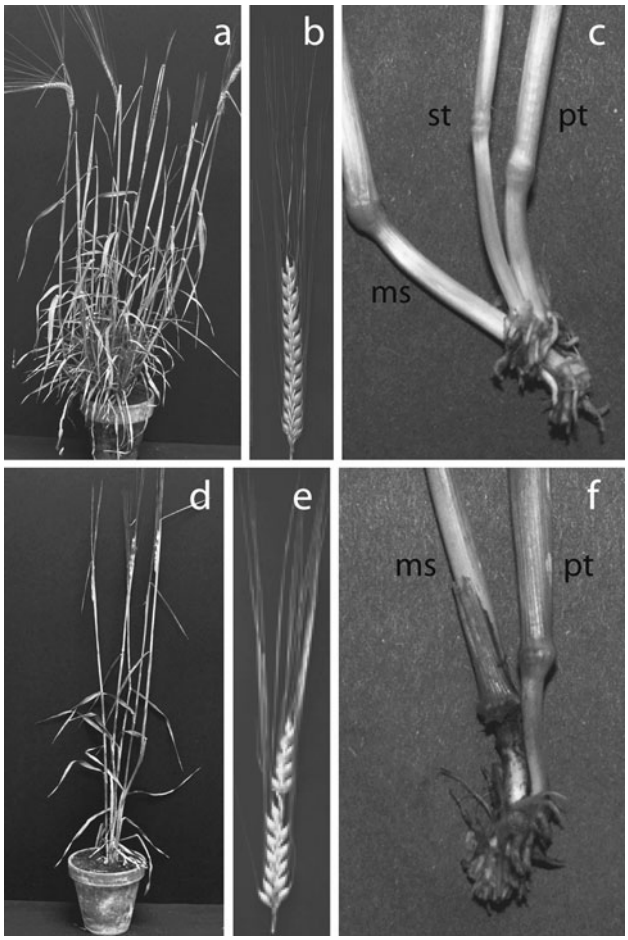

Fig. 1 Wild-type Bowman and *lnt1* mutant phenotypes. **a** Wild-type plant exhibiting many tillers. **b** Wild-type inflorescence. **c** Wild-type plants exhibiting primary and secondary tillers. **d** *lnt1.a* mutant plant exhibiting few tillers. **e** *lnt1.a* mutant inflorescence. **f** *lnt1.a* mutant plant exhibiting only a primary tiller. *ms* main stem, *pt* primary tiller, *st* secondary tiller

We next examined vegetative axillary bud and tiller development in one to 6-week-old *lnt1.a* and wild-type Bowman plants. Wild-type seedlings had an average of three axillary buds in 1-week-old plants (data not shown). The shoot apical meristems of 1-week-old wild-type seedlings were still developing vegetatively. By the second week of development, a single axillary bud was seen in the first, second, third and fourth leaf axils of wild-type plants, and the shoot apical meristem had transitioned into the reproductive stage of development by week 2 (Fig. 2a). Wild-type plants had five developing tillers by the third week and the number of tillers increased to approximately seven by week six (Table 2).

Plants carrying the *lnt1.a* allele generally produced two or three axillary buds that developed into tillers. At 1 week, no axillary buds were observed, indicating a delay in the timing of axillary bud development compared to wild-type (data not shown). By the second week, axillary buds were evident in the leaf axils of *lnt1.a* mutant plants. In Fig. 2b, we show a 2-week-old *lnt1.a* mutant plant with a single axillary bud. There were two developing tillers on *lnt1.a* plants after 3 weeks, and two to three tillers at later timepoints (Table 2). Secondary tillers were not found in *lnt1.a* plants.

Genetic analysis of *lnt1.a*

Genetic interactions between *lnt1.a* and six other tillering mutants were studied. The two low tillering mutants, *int-b* and *uzu,* produce more tillers than *lnt1.a*, but significantly less tillers than wild-type. The high-tillering mutants, *den6*, *gra-a*, *int-m* and *mnd1*, produce significantly more tillers than wild-type.

