Genetic and morphological analysis of the barley low-tillering mutant *intermediium-b*

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Minor master-thesis of Adriaan Westgeest

Department of Plant Breeding and Genetics of Dr. M. Koornneef
Max Planck Institute for Plant Breeding Research
Carl-von-Linné Weg 10
50829 Cologne

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Max-Planck-Institut
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Max Planck Institute for Plant Breeding Research

Supervisors

Professor M. Koornneef, Max Planck Institute for Plant Breeding Research

Professor H. de Jong, Wageningen University and Research

Junior-professor M. von Korff Schmising, Max Planck Institute for Plant Breeding Research

Doctor W. van Esse, Max Planck Institute for Plant Breeding Research

Student

Adriaan Westgeest, master student Plant Biotechnology, Wageningen University and Research

Pictures at the front page represent a wild type barley plant (cultivar Bonus) on the left, and the intermedium-b mutant (background cultivar Bonus) on the right.

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Acknowledgement

I would like to thank my colleagues of the Koornneef group and the von Korff group for their contributions and support during group meetings, coffee breaks and lunch. Especially, I would like to thank both Prof. Dr. Koornneef for the opportunity to work in his lab, and Jun.-Prof. M. von Korff for their supervision on the shared project. Special thanks to my supervising tutor, Dr. W. van Esse, who assisted me in and outside the institute. The help of technicians, gardeners and lab assistants in my project made it possible to obtain the results discussed in this report. Thanks a lot!

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Abstract

Barley (*Hordeum vulgare L.*) carrying the recessive mutation *intermedium-b* (*int-b*) shows a low number of tillers and an irregular inflorescence development. It is a neutron-induced mutant and the *int-b* locus was previously mapped on chromosome 5HL between 91.8 and 139.9 cM. In this project the *int-b* mutant was analyzed morphologically, by a meristem dissection experiment and genetically, by mapping the locus in an segregating F2 population. Also, a Bulk Segregant Analysis on RNAseq was performed to obtain SNP markers and to reduce the size of the introgression. Afterwards, the expression data of different allelic variants of *int-b* and wild types was compared to obtain more insights in possible involved pathways and the selection of candidate genes. Meristem dissection revealed that in mutants, bud outgrowth in the first leaf axil was hampered after 2 weeks of growing. At the same moment, the shoot apical meristem (SAM) developed faster in the mutant than in wild type. Mapping the *int-b* locus in a segregating F2 population between *int-b* and Proctor resulted in a new introgression from 95.9 to 99.9 cM on the long arm of chromosome 5H between markers R2324 and GBM1227. BSA-RNAseq analysis confirmed the result of the mapping with a peak on 97 cM on chromosome 5H. SNP data derived from expression data did not reveal a gene with SNP between *int-b* and wild type. However, expression data did show two strongly downregulated genes in the introgression. Based on BSA RNAseq, four genes were found which did not had reads in the mutant. One of these is a MYB protein transcription activator, and the rice mutant ANTHER INDEHISCENCE1 exhibiting a mutation in a MYB protein exhibits the same phenotype as the *int-b* mutant.

Introduction

The most important cereal crops are maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* spp. *Vulgare*). The total annual production of these crops was 2.6 billion tons in 2013 (FAOSTAT, 2013). The demand for cereals will double by 2050, which means that we need an annual yield increase of 2.4% per year. The yield in monocots is mainly determined by shoot branching (Sakamoto and Matsuoka, 2004). Each branch, called a tiller in barley, can produce an inflorescence and hence increase the yield. However, unproductive tillers cost the plant nutrients and can lead to a lower grain weight. The aim is to create a balance between grain weight and number of productive tillers. Research in model crops as rice and *Arabidopsis* has proposed that there exist a number of pathways which influence shoot branching, however little is known about tillering pathways in temperate cereals as wheat and barley.

The complex architecture of the plant is determined by the arrangement of growth axes. The first apical-basal axis is developed during embryogenesis and consists of a shoot apical meristem (SAM) and root apical meristem. The above-ground architecture is developed by the repeated formation of new growth axes in the leaf axils (Oikawa et al., 2009). The resulting side-branch is called a tiller when originated from a basal leaf axil, whereas a branch developed from a leaf axil of an elongated internode is called an aerial branch (Kebrom et al., 2013). Barley only exhibits basal branches, called primary tillers when emerged from leaf axils of the main shoot, secondary tillers when emerged from the leaf axils of primary tillers and so on (Kirby and Appleyard, 1987). The development of a tiller is characterized by three general steps: initiation of an axillary meristem (AM), the development of a dormant axillary bud (AB), and the outgrowth of the AB into a tiller (Schmitz and Theres, 2005). The development of each next step is determined by a network of different factors.

In barley, the first AM's are initiated during embryonic development. In the vegetative growth stage the AM produces a few leaf primordia, resulting in an AB. In different species, a number of mutants are found with a defect in the initiation of an AM. Their phenotypes can be divided in two classes (Oikawa et al., 2009). The first class exhibits a defect in AM formation in combination with other abnormalities found in *Arabidopsis thaliana*, such as defects in SAM formation (*pinhead* mutant), leaf polarity and vascular development (McConnol and Barton 1995; Raman et al., 2008). The other class shows AM specific defects, such as: *Lateral suppressor* (*LAS*) and *regulator of axillary meristems* (*RAX*) mutants in *Arabidopsis thaliana*, *Lateral suppressor (Ls*) and *blind* (*Bl*) in tomato (*Solanum lycopersicum*), *barren stalk1* (*BA1*) in maize (*Zea mays*), and *monoculm1* (*MOC1*) and *lax panicle1* (*LAX1*) in rice (*Oryza sativa*), which are important for AM formation (Schumacher et al., 1999; Schmitz et al., 2002; Greb et al., 2003; Li et al., 2003; Keller et al., 2006; Müller et al., 2006; Oikawa et al., 2009). The identified loci can have an effect at a particular developmental stage. *MOC1, Bl* and *BA1* mutants lack any sign of AM development, meaning that they play an important role at the early beginning of AM formation. However, *LAS* and *Ls* are required for AM formation at the vegetative stage, but not during reproductive development (Greb, 2003).

After the initiation of a bud, the outgrowth is determined by the outcome of a network of hormonal, genetic and environmental factors (Fig. 1, Kebrom et al, 2013). A main role is played by the hormone auxin, transported basipetally from the SAM, which is known as apical dominance (Agusti and Greb, 2013). Auxin upregulates the biosynthesis of strigolactones, which block the bud outgrowth (Gomez Roldan et al., 2008). At the same time, auxin downregulates the biosynthesis of cytokinin, a hormone that stimulates the bud outgrowth (Ferguson and Beveridge, 2009). Cytokinin and strigolactone both control the bud outgrowth by influencing the biosynthesis of downstream proteins like TEOSINTE BRANCHED 1 (TB1), which integrates the signals in the bud and inhibits the outgrowth of the bud into a tiller. There are general two classes of genes involved in the regulation of bud outgrowth (Bennet and Leyser, 2006). The first-class inhibits branching, like: *TB1* in maize, orthologs of *TB1* in rice (*OsTB1*) and barley (*INT-C*), *GRASSY TILLERS1* (*GT1*), *BRANCHED1* (*BRC1*) and *MORE AXILLARY GROWTH 1* (*MAX1*), *MAX3* and *MAX4* in *Arabidopsis thaliana*. Loss-of-function mutations

in these genes lead to increased branching (Takeda, 2003; Ramsay, 2011; Aguilar-Martinez, 2007; Bennet, 2006; Whipple et al., 2011). The second-class exhibit the outgrowth, where next to genes involved in cytokinin synthesis, a role is played by sucrose, when found in higher concentration in the bud, causes outgrowth. The intense demand for sugars by the SAM can limit the sugar availability for the buds, causing apical dominance. Also the expression of the *BRC1* transcription factor is substantially reduced when supplying sucrose, causing bud outgrowth (Mason, 2014). Precocious internode elongation have also been proposed to act as a sink for sugars, thereby reducing tillering in the *tin* mutant in wheat (Kebrom, 2012).

