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ORIGINAL PAPER

The genetics of barley low-tillering mutants: *absent lower laterals* (*als*)

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Abstract Barley (*Hordeum vulgare* L.) carrying the recessive mutation *absent lower laterals* (*als*) exhibits few tillers and irregular inflorescence development. To gain an increased understanding of the genetic control of tillering in barley, we conducted morphological, genetic, and transcriptome analysis of the *als* mutant. Axillary buds for primary tillers, but not for secondary tillers, developed in *als* plants. Double mutant combinations of *als* with one low-tillering and four high-tillering mutants resulted in a tillering phenotype similar to *als*, indicating that *als* was epistatic to these tillering genes. However, double mutant combinations of *als* with another low-tillering mutant, *intermedium-b*, reduced tiller numbers, indicating there were at least two genetic pathways regulating tillering in barley. Next, we used simple sequence repeat markers to map the *Als* gene on the long arm of barley chromosome 3H, Bin 11. Finally, the Affymetrix Barley1 GeneChip was used to identify differentially accumulated transcripts in *als* compared to wild-type. Forty percent of the transcripts with twofold or greater accumulation in *als* tissues corresponded to stress and defense response genes. This finding suggested that a tillering pathway may modulate the stress response.

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Introduction

Shoot architecture is largely determined by the actions of the shoot apical meristem (SAM) and axillary meristems (AMs). The SAM gives rise to all above-ground structures of plants, including the primary shoot, leaves, nodes, internodes, inflorescence, and the AMs. The AMs are initiated in the leaf axils and give rise to axillary buds (ABs) that develop into axillary (lateral) shoots. Axillary shoots in barley and other grasses are modified branches, which develop at the crown of the plant independently of the primary shoot. They are referred to as tillers (McSteen and Leyser 2005). Primary tillers develop in the leaf axils of the SAM, and secondary tillers develop from AMs found in the axils of primary tillers (Counce et al. 1996). Tiller development has been described as having three stages: (1) initiation of an AM; (2) development of an axillary bud; and (3) the outgrowth of the axillary bud (Schmitz and Theres 2005). These stages are under genetic, environmental, and hormonal control (Doust 2007).

Genes controlling axillary shoot production fall into two general classes (Bennett and Leyser 2006). One class

promotes axillary shoot development. Examples include the tomato *LATERAL SUPPRESSOR* (*LS*) gene (Schumacher et al. 1999) and the *REGULATOR OF AXILLARY MERISTEMS* genes in *Arabidopsis* (*RAX1*, *RAX2*, and *RAX3*) (Müller et al. 2006). The second class represses tillering and branching. Examples include the maize *TEOSINTE BRANCHED1* (*TB1*) gene (Doebley et al. 1997) and genes in the *Arabidopsis MORE AXILLARY BRANCHING* (*MAX*) pathway including the *MAX1*, *MAX2*, *MAX3*, and *MAX4* genes (Bennett et al. 2006). Genes controlling AM development and branching appear to be conserved among dicots and monocots as orthologous genes or closely related genes to *LS* and *TB1* have been identified in the model systems of *Arabidopsis* and rice. The tomato *LS* gene is homologous to the *Arabidopsis LATERAL SUPPRESSOR* (*LAS*) gene (Greb et al. 2003) and the rice *MONOCULM1* gene (Li et al. 2003b). The maize *TB1* gene is homologous to the *Arabidopsis BRANCHED1* gene (Aguilar-Martinez et al. 2007), and the rice *FINECULM or OsTB1* (Hu et al. 2003; Takeda et al. 2003). Rice genes orthologous to *MAX2*, *MAX3*, and *MAX4* have also be described in recent years (Ishikawa et al. 2005; Zou et al. 2006; Arite et al. 2007). These and other genes regulating branching and tillering have been described in multiple reviews (e.g., Wang and Li, 2006; McSteen and Leyser 2005; Bennett and Leyser 2006). This apparent conservation of genes suggests that genetic pathways controlling branching and tillering are also conserved.

Mutant analysis is unraveling the relationships between the genes controlling AMs and branching. For example, genes with similar phenotypes may act in the same pathway or different pathways. The *Arabidopsis MAX* genes are part of a genetic pathway that is hypothesized to control branching by regulating auxin transport via a novel plant hormone (Bennett et al. 2006; Gomez-Roldan et al. 2008; Umehara et al. 2008). Plant mutants for a single *MAX* gene have a similar appearance to plant mutants for two different *MAX* genes, blocking a pathway at one place or in two places leading to similar phenotypes (Booker et al. 2005). In contrast, mutations in genes in two different pathways often lead to a more extreme phenotype than seen in either single mutant. Mutation of either the *Arabidopsis RAX1* or *LAS* genes has little effect on branching in the flowering stem; branching is reduced in the vegetative rosette. Plant mutants for both genes show a strong reduction of branching in the flowering stem, suggesting that *RAX1* and *LAS* function in separate genetic pathways (Müller et al. 2006). At least two pathways are believed to control branching in dicots, and it is reasonable to expect similar pathways to be operating in monocots.