The *lnt1.a* mutant was epistatic to four of the six tillering mutants for the tillering phenotype. Double mutant combinations between *lnt1.a* and the low tillering mutant *uzu* gave plants with two to three tillers, as did combinations between *lnt1.a* and the high tillering mutants *gra-a*, and *mnd1* (Fig. 3). In addition, double mutant combinations of *lnt1.a* and *den6* also exhibited two to three tillers, indicating that this was also an epistatic interaction (data not shown). A synergistic interaction was seen between *lnt1.a* and the *int-b* low tillering mutation (Fig. 3b). Plants with the *lnt1.a/lnt1.a; int-b/int-b* genotype did not produce any tillers. Double mutant combinations of *lnt1.a; int-m* exhibited a few more tillers than *lnt1.a*, indicating that this was an additive interaction (Fig. 3d).

Epistatic and synergistic interactions were seen for plant height. The *gra-a* mutant produces short plants with many tillers (Fig. 3c). The *lnt1.a* mutant was epistatic to *gra-a.1* allele for the tillering trait however, *gra-a.1* was epistatic to *lnt1.a* for plant height. The *uzu* mutant also exhibited short plants (Fig. 3a), but here there was a synergistic interaction between *lnt1.a* and *uzu1.a* resulting in plants shorter than both parents. There was also a synergistic interaction between *lnt1.a* and *int-m.85* for plant height (Fig. 3d). In this case, neither parent was dwarfed, but double mutant plants were short.

Differential transcript accumulation in *lnt1* and wild-type

The Affymetrix Barley1 GeneChip® was used to measure the transcript accumulation of 22,792 barley genes in Bowman-*lnt1.a* and wild-type Bowman plants. RNA was isolated from four tissue types: seedling, crown, immature inflorescence and embryo. These tissue types represent regions of the developing plant with active axillary bud development. During the initial stages of development, the embryo in wild-type plants develops an axillary meristem in the first leaf axil. Upon germination, the embryo develops into a seedling and new axillary meristems develop in the subsequent leaf axils. By the second week of development,

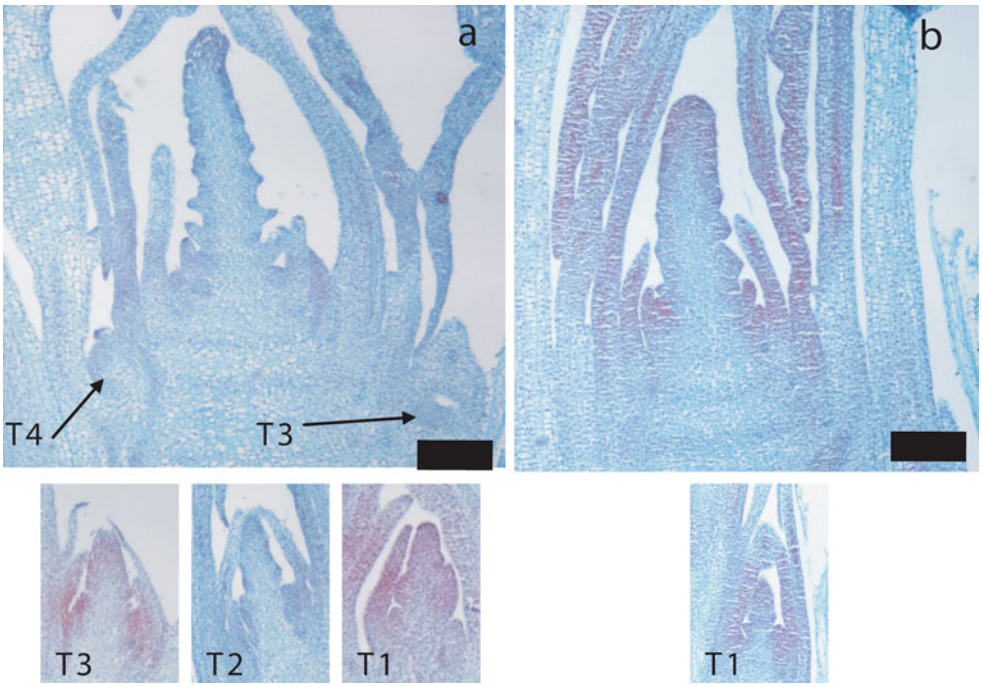

Fig. 2 Shoot apices and axillary buds from 2-week-old wild-type Bowman and *lnt1.a* seedlings. **a** Wild-type shoot apex. **b** Shoot apex from *lnt1.a* mutant seedling. T1, T2, T3, and T4 are the developing axillary buds developing under the leaf axils. Axillary buds that were not in the section containing the cross-section of the shoot meristem are shown below. The T3 axillary bud in **a** is not in the correct plane to view the axillary meristem, thus the T3 axillary bud is shown below in a section that shows the axillary meristem. *Black bars* represent 300 μm for each picture. The picture of the wild-type shoot apex was previously published in Dabbert et al. (2009)