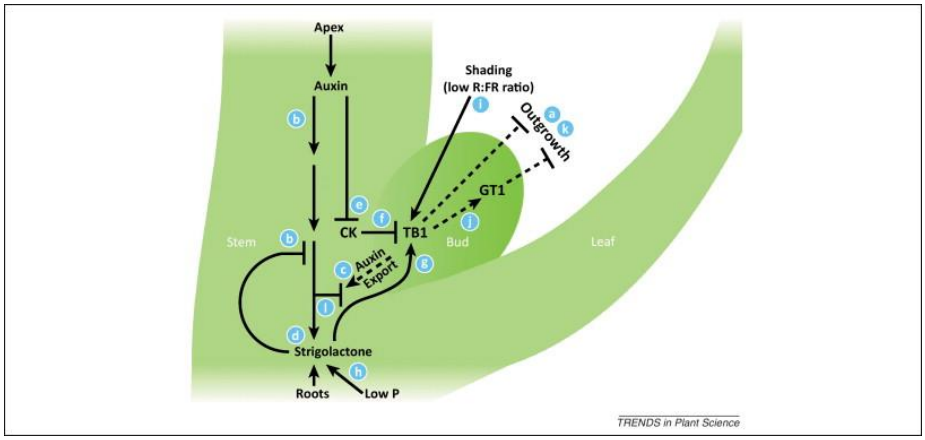


Figure 1: Generic model of bud outgrowth mechanisms. Auxin is transported basipetally form the SAM (b), and promotes the biosynthesis of strigolactones (d), and inhibits the biosynthesis of cytokinin (e). Cytokinin promotes bud outgrowth by inhibiting respressor proteins of lateral bud outgrowth as TB1 (f). Strigolactones are produced in the roots and transported upwards and enhances respressor proteins like TB1 (g). Other genetic factors, as shading (i) and *GT1* (j) influence the bud outgrowth (Figure from Kebrom et al., 2013).

Axillary or lateral meristems are not only important for shoot branching, they also play a crucial role in the establishment of the structure of the inflorescence, that ultimately lead to the formation of flowers. This explains why tillering mutants often have a pleiotropic effect on the inflorescence phenotype. In barley, the inflorescence is composed of two rows of triplets with respectively three spikelets at each rachis internode. Based on the spike architecture, barley can be divided into two-rowed and six-rowed cultivars. In two-rowed cultivars, the central spikelet of a triplet is fertile and lateral spikelets are sterile. In six-rowed cultivars, the two lateral spikelets are also fertile, resulting in six rows of spikelets (Komatsuda et al., 2007; Fig. 2). The six-rowed phenotype is the result of a homozygous mutation in the barley gene *six-rowed spike 1* (*Vrs1*), which is located on the long arm of chromosome 2H (Franckowiak et al., 1997). *Vrs1* encodes a transcription factor that includes a homeodomain-leucine zipper motif (HD-ZIP) class I (Chan et al., 1998). In addition there were more alleles found to affect the row- and tillering-type of barley such as *Vrs2, Vrs3, Vrs4, Vrs5*, *Intermedium-a (Int-a*), *Intermedium-b* (*Int-b*), *Intermedium-c (Int-c*), *Intermedium-e* (*Int-e*)(Gustafsson and Lundqvist, 1980; Lundqvist, 1988; Komatsuda et al., 2007; Ramsey et al., 2011; Koppolu et al., 2013). All these mutants show a different row-type phenotype, but not all mutants show a tillering phenotype.

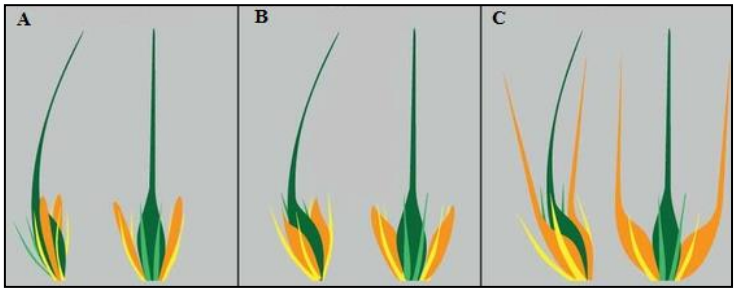


Figure 2: Different spike phenotypes in barley. A) Two-rowed spike, B) Intermedium, C) Six-rowed. Green denotes the central spikelet, yellow the lateral spikelets (Modified from Ramsay et al., 2013).

Mutations at the *int-b* locus result in irregular shaped spikes with fertile central spikelets, but partially filled lateral spikelets. In addition, *int-b* mutants are tall plants that show a reduced tiller number (Lundqvist, 1988; Babb and Muehlbauer, 2003). Double mutant combinations of *int-b* with other barley mutants gives more insight in the tillering pathways in barley. Synergistic interactions between the *absent lower laterals* (*als*) mutant and *int-b* were determined by a double mutant which had only one tiller, which is fewer than each single mutant. Stress and defense transcripts were two-fold upregulated in *als* tissues, meaning that it acts in a tillering pathway that modulates a stress response (Dabbert, 2009). Environmental stresses as low nitrogen conditions or low light have been shown to have an effect on *TB1* (Kebrom et al, 2006). A synergistic interaction was also reported for the double mutant of *int-b* and the mutant *low number of tillers1 (lnt1*). Normally *lnt1* has 2 or 3 tillers, but a double mutant with *int-b* contained only 1 tiller (Dabber and Muehlbauer, unpublished data). Gene expression, mapping and sequence analysis supported the *JuBel2* gene as a candidate for *lnt1* (Dabbert et al, 2010). The JuBEL2 protein is a homeodomain transcription factor, part of the BELL family (Muller et al, 2001). In *Arabidopsis* the *BELLRINGER* (*BLR*) transcription factor shows high sequence similarity, and the *blr* mutant exhibits a reduced height, additional axillary branches and disordered inflorescence. Double protein actions have shown that BELL transcription factors are important for meristematic identity of SAM and AM. The partially overlapping phenotypes may indicate differences in pathways between monocots and dicots. However, it is clear that *JuBel2* in barley is important for tiller development. The barley mutant *cul2* does not develop any tillers, therefore a synergistic combination with *cul2* could not be seen for tillering. Yet, a synergistic interaction could be observed for the spike phenotype where spikelets failed to form along the sides of the inflorescence. So, in the absence of *Cul2* and *Int-b* AM's fail to develop into spikelets (Dabbert et al., 2009). These synergistic interactions suggest that there are at least two separate pathways that promote tillering in barley (Dabbert et al., 2009, 2010).

In this project the mutant *int-b* has been studied in detail, as part of a research project that attempts to obtain an increased understanding of the control of tillering in barley. Potentially, this information can be used by breeders to improve the crop yield of barley. The macroscopic phenotype of this mutant has been characterised as having a reduced number of tillers and an intermedium spike phenotype. *Int-b* is a recessive mutation which has been identified in the spring cultivar Bonus after a X-irradiation treatment (Babb and Muehlbauer, 2003). There are several allelic variants of *int-b* available, introgressed in different backgrounds: *int-b3* (Bonus), *int-b3* (Bowman), *int-b6* (Bonus) and *int-b75* (Bonus). *Int-b3* has been backcrossed in Bowman for several generations, and this near isogenic line (NIL) was genotyped with 3000 SNP markers to locate the introgression of the mutant gene (Druka, 2011). *Int-b* is localized on chromosome 5H between Bopa marker 2_0134 and 2_1355 (Druka, 2011). The gene underlying the *int-b* locus has not yet been identified and therefore was the most important aim of this project was to identify the gene. To this end, we performed a dissection experiment to obtain a detailed phenotypic describtion of the mutant. In addition, we have performed a mapping of the locus with use of a segregating F2 population between *int-b* and Proctor and a RNAseq bulk segregant analysis (BSA RNAseq) to reduce the size of the introgression. RNA sequencing (RNAseq) on crown tissue of *int-b* and wild type at one week after emergence was used to evaluate which known tillering genes were differentially regulated. Finally, based on the results of these experiments potential candidate genes were selected which were located in the introgression.