There are barley genes promoting or repressing tillering that may act on AM initiation, AB development or AB outgrowth (Babb and Muehlbauer 2003; Bossinger et al. 1992; Lundqvist et al. 1996). Low-tillering mutants belong to the class of genes that promote tiller development. Low-tillering mutants include, *als*, *low number of tillers1* (*lnt1*), *uniculm2* (*cul2*), *uniculm4* (*cul4*), *intermedium spike-b* (*int-b*) and *semi brachytic* (*uzu*). The second class of genes represses tiller development; these are the high-tillering mutants. Mutations in the *densonidosum6* (*den6*), *granum-a* (*gra-a*), *intermedium spike-m* (*int-m*) or the *many noded dwarf1* (*mnd1*) genes result in plants with more tillers than wild-type. One mutant, *cul2*, has been described in detail (Babb and Muehlbauer 2003). AM initiate, but then fail to develop in plants carrying the *cul2* mutation. The morphology and genetics of the other barley tillering mutants is largely unexplored. In addition, the relationships of these mutants to the branching mutants in other plants are unknown. To develop an understanding of the genetic control of tillering in barley, our objectives were to (1) morphologically describe and characterize the *als* mutant during tiller development, (2) understand the genetic interactions of *als* with other tillering mutants, (3) identify the genetic map position of the *Als* gene and (4) compare the RNA profiles of wild-type and *als* mutant plants.

Materials and methods

Genetic stocks

Als1.a is a single gene recessive mutation that was induced by gamma rays in the cultivar Montcalm (Kasha and Walker 1960). For this study, we used a near-isogenic line containing the *als1.a* allele in the Bowman (wild-type) genetic background that was developed through seven backcrosses. All 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. 1996). A list of all mutant genetic stocks used, known map positions, and the number of backcrosses into the Bowman genetic background are shown in Table 1.

Genetic analysis

The *als* mutant was crossed to six other tillering mutants. Crosses between each homozygous single mutant individual were advanced to create F_2 segregating populations. Double mutants were identified in the F_2 families. The segregation ratios did not deviate from 9:3:3:1. The double mutants were distinguished by a combination of whole plant phenotypes including tiller number, plant height, leaf number, leaf shape and spike characteristics. Double mutant phenotypes were confirmed by examining F_3 families that were segregating for one of the mutants and

Table 1 Barley genetic stocks

Genetic stocks	Tillering habit[a]	Chromosome location	Backcrosses to Bowman	Source
als1.a	Low	3HL	7	GSHO1990[b]
int-b.3	Low	5HL	6	Franckowiak[c]
uzu1.a	Low	3HL	7	GSHO1963
den6	High	Unknown	7	GSHO2235
gra-a.1	High	3HL	7	GSHO1980
int-m.85	High	Unknown	5	Franckowiak
mnd1.a	High	Unknown	9	GSHO2038

[a] Tiller habit as compared to wild-type

[b] GSHO were obtained from the USDA-ARS National Small Grain Germplasm Research Facility, Aberdeen, ID

[c] A gift from J. Franckowiak, Department of Plant Sciences, North Dakota State University, Fargo, ND

homozygous for the other. Also, putative double mutants from each cross were examined in F_3 families. The phenotypes of double mutants from the segregating homozygous single mutant families and those from the putative double mutant families were compared. Vegetative phenotypes of the resulting double mutants were categorized as exhibiting an epistatic, synergistic or additive interaction.

Morphological analysis

Tiller development was examined at 3, 4, 5 and 6 weeks after planting. Shoots were dissected from *als* mutant and wild-type plants and the number of tillers (tillers plus developing axillary buds) at the crown was recorded. A minimum of four plants were dissected from the *als* mutant and wild-type plants. *t* tests were used to compare the number of developing tillers in *als* mutant and wild-type plants.

Shoot apices from 1- and 2-week-old seedlings of *als* and wild-type plants were fixed in FPA [a solution of 50% ethanol, 5% propionic acid and 10% formaldehyde (37%)] at 4°C overnight (Ruzin 1992). Tissue was passed through an ethanol/tert-butyl alcohol dehydration series and embedded into paraffin wax (Paraplast Plus, Sigma, Oxford, St. Louis, MO). A microtome was used to obtain 10 µm thick serial sections that were mounted on Fisher Probe-On Plus (Fisher Scientific, Pittsburgh, PA) microscope slides. Tissue was stained using safranine-O and fast green. Glass cover slips were mounted on the slides with Permount (Fisher Scientific, Pittsburg, PA). Tissue sections were photographed under a bright field microscope.

To examine root phenotypes, seeds of *als* and wild-type plants were planted in 100% vermiculite in the greenhouse. The root phenotypes were examined from a minimum of five plants at two time points, 1- and 4-weeks after planting.