Table 2 Number of tillers and axillary buds (AB) present in *lnt1.a* and wild-type Bowman plants 1 to 6 weeks after planting

Genotype	Time point (weeks)	No. of plants examined	Mean number of tillers	Mean number of AB[a]	Mean number of developing Tillers (Tillers + AB)
Wild-type	1	5	ND	3.0	3.0
	2	4	ND	4.3	4.3
	3	8	2.9	2.1	5.0
	4	5	3.0	2.8	5.8
	5	8	2.9	3.8	6.4
	6	7	3.9	2.9	6.7
lnt1.a	1	5	ND	ND	ND
	2	5	ND	1.3	1.3
	3	8	2.0	ND	2.0*
	4	5	3.0	ND	3.0*
	5	10	2.1	ND	2.1*
	6	10	1.9	ND	1.9*

ND none detected

* Number of developing tillers in *lnt1.a* mutant plants is significantly different at $p \leq 0.0001$ compared to wild-type at the indicated time point

[a] Axillary bud reduction at 3 weeks due to outgrowth of previous axillary buds into tillers

new axillary meristems develop at the crown of the plant as the first axillary bud begins to develop into a tiller. During inflorescence development, multiple axillary meristems give rise to spikelets, which include the glumes, lemma, palea, pistil and style.

A total of 70 transcripts with at least a twofold difference in transcript levels between the wild-type and *lnt1.a* mutant were identified. Fifty transcripts had increased levels in *lnt1.a* tissue compared to wild-type (Supplemental Table 2), and 20 transcripts exhibited reduced levels in