Material and methods

Genetic stocks

Intermedium-b is an X-irradiation induced mutant in Bonus (U. Lundqvist and J.D. Franckowiak, 1997). Different mutational events have created *int-b3*, *int-b6* and *int-b75* in Bonus and crossing showed that they contain a mutation at the same allele. *Int-b3* has been introduced in Bowman (GSHO 2129). For the linkage analysis, a segregating F2 population was used consisting of 393 individuals derived from the cross between Proctor and *int-b3* (Bonus).

Microscopic analysis

The morphological analysis was carried out on the accessions *int-b75* (Bonus) and *int-b3* (Bowman). Plants were grown in trays at greenhouse conditions at the Max Planck Institute in Cologne in light conditions of 12/12 h (day/night) and at 18 degrees Celsius. For the morphological analysis, *int-b3* (Bowman), *int-b75* (Bonus), Bowman and Bonus were used for meristem dissection using the Leica MZ75 microscope.

Genetic analysis

An F2 segregating population between Proctor and *int-b*3 (Bonus) was used to map the *int-b* locus. A subpopulation of 59 plants was grown in summer, and of this population the spike phenotypes could be scored. The other population (334 individuals) was grown in winter under greenhouse conditions: 12/12 h (day/night) and at 18 degrees Celsius. In the latter population, only the tiller number could be evaluated.

Genomic DNA extraction

For DNA extraction, approximately equal leaf samples were taken after two weeks after emergence. The material was freeze-dried and stored at -80 °C. DNA extraction was performed using the BioSprint 96 robot from Qiagen. After grinding the samples with a metal ball using the Tissue lyser of Qiagen, 300 µl Lyses buffer was added. After spinning down the lysate, 200µl was pipetted in a well of a S-block. There was 200 µl ispropanol and 20 µl Mag-Attract suspension G added. The DNA binds to the magnetic particles and the robot performed different washing steps with the buffer RPW, two times with 95% ethanol and in distilled water containing 0.02% tween. At the end the DNA was eluted in 200 µl buffer AE and stored at 4 °C.

SNP selection and marker development

The marker 4771-380 from BOPA1 at 110.26 cM (Close at al., 2009) with the primer pair R23 and R24, was known to cosegregate with the phenotype (Vanesse, unpublished). Based on the recombination frequency, the *int-b* locus is located about 8 cM from the 4771-380 marker. Primers were designed based on their location on the Popseq map (Mascher, 2014), in the flanking region of the marker 4771-380 (R2324). Next to this, RNAseq data was used to see which genes could be potential candidate genes, based on their expression and function. The RNAseq data was derived from the SAM at Waddington stage 0, and available for *int-b6* (Bonus), *int-b3* (Bowman), *int-b3* (Bonus) and Bonus and Bowman. After designing primers, the PCR products of 13 candidate genes in this region were Sanger sequenced and 1 SNP could be used as a polymorphic marker.

For the marker A2122, the digest was done with BsaJI, for 8 hours at 60 °C. For the marker R2324 the digest was done with the enzyme BsaHI, for 1 hour at 37 °C. Depending on cut and uncut fragments, the mapping population could be genotyped in homozygous for either one parent or heterozygous on a 1% agarose gel. Also, 20 SSR markers were tested, which were selected based on the paper of Varshney *et al.*(2007) at chromosome 5HL. Two markers, GBM1227 and Scssr10148 were seen to be

polymorphic between Proctor and *Int-b3* (Bonus). The marker Scssr10148 could be scored on a 4% agarose gel. The marker GBM1227 was evaluated using the LICOR 4200 DNA sequencer.

BSA-RNAseq analysis

Bulk Segregant Analysis on RNA was performed on the F2 mapping population of the cross between *int-b3* (Bonus) and Proctor grown in winter. Leaf samples of individuals were harvested based on their high or low tillering phenotype and genotypic information. Leaf samples of 53 low-tillering were independently pooled as one sample, while 43 leaf samples were pooled for high-tillering. Also of the parental leaf samples of 5 individuals were pooled as a control. From these groups RNA was extracted and sent for sequencing on an Illumina Hiseq2500 lane. The RNA was isolated using the Qiagen Mini Kit, according to the manufacturers manual.

The objective of sequencing RNA in bulks was to calculate the parental allele frequencies and compare that with the bulk frequencies. For each SNP in the bulk, the allele frequency was calculated by dividing the number of reference reads by the number of reads with the alternative base. Then the ratio was calculated of the allele frequencies between the two bulks, which is called the Bulk Frequency Ratio (BFR). The log (2) was calculated and the absolute value was plotted.

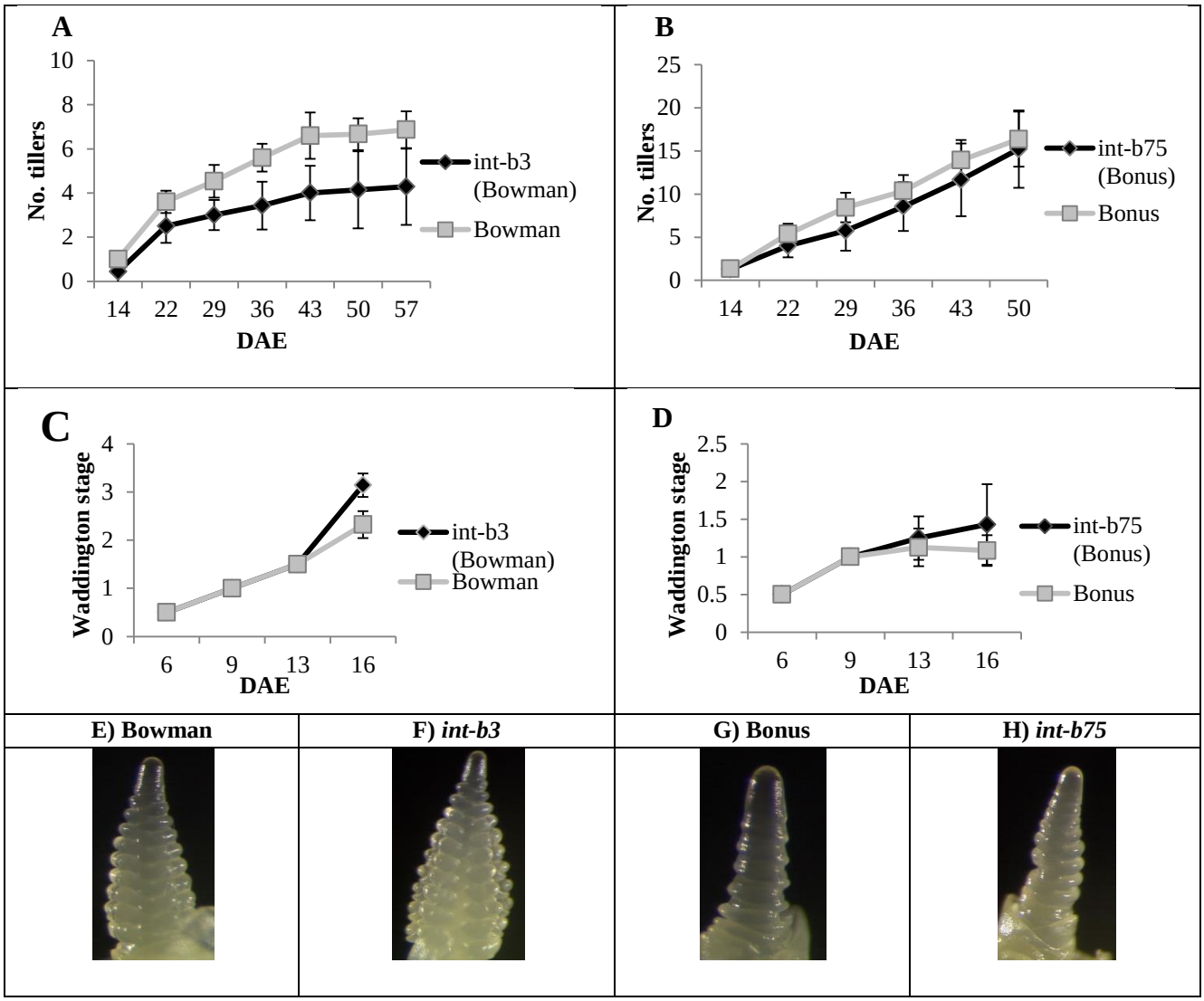
Allelism test

Six-rowed spike *vrs2e* (Fukuyama, 1972) is an X-ray induced mutant in Svanhals and located between marker ge00066s01 and ge00186s01 on the long arm of chromosome 5 (Youssef, 2014). It has been introduced in Bowman, and this one was crossed with *Int-b* (Bowman).