Linkage mapping

A segregating 122 member F_2 population was created by crossing Bowman-*als* to Morex. Individuals were phenotyped for tiller number in the F_2 generation. Genotypes of F_2 plants were inferred by scoring tiller number in F_3 families. Total genomic DNA was extracted from the parents and the progeny of the F_2 population using the method described by Riede and Anderson (1996). Parents of the mapping population were first screened for polymorphisms with 50 simple sequence repeat (SSR) markers that spanned chromosome 3HL (Li et al. 2003a). Polymorphic SSR markers were used to genotype the segregating mapping population. PCR conditions used were 94°C: 5 min, 35 cycles of 94°C: 30 s, 58°C: 30 s, 72°C: 45 s, then 72°C for 7 min. PCR products were run on 6% poly-acrylamide gels and silver stained using the modified silver stain protocol from Bio-Rad (http://www.biorad.com/LifeScience/pdf/Bulletin_9057.pdf). The poly-acrylamide gels were hand scored. Linkage analysis was performed using the Kosambi mapping function within Map Manager QTX (Manly et al. 2001).

Barley1 GeneChip analysis of *als* mutant and wild-type plants

A randomized complete block design with three replications was used for Bowman and Bowman-*als* for each tissue sampled. Seeds for each genotype were surface sterilized with bleach and planted in growth chambers. For each genotype-tissue combination, at least ten plants were sampled from each of the three replications. Samples taken were 2–3 day-old seedling tissue, crown tissue from 10 to 14-day-old plants, immature inflorescence and embryo 16–20 days after pollination. Seedling tissue was at growth stage "first leaf just emerging through the coleoptile" (GRO:0007059). Crown tissue was sampled at the "first leaves unfolded" growth stage (GRO:0007060). Inflorescence tissue was sampled at the "third node detectable" growth stage (GRO:0007084). The embryo tissue was from the "coleoptilar stage" (PO:0001094). Total RNA was isolated from pooled tissue (ten plants) from each genotype/replication/tissue combination. RNA isolation and labeling were conducted as previously described (Boddu et al. 2006).

The Barley1 GeneChip contains 22,792 probe sets that represent 21,439 genes (Close et al. 2004). Hybridizations and data generation were performed as previously described (Boddu et al. 2006). GeneChip data were deposited at the plant expression database (www.plexdb.org) with accession number BB47.

GeneChip data analysis was conducted using the Genedata Expressionist Pro v3.1 software (Genedata, San Francisco, CA). The refiner tool within the Expressionist

Pro software was used to normalize the raw signals. Signal normalization was performed using robust multichip analysis (Irizarry et al. 2003). The normalized signals were uploaded into analyst tool for further analysis. Correlation coefficients were determined to be >0.93 for all experiments. Primary transcript accumulation differences between *als* compared to wild-type were identified using two-way ANOVA (p value ≤ 0.001) and combining the genes obtained from genotype and genotype-tissue effects. A false discovery rate (FDR) of 2% was imposed (Benjamini and Hochberg 1995), and the transcripts showing $\geq$2.0-fold change difference between genotypes were selected. Finally, 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 $\leq 10^{-16}$.

In silico mapping

Map positions of transcripts corresponding to previously mapped SNPs were obtained from HarvEST (http://harvest.ucr.edu). Map positions of other transcripts were predicted from the positions of homologous sequences in the rice genome. Homologous sequences were identified using BLASTN searches of the TIGR rice pseudomolecules (Release 5 at URL: http://rice.plantbiology.msu.edu/), and syntenic regions in the barley genome were predicted using nearby markers mapped in barley.

Single feature polymorphism (SFP) detection was conducted for each tissue type between *als* and wild-type. The statistical analysis of microarrays feature in the Bioconductor with Siggenes package was used to detect the SFPs from our GeneChip hybridizations (http://bioconductor.org). SFPs were detected following the general algorithm in Cui et al. (2005) with modified probe level detection statistics from Rostoks et al. (2005).

Results

Growth of *als* plants in the greenhouse and field resulted in a lower number of tillers and altered inflorescence development compared to wild-type. Wild-type barley (cv. Bowman) plants generally exhibit up to 30 tillers under ideal conditions, and fewer tillers with limited resources or when under stress conditions. Mutant *als* plants produced three to four tillers with thick, rigid shoots (Fig. 1b). The inflorescence development of *als* plants displayed irregular floret phyllotaxy, such that the central and lateral florets developed in improper positions. In addition, the *als* mutant inflorescence exhibited the lack of lower lateral spikelets (Fig. 1d). No obvious differences in root branching were observed (data not shown).