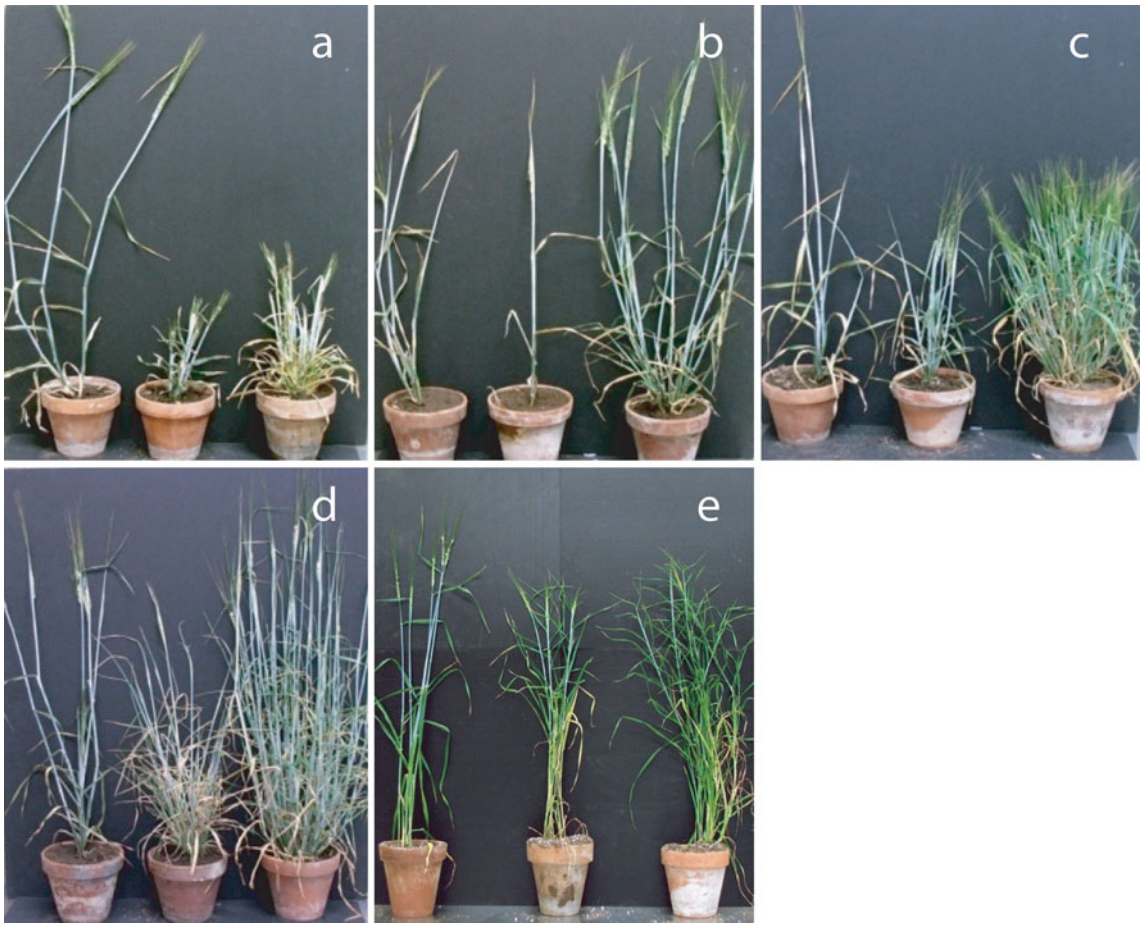

Fig. 3 *lnt1.a* double mutants with five different tillering mutants. *Panels* show the single mutant *lnt1.a* plant on the *left*, the double mutant plant in the *middle*, and other single mutant plant on the *right*. **a** *lnt1.a* and the low-tillering *uzu* mutant. **b** *lnt1.a* and the low-tillering *int-b.3* mutant. **c** *lnt1.a* and the high-tillering *gra-a.1* mutant. **d** *lnt1.a* and the high-tillering *int-m.85* mutant. **e** *lnt1.a* and the high-tillering *mnd1.a* mutant

lnt1.a compared to wild-type (Supplemental Table 3). Many functional categories were represented among the transcripts identified. Sixty-nine of the 70 transcripts exhibited altered abundance in one or two different tissues. One transcript, Contig12274, had reduced transcript levels in all four tissues sampled.

Contig12274 encodes the barley Bell-like homeodomain protein, *JuBel2* (Müller et al. 2001). Homologous genes in *Arabidopsis* have numerous roles in development, including meristem function (Cole et al. 2006; Rutjens et al. 2009). The *Lnt1* gene is located on the long arm of barley chromosome 3H (Franckowiak and Lundqvist 1997), and a SNP for the *JuBel2* gene was mapped to barley chromosome 3H near the reported position of *Lnt1* (Close et al. 2009).

JuBel2 is a candidate gene for *Lnt1*

Linkage analysis confirmed the previously reported map position of *Lnt1*, and demonstrated that *lnt1.a* co-segregated with *JuBel2*. Using a segregating F_2 population with 161 individuals, *Lnt1* was placed 5.8 cM distal from GBM1043 and 31.0 cM proximal of Bmag0013 (data not shown). This placed *Lnt1* on chromosome 3HL Bin11 of the barley genetic map (http://barleygenomics.wsu.edu/). This location was consistent with previous reports (Nonaka 1973). Next a CAPS marker was developed for *JuBel2*. This marker was used to genotype individuals from the *lnt1.a*/Morex F_2 population. The *JuBel2* allele present in *lnt1.a* co-segregated with the *lnt1.a* phenotype in the 161 individuals comprising the F_2 population (data not shown).

A region extending from approximately 1 kb upstream of the *JuBel2* coding region to 0.25 kb downstream was amplified and sequenced from *lnt1.a*, Bowman, and Steptoe. Indels and single nucleotide polymorphisms distinguished these alleles from each other and from the previously published *JuBel2* sequence (GenBank Accession Numbers GQ853057, GQ853058, and GQ853059; Supplemental Figure 1). Compared to the two non-mutant alleles, there was a one-basepair deletion in the *JuBel2* coding region