Results

***Intermedium-b* mutants showed a faster SAM development**

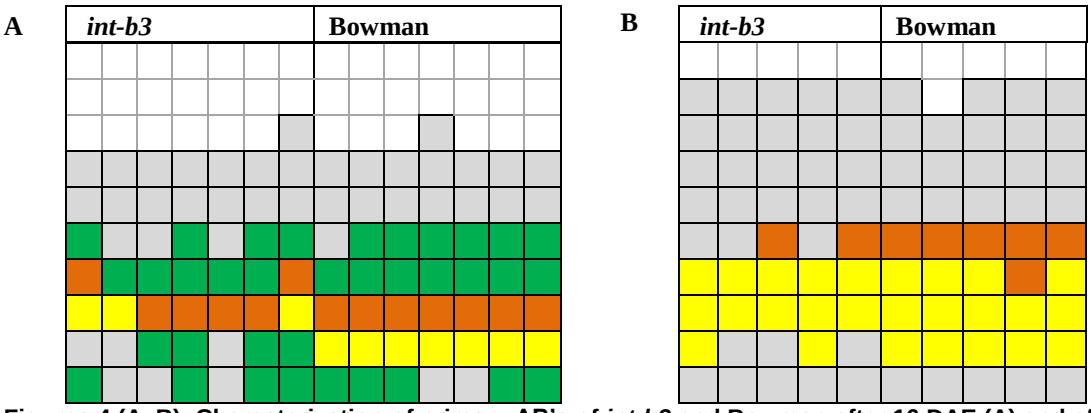
In an experiment only the tillers were scored and both mutants exhibited a reduction in tiller number (Fig. 3A, B). However, the difference in tiller number between Bonus and *int-b75* was less as the difference between Bowman and *int-b3.* A meristem dissection experiment was performed to know at which time point this reduction in tillers was seen. The number of leaves, tillers and stage of the SAM were counted at different time-points. The development of the SAM was checked to see the effect of the reduction in tillers, because it is known that tillers compete with the main culm for nutrients (Gu, 1988). Two allelic variants of *int-b* were used for dissection: *int-b3* (Bowman) and *int-b75* (Bonus). Already at the first time-point (14 days after emergence (DAE)) a difference in tiller number was observed between mutant and wild type. During the time-points, the standard deviation for tiller number was higher in the mutant, compared to wild type (Fig. 3 A, B). After 16 DAE the SAM of *int-b3* developed significantly faster when compared to the wild type (Fig. 3 C). In this stage the SAM of *int-b3* transitioned into the reproductive phase of development (Waddington stage 2.25-3). A faster development of the SAM was also seen for *int-b75,* but this was not significant and was not seen around the double ridge stage of Waddington, the transition into the reproductive phase of development (Fig. 3 D). The result of an earlier flowering time for *int-b* could not be scored during the winter, but there was no difference in flowering time observed in the field between *int-b75* and Bonus (Liller et al., in preparation). In an earlier dissection experiment of *int-b75* grown in a climate chamber, the differences in tiller number, AM's and Waddington stage of the SAM between *int-b75* and Bonus were seen to be significantly different (Supplementary Fig. 10).



Figures 3 (A-H): Morphological analysis of *intermedium-b* mutants. A) Tiller number of *int-b3* (Bowman) and Bowman at different DAE (n=14 plants) B) Tiller number of *int-b75* (Bonus) and Bonus at different DAE (n=9 plants). C) SAM development *int-b3* and Bowman at different DAE, staged according to the Waddington scale (Waddington *et al.,* 1983) D) Staging of SAM according to Waddington scale of *int-b75* in comparison with Bonus, at different time-points (DAE). Microscopically taken pictures of the SAM of E) Bowman, F) *int-b3* G) *int-b75* and H) Bonus were made after 16 DAE at magnitude of 4.0. Errors bars represent standard deviation.

Intermedium-b mutants showed an early cessation of tiller bud outgrowth

The tiller number data (Fig. 3 A,B) showed that the reduction in tiller number was made in an early developmental stage. The reduction in tiller number can occur due to the absence of AM formation or regulation in AB outgrowth. The latter one can take place during the transition of the shoot apex from the vegetative to reproductive growth. The tillers of barley are formed in a sequential order, starting with the first AB under the coleoptile. The development of the AB's in the axils of successive leaves can be studied by dissecting the plant at different developmental stages. To assess if *int-b* is deficient in bud initiation or bud outgrowth, the primary AB's were characterized as a dormant bud, outgrowing bud or tiller in each leaf axil in an early developmental stage (16 DAE).



Figures 4 (A, B): Characterization of primary AB's of *int-b3* and Bowman after 16 DAE (A) and after 30 DAE (B). Each column represents a single individual, with each square within a column representing an individual leaf axil, starting with the coleoptile. Green denotes a dormant AB, brown an outgrowing bud and yellow a tiller. Grey denotes the absence of an AB.

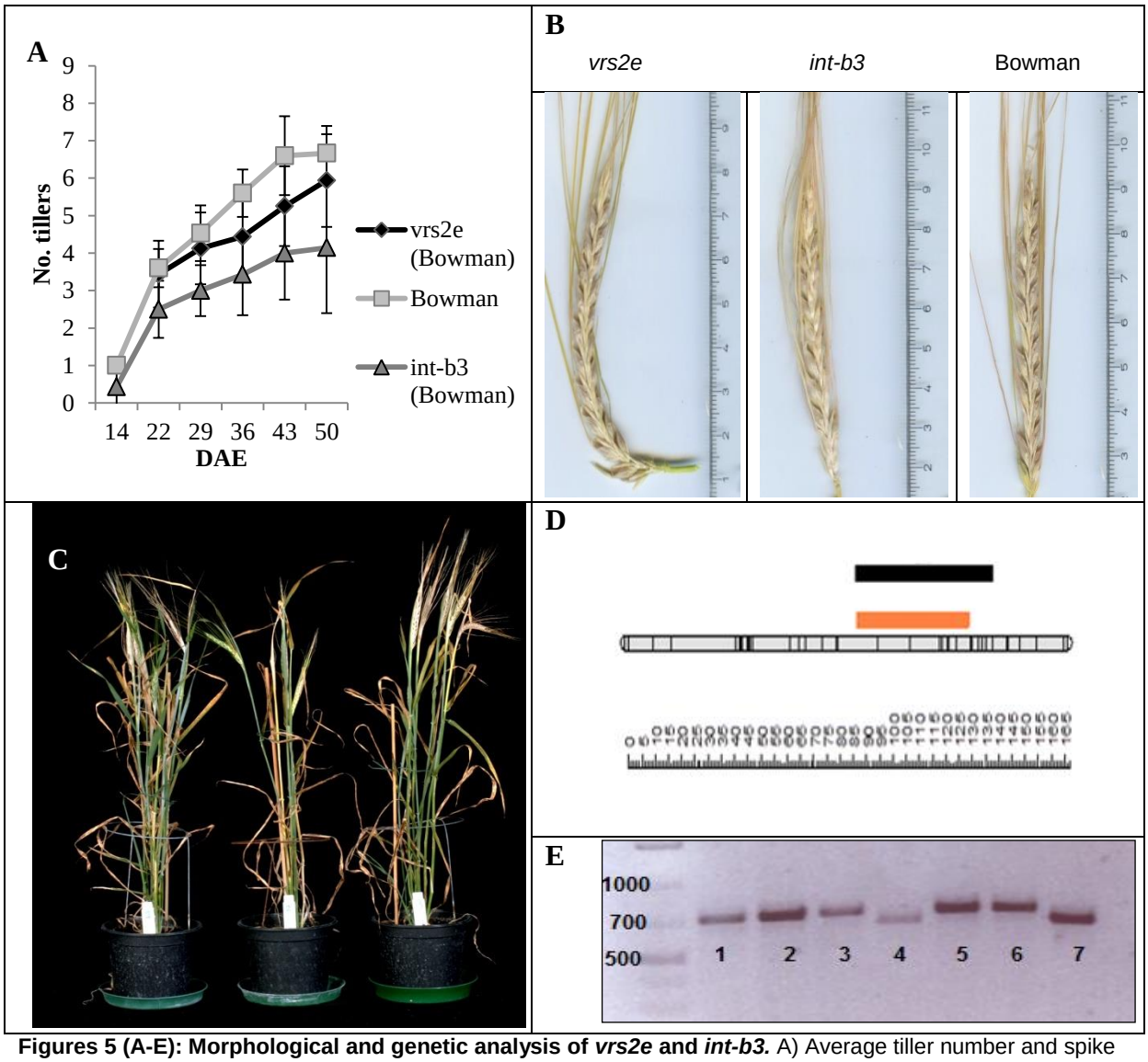
In *int-b3* mutants it was evident that the bud outgrowth rather than number of primary AB's was affected (Fig. 4A). In the coleoptile the AB's did not grow out in mature tillers in wild type and mutant. However, in the first leaf axil of the mutant, buds were dying off (grey) or stayed dormant (green) after 16 DAE, while in wild type tillers grew normally out (Fig. 4A). This took place during the transition of the SAM to the reproductive stage (Waddington stage 2.25). After 30 DAE, some of the dormant buds in the mutant's first leaf axil did grew out in tillers (Fig. 4B). Also, the total number of AB's was lower in the mutant, which may be due to apical dominance resulting of a faster SAM development (Fig. 3C) or there might be also a role for *int-b* in initiation of an AM. The buds in the *int-b75* mutant were also hampered in their outgrowth in the first leaf axil after 16 DAE, but did all grew out after 30 DAE (Supplementary, Fig. 11). This suggests that the outgrowth of AB's rather than AM initiation is affected in *int-b* mutants in an early stage of development.