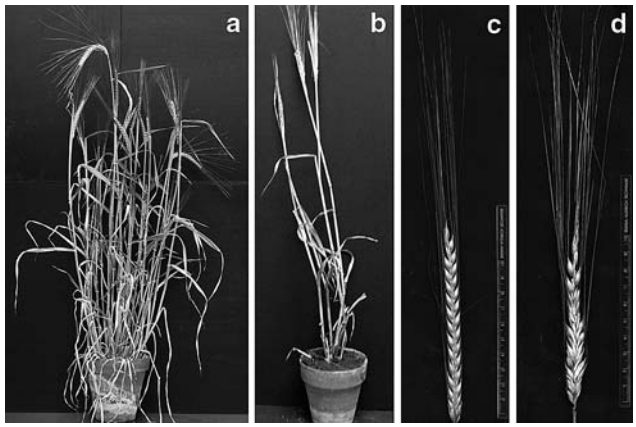

Fig. 1 Vegetative and inflorescence phenotypes **a**, **c** wild-type, and **b**, **d** *als*

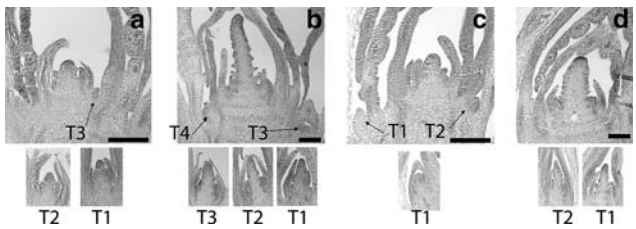

Fig. 2 Histological sections of developing *als* mutant and wild-type shoot apices. Shoot apices with axillary buds of **a** wild-type after 1 week of development, **b** wild-type after 2 weeks of development, **c** *als* mutant after 1 week of development and **d** *als* mutant shoot apices with axillary buds after 2 weeks of development. Below each *picture* are the axillary buds from other serial sections of the same apex. *Black bars* represent 300 μm of both the main-frame and frames below. *T1*, *T2*, *T3* and *T4* are the developing axillary buds under the first, second, third and fourth leaf axils, respectively

To determine the stage of tiller inhibition in *als* plants compared to wild-type plants, we examined vegetative AB and tiller development in sectioned material from 1- and 2-week old seedlings, and followed tiller development in 3–6 week old seedlings. Wild-type seedlings exhibited an average of three AB on 1-week-old plants (Fig. 2a). The SAMs of 1-week-old wild-type seedlings were still developing vegetatively. Wild-type seedlings exhibited a single AB in the first, second, third, and fourth leaf axils at the end of 2 weeks (Fig. 2b). By the second week, the SAM had transitioned into the reproductive stage of development (Fig. 2b). Visual inspection of wild-type plants found five developing tillers by the third week and six to seven developing tillers by the sixth week (Table 2). Crowns from plants 3, 4, 5, and 6 weeks old were dissected, and secondary tillers were seen developing in primary tiller axils (data not shown).

Als mutant plants made three to four tillers. At one week, *als* seedlings exhibited ABs in the first and second leaf axils (Fig. 2c). By the second week, only two ABs were observed in most seedlings, but occasionally a third AB

Table 2 Number of tillers and axillary buds present in *als* and wild-type 1–6 weeks after planting

Genotype	Time point (weeks)	No. of plants	Tiller no.	AB present[a]	Developing tillers (tillers + AB)
Wild-type	1	5	–	3.0	3.0
	2	4	–	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
als	1	5	–	1.7	1.7
	2	4	–	2.7	2.7
	3	8	2.5	0.0	2.5***
	4	4	2.5	0.0	2.5***
	5	7	2.7	0.0	2.7***
	6	8	3.6	0.0	3.7***

AB axillary bud

*** Significantly different at $p < 0.0001$ compared to wild-type

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

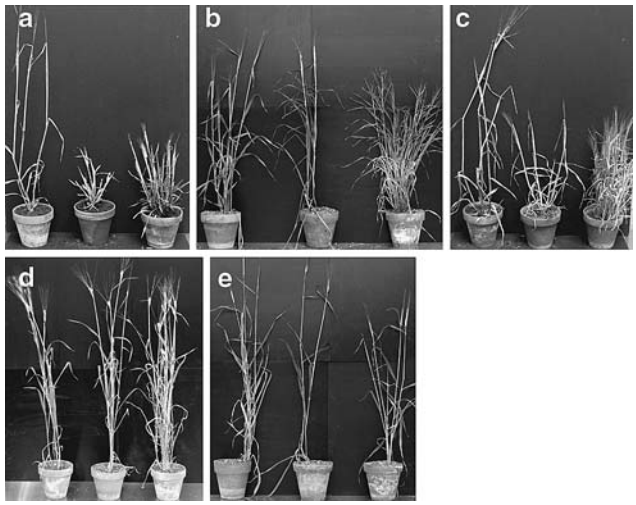

Fig. 3 Vegetative phenotypes of double mutant combinations of *als* with five other mutants: **a** *als;uzu,* **b** *als;mnd1*. **c** *als;gra-a*, **d** *als;int-m*, **e** *als;int-b*. In each frame, *als* is located on the *left*, the double mutant is in the *middle*, and the other tillering mutant parent is located on the *right*. Tiller numbers for the *als* mutant and *als-mnd1* double mutant plants ranged from 1 to 4 in this field season

was seen in the third leaf axil (Fig. 2d). The timing of AB development was slightly delayed compared to wild-type seedlings. The SAM in *als* plants was shorter than in wild-type plants (Fig. 2d). By the sixth week, *als* plants had three to four developing tillers (Table 2). Secondary tillers were not seen in the axils of the primary tillers in *als* mutant plants between the third and sixth weeks (data not shown).