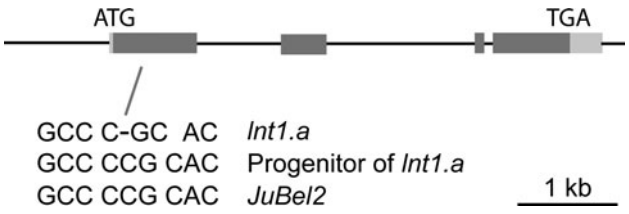

Fig. 4 *JuBel2* sequences. The structure of the *JuBel2* gene is illustrated with the *dark gray boxes* indicating coding sequences and *light gray boxes* indicating the 5′- and 3′-untranslated regions. The sequence flanking the *lnt1.a* deletion is shown below, and the *line* indicates the location of the deletion in the *JuBel2* gene. Sequences flanking the deletion in the progenitor allele (Chikurin-Ibaraki2 and Miho-Harada) were identical to the published *JuBel2* sequence (Müller et al. 2001)

from the *lnt1.a* allele (Fig. 4). This frameshift mutation results in a truncated polypeptide (Supplemental Figure 1).

Additional evidence that *Lnt1* encodes *JuBel2* came from examining the *lnt1.a* progenitor allele and from an independent *lnt1* allele. The *lnt1.a* allele was a spontaneous mutation recovered from a bulk of a cross of Chikurin-Ibaraki2 and Miho Hadaka, resulting in the cultivar Mitake (Nonaka 1973). Both parental lines and Mitake were obtained from the USDA-ARS National Small Grains Germplasm Research Facility, and *JuBel2* sequences from Chikurin-Ibaraki2, Miho Hadaka and Mitake were amplified and sequenced. *JuBel2* sequences from Chikurin-Ibaraki 2 and Miho Hadaka (Genbank accession numbers GU722209 and GU722210) were identical to *lnt1.a* except for the frameshift mutation found in Bowman-*lnt1.a* (Supplemental Figure 1). Mitake contained the frameshift mutation at the same location (data not shown). The absence of this deletion in the progenitor alleles points to this frameshift mutation as the causative lesion in *lnt1.a*. The *int-l.81* allele of *lnt1* appears to be a deletion of the entire locus. Attempts to detect *JuBel2* sequences using PCR were unsuccessful, and Southern blot analysis using probes specific to the 5′-untranslated region, intron 1 and the 3′-untranslated region did not detect *JuBel2* sequences in *int-l.81* (Supplemental Figure 2). Together, these results argue that the *Lnt1* locus most likely encodes *JuBel2*.

Discussion

The *lnt1* mutant inhibits axillary bud development

Axillary branching may be controlled during axillary meristem initiation, axillary bud development, and during bud outgrowth. Morphological studies indicate that *Lnt1* functions before the bud outgrowth stage, as the number of axillary buds found in *lnt1.a* mutant seedlings is strongly reduced and is consistent with the number of tillers present in the adult plant. On average, *lnt1.a* plants had two tillers, and one or two axillary buds were seen in histological sections of 2-week-old *lnt1.a* seedlings. Additional axillary buds were not observed on the dissected crowns from older *lnt1.a* plants, indicating a block in secondary tiller bud development. In contrast, wild-type plants had four to five axillary buds at 2 weeks, and additional buds developed in subsequent weeks. Thus, *Lnt1* may influence axillary meristem initiation, like the rice *monoculm1* gene and its dicot homologs (Li et al. 2003; Schumacher et al. 1999; Greb et al. 2003), or axillary bud development, like the rice *floral organ number1* gene and its dicot homologs (Moon et al. 2006; Clark et al. 1997). These observations also distinguish *Lnt1* from genes that control bud outgrowth such as the *Tb1* and the *MAX* genes (Doebley et al. 1997; Brewer et al. 2009).

Genetic interactions help define pathways controlling tiller development

The reduction in the number of axillary buds in *lnt1.a* plants explains the epistatic interaction between *lnt1.a* and the high-tillering mutants *den6*, *gra-a*, and *mnd1*. It is likely that these high tillering mutants are the result of the lack of control of bud outgrowth. The loss of bud outgrowth would not be expected to affect tiller number in double mutants with *lnt1.a* because only a few axillary buds develop in *lnt1.a* plants. This relationship between the number of axillary buds formed and bud outgrowth may also explain the epistasis of *lnt1.a* over the *uzu* low-tillering mutation.