To see if *int-b* also affects secondary (develop from the leaf axils of primary tillers) and tertiary AB's, the secondary AB's were counted at Waddington stage 3.5 in *int-b75* and Bonus (Supplementary, Table 3). In the mutants, a delay was seen in bud outgrowth, based on the lower number of secondary and tertiary AB's. This data was not collected for *int-b3.*

Int-b maps to the same introgression as *vrs2e*

Int-b mutants have a reduced number of tillers at full maturity and an intermedium spike phenotype (Babb and Muehlbauer, 2003). Several allelic variants have been identified, originating from mutagenesis studies (Lundqvist, 2014). To asses if there are more allelic variants, we aimed to find mutants which exhibit similar phenotype and were both introgressions overlap. One interesting candidate is six-rowed spike *vrs2e* which shows a reduced tiller number and an abnormal spike phenotype (Fukuyama, 1972). To compare the phenotype of *int-b3* and *vrs2e*, plants were grown simultaneously and spike and tillering phenotypes were analysed. The average tiller number of *int-b3* and *vrs2e* was reduced when compared to the wild type (Fig.5A). However, at 57 DAE *vrs2e* does not show a significant reduction in tiller number (Fig. 5A). In contrast, *int-b3* has a reduced number of tiller throughout all time-points recorded. In addition, the intermedium spike phenotype with partially developed lateral spikelets was clearly seen in *int-b3,* but not in *vrs2e* (Fig. 5B). In conclusion, the observed morphological differences between *vrs2e* and *int-b3*, suggests that they are not caused by the same gene. In addition, Lundqvist (1992) describes that *vrs2e* is not allelic to any of the *int* loci, suggesting that *vrs2e* and *int-b* are not allelic. To verify this, an allelism test between *vrs2e* and *int-b* was performed. *Vrs2e* is located between marker ge00066s01 and ge00186s01 on the long arm of chromosome 5 (Youssef, 2014). The introgression shows an overlap with the *int-b* introgression (Fig. 5D), therefore it may be another allelic variant of *int-b*. To test if the same locus is affected in both mutants, a cross was made between *int-b3* (father) and *vrs2e* (mother), both in Bowman background. The *int-b3* introgression in Bowman contains genetic material of Bonus. As the introgressions in both

mutants do not completely overlap, a SNP between Bowman and Bonus could be used to design a polymorphic marker. The gel showed that the F1 had the same product size as *vrs2e* and Bowman (Fig. 5E). This indicates that the cross did not succeeded. Yet, based on phenotypical analysis and previous data (Lundqvist, 1992), it is unlikely that *vrs2e* and *int-b* are allelic.



Figures 5 (A-E): Morphological and genetic analysis of *vrs2e* and *int-b3.* A) Average tiller number and spike phenotype for *vrs2e, int-b3* and bowman. Tiller number was counted each week, starting 3 weeks after emergence (DAE). B) Pictures of spike phenotypes are shown for *vrs2e, int-b3* and bowman. C) Picture of *vrs2e*, *int-b3* and Bowman, respectively, at flowering stage after 12 weeks of growing. D) Linkage map of Barley with the introgression given of *vrs2e* in orange and *int-b* in black on the long arm of chromosome 5H.. E) Gel picture shows the result of the polymorphic marker for the allelism test. From left to right: 1, 2 and 3 represent the F1; 4) *int-b* (Bowman); 5) *vrs2e* (Bowman); 6) Bowman and 7) Bonus. On the left the latter is shown with the product sizes in number of base pairs

Differentially regulated conserved genes in *int-b*

It is clear that *int-b* has an effect on tillering, now we want to know in which tillering pathway it is possibly involved. To this end, the SAM at Waddington stage 0 of *int-b3* (Bowman), *int-b3* (Bonus) and *int-b6* was collected to look if transcripts of conserved genes with a known function in tillering in plants were differentially regulated in the *int-b* mutants (Table 1). This was done to obtain an indication about the possible downstream targets of *int-b*, and the related tillering pathway based on literature.

Table 1: Differentially expressed conserved genes in the SAM of 3 *int-b* mutants. The differential regulation of these genes was seen to be the same in all mutants. Their annotation, location and function are given. Log(FC) values are calculated in comparison with their background and p-values are showed.

Annotation	Chromosome	Function	Expression	Log(FC) Int-b3 (Bonus)	p-value	Log(FC) Int-b3 (Bowman)	p-value	Log(FC) Int-b6 (Bonus)	p-value
MLOC_73076.2	4	*CEP (C-TERMINALLY ENCODED PEPTIDE)*	Down	-3.532	0.055	-5.965	0.000	-5.875	0.000
MLOC_72157.3	3	*SERK1 (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1)*	Up	2.797	0.517	-0.646	1.000	3.660	0.001
MLOC_4614.1	5	*TB1-like*	Down	-2.950	0.099	-0.013	1.000	-1.614	0.403
MLOC_71958.2	0	*MAX1*	Up	5.131	0.047	0.009	1.000	4.219	0.014
MLOC_70116.1	4	*TB1/ INT-C*	Down	-2.667	0.067	-0.519	1.000	-1.130	0.813

A *CEP* gene was seen to be significantly downregulated and encodes a gene of the CEP family that produce peptides that negatively regulate plant growth, especially under stress, and affect other important developmental processes (Ogilvie, 2014). *SERK1* was upregulated and acts as a negative regulator of abscission, regulating organ separation in *Arabidopsis* flowers (Pandey, 2013). The overexpression of the orthologous gene in rice (*OsSERK1*) showed a positive effect on somatic embryogenesis in cultured cells (Hu, 2005). *SERK1* may act as stimulus for lateral spikelet initiation in barley. Moreover, the spike phenotype in barley is imposed by *VRS1* alleles that are modified by alleles at the *INT-C* locus encoding an ortholog of the maize domestication gene *TB1* (Ramsay, 2011). In two-rowed cultivars, mutations at the *INT-C* locus lead to partially developed lateral spikelets. In all the *int-b* mutants *INT-C* was downregulated, which is expected as *int-b* shows also the intermedium spike. The barley ortholog of *MAX1* is strongly upregulated in two of the three mutants and is known to have a bud inhibiting function. When *int-b* acts upstream as an repressor of *MAX1*, a mutated *int*-b gene would increase the expression of *MAX1* and thereby inhibiting bud outgrowth.