Genetic interactions of *als* with six other tillering mutants

Double mutant combinations between *als* and other tillering mutants were examined to assess the genetic role of *Als* during tiller and inflorescence development. The low-tillering mutants, *int-b* and *uzu* produced more tillers than *als*, but significantly fewer tillers than wild-type. The high-tillering mutants used were *den6*, *gra-a*, *int-m* and *mnd1*. Vegetative phenotypes of the double mutant combinations, except *als;den6*, are shown in Fig. 3. Chi-square analysis was conducted in the F_2 families segregating for the mutations. None of the segregation ratios differed significantly from the expected 9:3:3:1 ratio (data not shown).

For the tillering phenotype, the *als* mutant was epistatic to all four high-tillering mutants tested and the low-tillering *uzu* mutant. For example, *als;gra-a* plants had three to four tillers like *als* plants (Fig. 3c). Neither *gra-a* nor other high-tillering mutations tested suppressed or modified the *als* tillering phenotype. Similarly, *als;uzu* plants had three to four tillers like *als*, which was fewer than *uzu* plants (Fig. 3a). In contrast to these phenotypes, *als*;*int-b* double mutants exhibited a single tiller (Fig. 3e), indicating that there was a synergistic interaction between *int-b* and *als*.

Additive, epistatic, and synergistic interactions were seen for plant height and inflorescence traits in the double mutants. *uzu* mutant plants were dwarfed, and *als* had normal height. However, *als;uzu* double mutants were shorter than either single mutant (Fig. 3a), indicating a synergistic interaction between *als* and *uzu* for plant height. Plant height of the other combinations resembled one of the single mutants. Interactions for inflorescence traits were complex, and additive and synergistic interactions were common (data not shown).

Genetic map position of the *Als* gene

Simple sequence repeat markers were used to determine the map location of the *Als* locus on the barley genetic map. A total of 15 SSR markers located on the long arm of chromosome 3H were identified as polymorphic between the mapping population parents Bowman-*als* and Morex. Genotyping the F_2 population with these 15 SSR markers positioned the *Als* gene 16.7 cM proximal of GBM1050 and 31.3 cM distal of HVM60. This placed *Als* on chromosome 3HL, Bin11 of the barley genetic map (http://barleygenomics.wsu.edu/) (Fig. 4).

Transcript accumulation in the *als* mutant compared to wild-type

The effect of *als* on transcript levels was most apparent in seedling tissue. There were 86 genes whose transcript

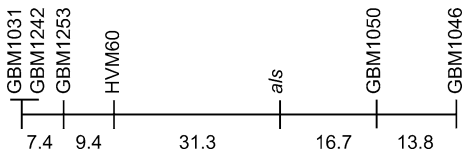

Fig. 4 Genetic map position of *Als* on chromosome 3HL, Bin 11 of the genetic map. Bin designations were derived from the barley genomics website (http://barleygenomics.wsu.edu/). All distances are expressed in centimorgans

levels were at least twofold higher ($p \leq 0.001$, FDR of 2%) in samples from *als* tissue as compared to wild-type tissue (Supplementary Table 1). Of the 86 transcripts, 50 transcripts had elevated levels in seedling tissue. Transcript levels for 77 transcripts were elevated in a single tissue. When only these transcripts were considered, 43 transcripts were identified in seedling tissue.

Annotations for the transcripts are given in Supplementary Table 1. The striking observation here was the large number of transcripts associated with a stress response. Thirty-three of 72 annotated transcripts belonged to gene families, whose members have been linked to a stress or defense response (Fig. 5). These transcripts included chitinases and other defense proteins, peroxidases and other proteins needed to process reactive oxygen species, and stress-induced proteins.

Transcripts with reduced accumulation in *als* plants versus wild-type are candidate genes for *Als*. Only 21 transcripts showed lower levels in *als* than in one or more of the wild-type tissues (Fig. 5; Supplementary Table 2). About 11 of these transcripts contained a SFP, and their lower