Genetic analysis also identified a strong synergistic interaction between *lnt1.a* and the *int-b* low-tillering mutant. The *lnt1.a; int-b* double mutant plants did not produce any tillers. Mutations in separate pathways regulating tillering could produce this synergistic response, and separate pathways were used to explain synergism for axillary branching seen in the *Arabidopsis las; rax1* double mutant (Müller et al. 2006). Genetic redundancy has been invoked to explain the synergistic response, and reduction of axillary branching, in the *RAX* genes (Müller et al. 2006). *RAX1*, *RAX2*, and *RAX3* encode closely related basic helix-loop-helix proteins. A mutation in one *RAX* gene has a small effect on branching and there is little overlap in the regions affected by each individual *rax* mutant (Müller et al. 2006). Double mutants, for example *rax1; rax2*, exhibit a more extreme phenotype than either single mutant, and the triple mutant has almost no branches. In a study from rice where redundancy was suggested as a possible explanation for synergism between two mutants, the *lax; spa1* double mutant had fewer tillers and neither single mutant alone affected tiller number (Komatsu et al. 2003). In these cases it is obvious how the loss of two genes could

have a larger effect than a single mutant. However, unlike the model that had been proposed for *lax* and *spa*, both *lnt1.a* and *int-b* reduce the number of tillers produced. And unlike the *rax* mutants, *lnt1.a* and *int-b* appear to function at similar times in development. Genetic redundancy does not appear to explain the lack of tillers in the *lnt1.a; int-b* double mutant. Therefore, *Lnt1* and *Int-b* appear to function in separate genetic pathways that regulate tillering in barley.

lnt1 and *als* mutants exhibit similar phenotypes, but disparate gene expression patterns

The *lnt1.a* tillering phenotype closely resembled the tillering phenotype of the *als* mutation (Dabbert et al. 2009). One to four tillers develop in *lnt1.a* and *als* mutant plants. Interestingly, secondary tillers did not develop on either mutant. In *als* mutant plants, two or three axillary buds developed by week two, compared to one or two axillary buds for *lnt1.a*. Similarities between *lnt1.a* and *als* extend to the genetic interactions seen in the double mutants. *Als* and *lnt1.a* both exhibited a synergistic interaction with the *int-b* low-tillering mutant, and were epistatic to the *uzu* low-tillering mutant (Dabbert et al. 2009). *Als* was epistatic to all four high-tillering mutants tested (Dabbert et al. 2009). *Lnt1.a* was epistatic to *den6*, *gra-a*, and *mnd1*; *lnt1.a* showed an additive interaction with *int-m*.

Despite the phenotypic and genetic similarities between *als* and *lnt1.a*, these similarities were not reflected in their transcriptomes. The *als* and *lnt1.a* plants used for these experiments were grown in the same growth chamber and sampled at the same time. Figure 5 presents the distribution of differentially accumulated transcripts into functional categories from transcriptome analysis of *als* and *lnt1.a*. The dominant feature of the *als* transcriptome is the increased transcript levels of stress and defense genes (Dabbert et al. 2009). Thirty-three defense and stress-related transcripts including chitinases, pathogenesis-related proteins, and glutathione transferases had higher accumulation in *als* tissues. Nearly 40% of the transcripts with increased accumulation in *als* were defense and stress-related, versus one from *lnt1.a*. One probe set representing a stress related transcript, Contig2320_s_at, detected increased accumulation in both *lnt1.a* and *als*. Another 11 probe sets detected increased transcript accumulation in both *lnt1.a* and *als*, and five other probe sets detected decreased transcript accumulation in both *lnt1.a* and *als* (Supplemental Tables 2 and 3). Nevertheless, the *lnt1.a* mutant did not show the widespread activation of defense and stress-related genes seen in the *als* mutant.

This transcriptome analysis suggested that *Als* and *Lnt1* function independently. For example, if *Lnt1* regulated *Als*, then transcripts with greater abundance in *als* would also have higher abundance in *lnt1.a*. However, the stress