From the RNAseq data of the 3 *int-b* mutants, it was possible to see that 9 genes were significantly differentially regulated in all the mutants (Supplementary, Table 4). These genes comprise two glucan-endo-1,3-beta-glucosidases which were upregulated and are involved in cell wall removal and resistance against microbial pathogens. Also, three protein kinases were upregulated. One unmapped gene was seen to be downregulated, which encodes a FAD-binding berberine family protein.

Mapping of *int-b* limited the introgression to 4 cM

Our interest is to know what gene causes the severe *int-b* phenotype. The *int-b* locus was previously mapped on chromosome 5HL between 91.8 and 139.9 cM (Van Esse, unpublished data). To reduce the size of the introgression, the *int-b* locus was mapped using a F2 population of a cross made between Proctor and *int-b3* (Bonus). Within this population, which consisted out of 383 individuals, the number of tillers was scored for each individual plant and used as the phenotypic marker because flowers did not develop. Growth of the F2 population showed a clear segregation of the mutant phenotype (Fig. 6). The amount of tillers of the *int-b* mutant showed 1-3 tillers, while Proctor showed generally up to 15 tillers. Mutant plants in the population could be clearly distinguished by exhibiting a lower tiller number and thick and rigid shoots.

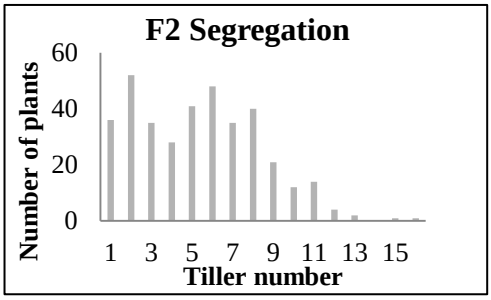
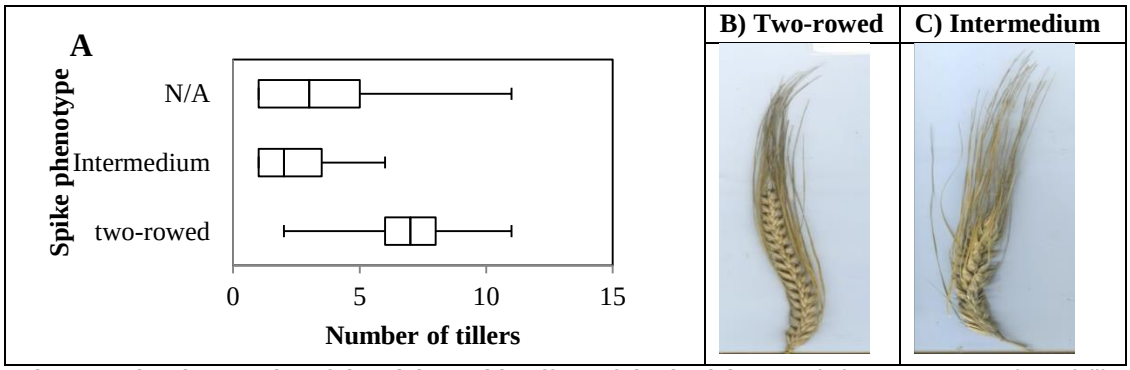
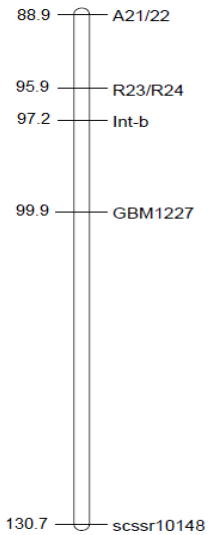


Figure 6: Frequency plot of the F2 population, which shows the number of plants having a certain number of tillers. Tillers were counted after 2 months.

In a subpopulation, the spike phenotype could be determined of 59 individuals. The mutant plants showed an intermedium inflorescence, where the lateral spikelets irregularly developed into fertile seeds (Fig. 7C), while in wild type stayed sterile (Fig. 7B). In this subpopulation, a clear cosegregation was seen between the spike phenotype and tillering phenotype of the mutant (Figure 7A). The segregation rate presented the expected 3 : 1 (high tillering : low tillering) of a single recessive gene.



Figures 7 (A-C): Box plot of the pleiotrophic effect of the *int-b* locus. A)The average number of tillers per spike phenotype characterized as two-rowed (B) or intermedium (C) in a subpopulation, consisting 59 individuals of the cross between Proctor and *Int-b* (Bonus). There were 5 plants which could not be scored (N/A), 43 as two-rowed and 11 plants were scored as intermedium.



The marker 4771-380 from BOPA1 at 110.26 cM (Close at al., 2009) with the primer pair R23 and R24 was known to cosegregate with the phenotype. Based on the recombination frequency, the *int-b* locus is located about 8 cM from the 4771-380 marker. To validate this, more markers were tested to find the flanking markers of *int-b.* SNP data was not present, so primers were designed on potential candidate genes in the region. The PCR products of these genes were Sanger sequenced and only 3 showed SNP's between the parental lines and 1 SNP could be used for a polymorphic marker using the primers A21 and A22. Also 20 SSR markers were tested, which were selected based on the paper of Varshney *et al.* (2007) at chromosome 5HL. Out of the 29 tested SSR markers located in the introgression, only 7 were polymorphic between the lines. Two SSR markers were used to genotype the population, GBM1227 and Scssr10148. Primer sequences can be found in the Supplementary, Table 5. The interval of the new introgression was mapped to a region of 4 cM (Fig. 8). The flanking markers were R23/R24 and GBM1227.

Figure 8: Molecular map position of *int-b* on Barley chromosome 5HL. Molecular markers are shown on the right. All distances are expressed in centimorgans on the left (*Linkage analysis and map making was done with Jointmap)*.

Bulk segregant analysis shows a peak at 97.4 cM

For the molecular mapping there was a strong need for polymorphic markers between Proctor and *int-b*, because of the low number of SNPs found with Sanger sequencing. Therefore, a RNAseq Bulk Segregant Analysis (RNAseq-BSA) was carried out to find SNPs between the parental lines and to possibly limit the introgression. From our F2 mapping population leaf samples were harvested and afterwards bulked according to high or low tillering phenotype. RNA was extracted and sent for sequencing on an Illumina Hiseq lane. The results showed that there was a peak on chromosome 5HL, with the gene with the highest bulk frequency located on 97.35 cM, which confirmed the genetic mapping (Fig. 9B). The flanking markers of the peak were located at 96.59 cM and 98.54 cM. A number of 27 genes contained high quality SNPs. A number of 5 genes on chromosome 0, had a higher bulk frequency ratio as 5, and were considered to be located in the introgression. However, these genes were not considered as candidate genes (Supplementary, Table 6)