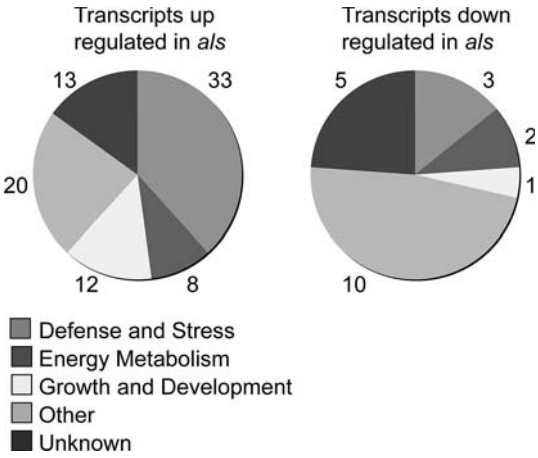

Fig. 5 Summary of Barley1 GeneChip analysis of expression differences. Eighty-six transcripts were up-regulated in tissues from *als* plants; 33 of these transcripts have been associated with defense and stress responses. Twenty-one transcripts were down-regulated in *als* tissue, with only three transcripts annotated as a defense or stress response. The category, other, includes transcripts annotated as regulatory, transport, biomacromolecule recycling, general metabolism, and miscellaneous functions

abundance in *als* may reflect technical issues with quantifying transcripts containing SFPs (DeCook et al. 2006). For comparison, eight of the 86 transcripts up-regulated in *als* contained an SFP (Supplementary Table 1). There was no obvious pattern to the tissue specificity of expression differences, nor was there a preponderance of stress-related transcripts.

Candidate genes may be evaluated on the basis of their annotations and map positions in addition to the Barley1 GeneChip data. One transcript, contig 9296_at, was mapped near *Als* on barley chromosome 3H (Supplementary Table 2). This transcript was similar to a hypothetical rice protein. The rice ortholog of *Als* should be on chromosome 1 between 27.6 and 41 mbp based on the analysis of Rossini et al. (2006). Putative orthologs for five other transcripts were located on rice chromosome 1 in the region syntenic with *Als* (Supplementary Table 2). However, the annotations of these transcripts were not consistent with known tillering or branching genes.

Expression of the 29 *Als* candidate genes identified by rice–barley synteny (Rossini et al. 2006) were examined using the Barley1 GeneChip data (Table 3). Annotations of these genes were consistent with previously identified branching and tillering genes, and these candidates were located near *Als*, based on synteny with rice. Homologous barley EST contigs to 20 of these rice candidate genes were identified on the Barley1 GeneChip. None of these EST contigs were identified as having reduced accumulation in *als* plants (Table 3).

Discussion

The role of *Als* in axillary bud development

Morphological analysis revealed that both *als* mutant and wild-type plants produce ABs and primary tillers in the early stages of development. But, unlike wild-type plants, secondary tillers do not develop from the primary tillers in the *als* mutant. By the third week in *als* mutants, no new ABs were evident and the existing primary ABs were developing into tillers. Secondary AMs either did not initiate or failed to form ABs. This lack of secondary tiller and axillary bud development coincided with the transitioning of the SAM to a reproductive meristem at approximately the second week of development. Thus, the *Als* gene product appears to be required for secondary tiller development, but primary tiller development does not require *Als*.

In the barley *cul2* mutant, neither the primary nor the secondary tillers develop. AMs are initiated in the embryo and 1-week-old *cul2* seedlings, but these AMs fail to develop into ABs (Babb and Muehlbauer 2003). These results indicate that the *Cul2* gene product is required for

Table 3 *Als* candidate genes based on synteny with rice

Candidate[a]	Annotation[a]	Barley Contig[b]	BLASTN[b]	Expression[c]
BAB86447.1	GRAS family transcription factor Scarecrow-like	12,925	0	Not different
BAC06237.1	GRAS family transcription factor	9,447	0	Not different
BAB89766.1	Homeobox	Not present[d]		Not tested
BAB90117.1	Homeobox	Not present		Not tested
Os01g62920	Homeobox	12,274	6e-62	Not different
BAB92357.1	Homeobox	Not present		Not tested
BAB62586.1	Homeobox	20,612	e-138	Not different
Os01g66030	MADS box	10,383	2e-16	Not different
BAB86065.1	MYB-like	18,002	2e-48	Not different
BAB92284.1	MYB-like	14,337	2e-81	Not different
		35,495	8e-72	Not different
BAB89293.1	MYB-like	53,019	e-141	Not different
		9,250	e-141	Not different
Os01g63680	MYB-like	Not present		Not tested
Os01g49160	MYB-like	18,002[e]	2e-48	Not different
BAB84433.1	MYB-like	18,002[e]	3e-25	Not different
Os01g59660.2	MYB-like	53,021	0	Not different
		3,667	0	Not different
BAB89519.1	MYB-like	Not present		Not tested
Os01g64310.1	NAC/NAM	Not present		Not tested
BAC06883.1	Putative fasciated1	12,319	e-123	Not different
BAB92568.1	Putative terminal ear1	Not present		Not tested
BAB89547.1	Auxin pathway	Not present		Not tested
Os01g57610.1	Auxin pathway	Not present		Not tested
BAB63594.1	Auxin pathway	12,102	1e-66	Not different
BAC03319.1	Auxin pathway	8,128[e]	0	Not different
Os01g63770.1	Auxin pathway	8,128[e]	0	Not different
BAB93227.1	Auxin pathway	26,361	e-112	Not different
BAB86111.1	Auxin pathway	15,329	3e-29	Not different
Os01g58420.1	Ethylene response	6,636	1e-39	Not different
Os01g55240.1	GA pathway	10,718	0	Not different
Os01g64000.1	Putative ABA insensitive	8,163	1e-49	Not different