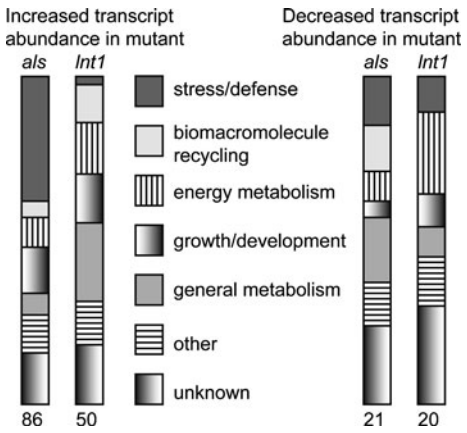

Fig. 5 Comparison of transcriptomes from *als* and *lnt1*. The fraction of transcripts in different functional categories are presented. Transcripts with increased abundance in the *als* or *lnt1* mutant versus wild-type are on the left, and transcripts with decreased abundance in the mutants are on the right. The category "other" includes the categories cell wall/membrane associated metabolism, regulatory, signal transduction, transport and miscellaneous. There were 86 and 50 transcripts with increased abundance in *als* and *lnt1*, respectively, and 21 and 20 transcripts with decreased abundance in *als* and *lnt1*, respectively. The *als* data was taken from Dabbert et al. (2009)

response seen in *als* was not seen in *lnt1.a*. Alternatively, if the *Als* locus regulated *Lnt* and if the *Lnt1* locus encodes *JuBel2*, then *JuBel2* transcript levels would be expected to be regulated by the *Als* gene. This was not seen either. These results imply that although *als* and *lnt1.a* have similar affects on vegetative axillary meristem development, they function independently.

JuBel2 is a candidate gene for *Lnt1*

Sequence analysis of the *lnt1.a* allele and its progenitors, in conjunction with the co-segregation analysis, and the results with the *int-l.81* deletion allele provide strong evidence that the *Lnt1* locus encodes *JuBel2*. The barley *JuBel2* gene encodes a *BELL-like* homeodomain transcription factor (Müller et al. 2001). RNA In situ hybridization experiments detected *JuBel2* transcripts in the shoot apical meristem, root and primordial leaves of embryos; the shoot apical meristem, lateral bud, leaves and roots of seedlings plants; and the lemma, palea, glume, anther, ovary, inflorescence and floral meristem of the inflorescence (Müller et al. 2001). Transformed Arabidopsis and tobacco plants carrying a 35S-*JuBel2* construct produced extra shoots giving rise to short bushy plants (Müller et al. 2001). These results suggested that *JuBel2* is likely *Lnt1*.

The barley *JuBel2* gene is related to the rice *QTL of seed shattering in chromosome 1* (*qSH1*) gene (Konishi et al. 2006) and the *Arabidopsis BELLRINGER*, *BLR*, gene (Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003). A nucleotide BLAST search of the GenBank non-redundant

sequences database identified *qSH1* ($e = 0$) and *BLR* ($e = 4e^{-34}$) as the closest gene sequences related to *JuBel2* in rice and *Arabidopsis*. Konishi et al. (2006) also identified *BLR* and *qSH1* as orthologous genes. The mutant *qSH1* allele in cultivated rice is a regulatory mutation that does not express *qSH1* in the pedicle at the base of the rice seed; expression of *qSH1* in other tissues was not detectably affected (Konishi et al. 2006). A *qSH1* loss-of-function allele has not been isolated in rice, and the *qSH1* locus may control other traits in addition to shattering (Konishi et al. 2006). Loss-of-function alleles exist in the *Arabidopsis BLR* gene, and these mutations are associated with a variety of traits (Byrne et al. 2003; Roeder et al. 2003; Smith and Hake 2003).

One of the functions of the *BLR* protein is to bind the *SHOOT MERISTEMLESS* (*STM*) protein and transport it to the nucleus (Cole et al. 2006; Rutjens et al. 2009). *STM* is required for axillary meristem development (Long and Barton 2000). Accumulation of the *STM* protein in the nucleus is necessary for *STM* function, and transport appears to require this interaction with *BLR* or another BELL-like protein (Cole et al. 2006; Rutjens et al. 2009). In vivo support for this mechanism comes from lines containing mutations in *BLR* and two related *BELL-like* genes, *POUND-FOOLISH* and *ARABIDOPSIS THALIANA HOMEOBOX GENE 1*. Together, these three mutations produce a phenocopy of the *stm* mutation, arguing that *STM* function requires one of these BELL-like proteins (Rutjens et al. 2009). The *JuBel2* protein binds to class 1 KNOX proteins including the barley homolog of *STM* (Müller et al. 2001). It is possible that *JuBel2* promotes secondary tiller development through its interactions with a barley *STM-like* gene. Studying the proteins that interact with *JuBel2* may lead to the identification of other genes regulating vegetative axillary meristem development in barley.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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