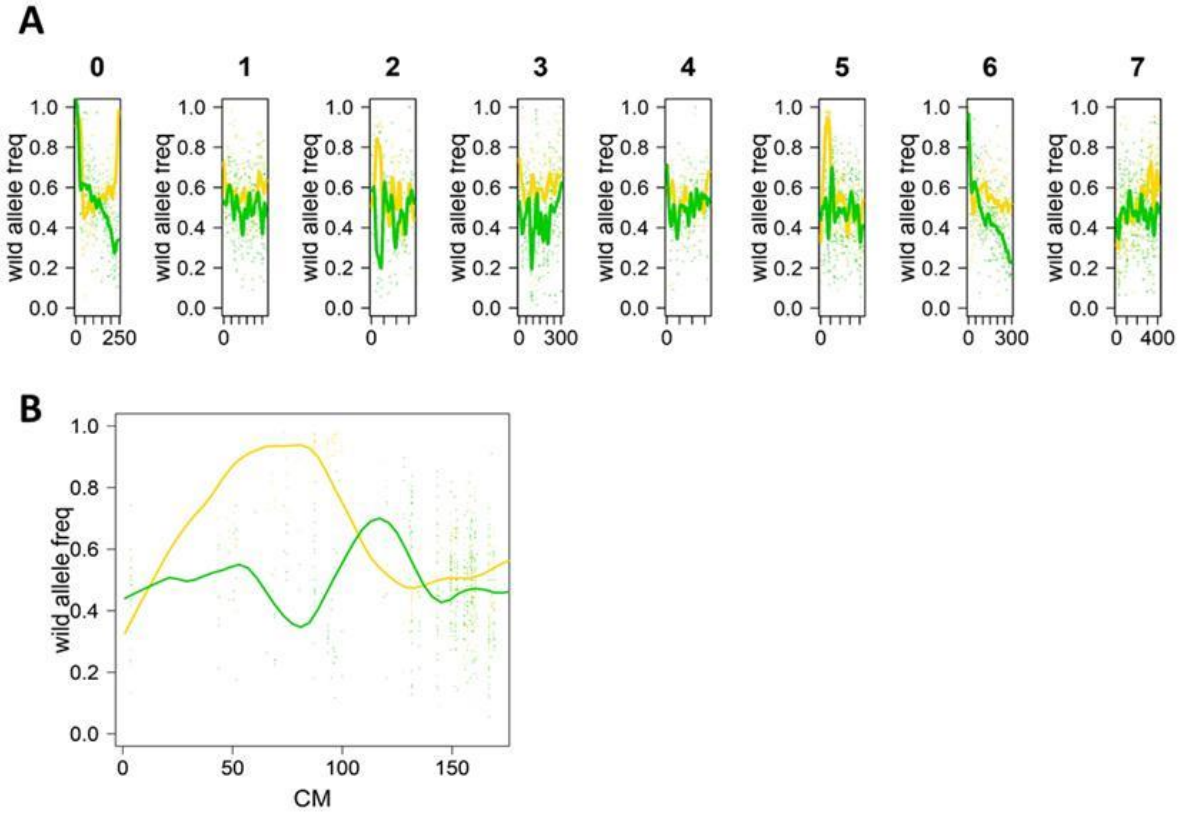


Figure 9 (A, B): Results of the BSR-seq analysis. A) Allele frequencies were plotted for wild type (green) and mutant (yellow) for each chromosome, including the non-mapped genes (Chr. 0). B) The highest peak was found on chromosome 5. Based on the bulk frequency ratios on chromosome 5, the *int-b* locus is located around 97 cM.

Candidate genes for *int-b*

The flanking markers of the gene with the higher BFR limited the introgression to a length of 1.95 cM and contains 108 high and low confidence genes based on the POPseq map (Masher et al., 2013). The information of the BSA-RNAseq was combined with the RNAseq data of the crown tissue to compare the SNPs in *int-b* with Bonus, Proctor and Bowman, but no common SNP could be found for the genes in the introgression. This may suggest that the gene is not expressed at all in the mutants, which is expected in an X-irradiated mutant. In the BSA-RNAseq, genes were found in the introgression that had no reads in the mutant comparing with wild type, but the expression was not seen to be different in the expression data (Table 2). Although an obvious candidate could not be

found based on SNP and 'no reads' data, a number of candidate genes have been selected that were significantly downregulated, or showed 'no reads' in the mutant from the BSA-RNAseq.

Table 2: Candidate genes in the introgression. Expression data of 3 allelic variants of *int-b* revealed that two genes were significantly downregulated. In the BSA-RNAseq experiment between Proctor and *int-b*, 'no reads' were found in four genes. (*****) indicates that the gene was significantly downregulated in one mutant, while (**) in two mutants.

Name	Chr.	Position	Reads BSA-RNAseq	Expression RNAseq	Annotation
MLOC_68487.1	5	97.3	-	down*	Lateral root primordium (LRP) protein-like protein
MLOC_9390.1	5	98.5	-	down**	unknown
MLOC_6696.1	5	97.3	no reads	Not diff.	MYB domain protein DNA-binding transcriptional activator
MLOC_38969.11	5	98.1	no reads	Not diff.	Alpha/beta hydrolase family
MLOC_52297.1	5	98.1	no reads	Not diff.	unknown
MLOC_70099.11	5	96.6	no reads	Not diff.	Unknown

In table 2 the selected candidate genes are listed. Based on the expression data and the BSA RNAseq, six genes were selected that were strongly downregulated or showed no reads in the mutant. The function of three of these candidate genes was not known, based on the IPK Barley BLAST Server, using ViroBLAST (Deng et al., 2007). The other genes are annotated as a Lateral root primordium (LRP) protein-like protein, a MYB domain protein and an Alpha/beta hydrolase family. LRP proteins are important for root branching and therefore the uptake of water, nutrients and anchorage by plants (Péret, 2009). MYB proteins are known for transcriptional activators and this gene is therefore an interesting candidate. The Alpha/beta hydrolase family contains enzymes with diverse catalytic functions and is earlier found to have an function in the signal transduction in the strigolactone pathway to the bud (Beveridge, 2010).

Discussion

The aim of the project was to acquire a detailed morphological representation of allelic variants of *int-b*, to perform a molecular mapping to locate the genetic position and combine this information with expression data to identify the gene at the *int-b* locus. We found that the reduced tillering in *int-b* mutant is mainly due to a delay in tiller bud outgrowth in the first leaf axil.

The morphological analysis of the allelic variants *int-b3* and *int-b75* showed variation in phenotype between the variants and interaction with their background. The variation may arise of the nature of the mutation in the gene, as seen in the *int-c* mutant (Ramsay, 2011). Additional background mutations, aroused during the chemical mutagenesis can also influence the expressivity and penetrance of the *int-b* phenotype, which was observed between *int-b3* in Bowman and *int-b3* in Bonus. Nevertheless, the conclusions presented here are based on similar observations noticed in the two allelic variants.

In our experiment, *int-b* exhibited a lower tiller number and showed an intermedium spike phenotype, as reported before by Babb and Muehlbauer (2003). It is known that tiller development is regulated by a complex network of hormonal, genetic and environmental factors. Genes involved in different pathways have been identified that either influence the bud initiation or bud outgrowth (Kebrom et al.,

2013). Plant dissection revealed that in *int-b* mutants the bud outgrowth in the first leaf axil was delayed or did not grow out at all, when compared to wild type. After a delay of two weeks, about half of the AB's in *int-b* under the first leaf grew out, whereas in wild type all the AB's developed directly in mature tillers. These results suggest that *int-b* is important for bud outgrowth at the early vegetative stage of the barley plant. Besides, fewer buds were counted in the higher leaf axils of *int-b* mutants, suggesting that *int-b* affects directly or indirectly bud initiation. There are other barley mutants known which exhibit a similar phenotype. The barley *als* mutant exhibits fewer tillers and an irregular spike phenotype (Dabbert, 2009). Their genetic relationship was tested in a double mutant combination were the double mutant plants had a lower number of tillers than each parent. This suggests that *int-b* is involved in a different pathway that controls tillering. A synergistic interaction was also seen in double mutant combinations of *int-b* with *lnt1* and *cul2* (Babb and Muehlbauer, 2003). In the latter one, there were no spikelets formed at all, which means that both products are needed to form fertile spikelets. Genetic interactions show that *int-b* more genes regulate the early steps of AB development but acting in different pathways.