[a] Candidates and annotation from Rossini et al. 2006

[b] Barley contigs from Assembly 25 HarvEST:Barley. BLASTN searches were performed on the HarvEST website to identify probes on the Barley1 GeneChip related to the candidate genes

[c] Expression data from the Barley1 GeneChip. *Not different* differences in transcript levels between wild-type and *als* were less than twofold. *Not tested* a barley EST contig homologous to the rice candidate gene was not on the Barley1 GeneChip

[d] A barley sequence closely related to the rice candidate gene was not present on the GeneChip

[e] Candidates Os01g49160 and BAB84433.1 were both similar to barley contig 18,002, and candidates BAC03319.1 and Os01g63770.1 were both similar to barley contig 8,128

AB formation in the early stages of development. However, because primary ABs do not develop in *cul2* plants, it is not possible to determine if *cul2* is required for secondary AB development, nor is it possible to determine the genetic relationship between *cul2* and *als*. Double mutants of *als* and *cul2* do not make any tillers, they are also taller than wild-type and *als* plants with reduced floret development on the lower part of the spike (Babb and Muehlbauer 2003).

The number of pathways controlling axillary bud development in barley

Two groups were defined in crosses between *als* and six other tillering mutants. Double mutant combinations between *als* and *den6*, *gra-a*, *int-m*, *mnd1*, and *uzu* exhibited three to four tillers, like *als* single mutant plants. The *Als* gene was epistatic to these genes. A second group was

defined by the synergistic interaction between *Als* and *Int-b*. The *als;int-b* double mutant plants had one tiller, fewer than either single mutant. These results indicate that the *Int-b* gene is in a pathway distinct from that involving the *Als* gene.

Synergistic interactions have also been observed between *int-b* and two other low-tillering genes. Mutant *low numbers of tillers1* (*lnt1*) plants typically have two or three tillers. The interaction between *lnt1;int-b* results in plants that do not make tillers (Dabbert and Muehlbauer, unpublished data). Mutant *cul2* plants do not make tillers, hence it was not possible to see a synergistic tillering response in *cul2;int-b* plants. However, there was a synergistic interaction between *int-b* and *cul2* for spikelet branching (Babb and Muehlbauer 2003). The *cul2* mutation disrupts the phyllotaxy in the top half of the spike (Babb and Muehlbauer 2003). The *int-b* spikes have developmental irregularities in the lower half of the spike with lateral spikelets that are reduced in size (Lundqvist et al. 1996). Inflorescence AMs in both the *cul2* and *int-b* single mutants initiated and developed into spikelets. But in the *cul2;int-b* double mutant, spikelets failed to form along the sides of the inflorescence. Thus, the absence of both the *Cul2* and *Int-b* gene products prevented AMs from developing into spikelets in the inflorescence.

Synergistic interactions between branching genes have been interpreted in two ways. Interactions between mutant alleles of the three *RAX* genes, and the interaction between a *rax1* mutant allele and the *las* mutation illustrate this (Müller et al. 2006). In *Arabidopsis*, *las* and *rax1* mutants have similar phenotypes, suppressing lateral shoot development in the rosette. *RAX1* encodes a *R2R3 myb* protein and *LAS* encodes a GRAS family transcription factor. Neither mutation alone affects lateral shoot development in the flowering stem. Yet lateral shoot development along the flowering stem is strongly suppressed in the *las;rax1* double mutant. This result coupled with the observation that *LAS* transcript level was not altered in a *rax1* mutant and *RAX1* transcript level was not altered in a *las* mutant, suggested to Müller et al. (2006) that *RAX1* and *LAS* functioned in separate genetic pathways. Mutations in homologous genes functioning in the same pathway can also give synergistic interactions. Homologous genes provide redundancy, and mutation in one gene may be masked by its homolog. This mechanism was proposed for the *LAX PANICLE* (*LAX*) and *SMALL PANICLE* (*SPA*) genes in rice (Komatsu et al. 2003) and observed with the *RAX* genes in *Arabidopsis* (Müller et al. 2006). The Arabidopsis, *RAX1*, *RAX2*, and *RAX3* genes encode similar *R2R3 myb* proteins. The *rax1* mutation reduces the number of AMs in the rosette. Neither the *rax2-1* nor the *rax3-1* mutant alleles individually affected AM development in the rosette or flowering stem, but double mutant combination between *rax2-1* and *rax3-1* reduced AM development in the rosette (Müller et al. 2006). Thus, synergistic interactions have been seen when there are mutations in two separate pathways, or between homologous genes with redundant functions.

It seems unlikely that *Int-b*, *Als*, and *Lnt1* are members of the same gene family and have redundant functions. First, unlike the *RAX* genes, which were initially identified as members of a gene family by sequence similarity (Müller et al. 2006), the *int-b, als*, and *lnt1* mutants were isolated on the basis of their phenotype. Second, the origin of gene redundancy is gene duplication, and the common fate of duplicated genes is either gene loss or sub-functionalization, where the duplicated genes split the functions previously shared by the ancestral gene (Prince and Pickett 2002). The *int-b, als*, and *lnt1* mutations have similar phenotypes, with no clear indications for functional separation. Thus, the genetic interactions between *int-b* and other low-tillering genes suggests the presence of more than one genetic pathway controlling tiller number.

Tillering and stress

Environmental stress is an important regulator of tillering. Low nitrogen conditions reduces tillering in spring wheat (Longnecker et al. 1993), as does competition from weeds in rice (Estorninos et al. 2005). UV light in wheat and oat (Barnes et al. 1990) can increase tillering, and mechanical stress in *Arabidopsis* (Pigliucci 2002) can increase branching. Recent work in sorghum demonstrated that regulation of tiller number by plant density and light quality is mediated by the action of phytochrome-B on the expression of the sorghum ortholog of *TB1* (*SbTB1*) tillering gene (Kebrom et al. 2006). This last result demonstrates how a tillering pathway can be regulated by environmental conditions.

Our finding that stress and defense response genes were the major group of transcripts showing elevated accumulation in *als* tissues suggests a different link between tillering and the environment. We do not believe that many transcripts were misclassified as stress response or defense genes due to annotation problems. The four chitinases and five pathogenesis-related proteins upregulated in *als* were also upregulated in *Fusarium graminearum*-infected barley plants (Boddu et al. 2006). Thus, fungal infection and the *als* mutation both induce some of the same defense transcripts.

Whereas the work on *SbTB1* showed how environmental conditions regulate tillering, here the *als* mutation altered expression of genes regulated by environmental conditions. Interestingly, not all tillering genes exhibit this interaction. The barley *lnt1* mutation has a plant phenotype very similar to *als* (Lundqvist et al. 1996), but few stress response or defense genes were up-regulated in the barley *lnt1* mutation

(Dabbert and Muehlbauer, unpublished data). Thus, it is not the reduction of tiller number that induces the stress response and defense genes. We propose that there is a step in a tillering pathway that interacts with the stress response. This interaction could be mediated by *Als* itself or by a downstream step in the tillering pathway.

Map position, expression, and prospects for cloning *Als*

A total of 21 genes had decreased accumulation in *als* plants versus wild-type. One of these genes may be *Als*, and other genes may be regulated by *Als* (Supplementary Table 2). However, none of the down-regulated genes in *als* plants showed decreased transcript levels in all tissues sampled, as would be expected by the *Als* gene. It was possible that the tissue specificity of the *Als* gene expression made it difficult to detect differences in transcript levels. For example, expression of the rice tillering gene *monoculm1* appears to be limited to ABs (Li et al. 2003b). Positional information identified six transcripts that mapped near *Als* (Supplementary Table 2). These contigs encoded a glucan endo-1,3-beta-glucosidase (1636_at), a beta-glucanase (1639_at), glutathione transferase F5(2456_at), a delta 1-pyrroline-5-carboxylate synthetase (3814_at), and an esterase (10373_at). One contig, 9296_at, was similar to a hypothetical protein in rice. Annotations of these genes did not suggest an obvious candidate gene for *Als*. Alternatively, it is possible that a probe set for the *Als* gene is not present on the Barley1 GeneChip and thus it is not possible to identify the *Als* gene using this technology.

Out of 29, 20 *Als* candidate genes identified by Rossini et al. (2006) were represented on the Barley1 GeneChip (Table 3). None of these EST contigs were identified as having reduced accumulation in *als* plants (Supplementary Table 2). Among the remaining nine candidates were several transcription factors. As noted above, expression of *Als* may be limited to ABs and differences in transcript levels could be difficult to detect by the Barley1 GeneChip analysis performed here.

There is a need to improve our search for candidate genes. *Als* along with *Cul2* are important regulators of the early stages of AB development. Additionally, *Als* or a downstream gene may help integrate plant growth and expression of stress response genes in the face of changing environmental conditions. Unfortunately, the location of neither *Als* nor *Cul2* corresponds to an obvious candidate gene from rice or maize. The probable syntenic location of rice *moc1* is on barley chromosome 7H (http://www.harvest-web.org/hweb/bin/wc.dll?hwebProcess~hmain~&versid=4); *Als* and *Cul2* are on barley chromosomes 3H and 6H, respectively. And the maize gene that likely integrates environmental signals with tillering, *TB1*, does not regulate the early stages of AB development as do *Als* and *Cul2*. Instead, *TB1* regulates AB outgrowth (Doebley et al. 1997). Our positioning of *Als* on barley chromosome 3H, Bin 11 represents a first step in efforts to localize *Als* to a small region and reduces the number of candidate genes to consider.

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