At the same time that the bud outgrowth was delayed, a faster development of the SAM was observed in *int-b* mutants when compared with wild type. After two weeks, the SAM of both mutant variants developed faster, irrespective of their Waddington stage. It is known that a faster SAM development can be the result of a lower tiller number, due to competition among tillers for minerals, carbon and water resources and hormones which can led to a higher nutrient allocation towards the main stem and improved shoot growth , which was shown in wheat (Gu and Marshall, 1988). In barley similar results were observed when tillers were removed and led to a bigger main shoot which contained an ear with more and heavier grains (Kirby and Jones, 1977). As the bud outgrowth is delayed in the mutant, more nutrients can be transported to the SAM, giving rise to a faster development. If the higher nutrient allocation to the SAM is caused by the SAM, it would mean that the SAM of *int-b* mutants require more nutrients after two weeks. This can be due to the fact that lateral spikelets grow out in *int-b* and therefore the SAM requires more nutrients. It is known that six-rowed varieties have a lower tillering capacity (Kirby et al., 1978). However, it is also known that a faster SAM development (early flowering) can itself reduce tillering (Gomez-Macpherson et al., 1998). Bud outgrowth is known to be controlled by signals from the SAM, known as apical dominance. This is done by the basal transport of auxin and by the intense demand for sucrose by the SAM (Mason et al., 2014). Auxin is known to inhibit the production of cytokinin, which exhibits lateral shoot outgrowth. In addition, the intense demand for sugars of the SAM decreases the bud outgrowth. Also, earlier internode elongation can act as a sink that diverts sugar away from the tillers, thereby preventing the development of tillers (Kebrom et al., 2012). But early flowering of *int-b* mutants was not seen in a field experiment and in our experiments the plants did not flower in winter, yet this needs to be confirmed by repeating the experiment during summer in the greenhouse. It suggests that SAM development is only faster at this specific time-point, and afterwards develops the same as wild type. Hence, it is likely that the delay in bud outgrowth and the faster SAM development have a relation towards each other. To determine what is directly affected by *int-b*, we need to know the causal factor which will hopefully be revealed by more detailed dissection experiments.

The introgression of the barley mutant *vrs2e* has an overlap with *int-b* and a lower number of tillers, therefore it was expected that it was another allelic variant of *int-b*. Morphological analysis of the two mutants showed observable differences in tiller number and spike phenotype. Genetic analysis revealed that the cross was not succeeded and so there was no evidence that they are allelic. Crossing of barley genotypes has proven to be difficult in winter. However, Lundqvist (1992) describes that *vrs2e* is not allelic to any of the *int* loci, suggesting that *vrs2e* and *int-b* are not allelic.

To see which genes where differentially regulated in *int-b* mutants, a RNAseq experiment was carried out on SAM's at Waddington stage 0. Conserved genes with a known function in a tillering pathway were checked upon their expression. The barley ortholog of *TB1*, *INT-C*, was downregulated in the *int-b* mutants, which might be not expected because of the known inhibiting function of TB1 on bud

outgrowth. However, it is known that *int-c* interacts with vrs1 resulting in a six-rowed inflorescence (Ramsay, 2011). Mutant alleles of *int-c* resulted in partially developed lateral spikelets and showed a higher tillering phenotype at early developmental stage, but few tillers were seen at later developmental stage (Liller, unpublished). The downregulation of *TB1* in *int-b* can explain the partially developed lateral spikelets, but would suggest that a different pathway would regulate the bud outgrowth. Recently, the class *more axillary branching* (*max*) mutants in *Arabidopsis thaliana* shows that in a different tillering pathway an undefined hormone inhibits branching and is transported upwardly as a mobile signal (Bennett, 2006; Zou, 2006). In our data, an ortholog of *MAX1* in barley is upregulated in *int-b* mutants. *MAX1* encodes a cytochrome P450 family member that acts downstream of *MAX3/4* to produce a branch-inhibiting hormone, so it is expected to be upregulated. In our data *MAX3/4* expression was not differentially regulated, suggesting that *int-b* might be another inhibiting factor which is acting upstream of *MAX1*. If *int-b* is acting in this pathway, it is difficult to detect the expression because the signal is mobile: it is produced in the roots and transported upwardly which means that in our RNAseq experiment the expression of the gene encoding *int-b* could not be detected. From the dissection experiment we can conclude that *int-b* has an effect after two weeks, which could be valuable information for further RNAseq experiments. In addition, if *int-b* is a mobile signal, it can be useful to perform an expression analysis in the roots.

Molecular analysis of the F2 population showed that the region of *int-b* is located between 95.9 and 99.9 cM on chromosome 5HL. The low amount of SNP's in the region of the introgression may indicate that the locus is conserved in barley cultivars and has been important during barley domestication (Pourkheirandish, 2007). BSA RNAseq analysis confirmed the mapping with a peak at 97.4 cM. With the information of the BSA RNAseq analysis, it will be more easy to find polymorphic markers to fine map the *int-b* locus. The syntenic region of our introgression in rice did not contain already identified tillering genes (Hussien, 2013). Expression data and SNP data was used to screen the region for candidate genes. *Int-b* is a neutron-induced mutant and this treatment often causes deletions. Therefore it is likely that *int-b* is an deletion mutant and strongly downregulated. Analysis of expression data of the SAM did not result in an obvious downregulated candidate gene. This can be an indication that the gene is active in specific tissues, which is also found for *TB1* (Hubbard, 2002). SNP data of RNAseq was compared between *int-b* and Bowman, Bonus and Proctor, and did not result in a common SNP. This might indicate that the gene is not expressed in these tissues or that the promoter region outside the open reading frame is affected. Integration of the data derived from RNAseq of the SAM and of the BSA-RNAseq, candidate genes in the introgression were selected based on function, expression and genes with no reads in *int-b* in the BSA-RNAseq experiment. The most interesting candidate is an MYB domain protein, which exhibits no reads in the mutant in the BSA-RNAseq. Recently, the MYB protein ANTHER INDEHISCENCE1 was found to be involved in anther development in rice. The recessive mutant has partial filled spikelets and fewer tillers and no expression was seen in the leaves (Zhu, 2004). To check if this is indeed the mutated gene, primers can be designed and Sanger sequencing of the PCR product will reveal if the coding sequence is affected in the mutant.

References

Aguilar-Martínez, J. A., Poza-Carrión, C., & Cubas, P. (2007). Arabidopsis BRANCHED1 acts as an integrator of branching signals within axillary buds. *The Plant Cell Online, 19(2), 458-472.*

Agusti, J., & Greb, T. (2013). Going with the wind–adaptive dynamics of plant secondary meristems. *Mechanisms of development, 130(1), 34-44.*

Babb, S., & Muehlbauer, G. (2003). Genetic and morphological characterization of the barley uniculm2 (cul2) mutant. *Theoretical and Applied Genetics, 106(5), 846-857.*

Bennett, T., Sieberer, T., Willett, B., Booker, J., Luschnig, C., & Leyser, O. (2006). The Arabidopsis MAX pathway controls shoot branching by regulating auxin transport. Current Biology, 16(6), 553-563.
Bossinger, G., Lundqvist, U., Rohde, W., & Salamini, F. (1992). *Genetics of plant development in barley. Barley Genetics IGBS VI (MUNCK, L., ED), 2, 989-1022.*

Beveridge, C. A., & Kyozuka, J. (2010). New genes in the strigolactone-related shoot branching pathway. *Current opinion in plant biology*, *13*(1), 34-39.

Bolle C (2004). The role of GRAS proteins in plant signal transduction and development. *Planta 218:683-692.*

Brewer, P. B., Dun, E. A., Ferguson, B. J., Rameau, C., & Beveridge, C. A. (2009). strigolactone acts downstream of auxin to regulate bud outgrowth in pea and Arabidopsis. *Plant Physiology, 150(1), 482-493.*

Close, T. J., Bhat, P. R., Lonardi, S., Wu, Y., Rostoks, N., Ramsay, L., ... & Waugh, R. (2009). Development and implementation of high-throughput SNP genotyping in barley. *BMC genomics, 10(1), 582.*

Dabbert, T., Okagaki, R. J., Cho, S., Heinen, S., Boddu, J., & Muehlbauer, G. J. (2010). The genetics of barley low-tillering mutants: low number of tillers-1 (lnt1). *Theoretical and applied genetics, 121(4), 705-715.*

Dabbert, T., Okagaki, R. J., Cho, S., Boddu, J., & Muehlbauer, G. J. (2009). The genetics of barley low-tillering mutants: absent lower laterals (als). *Theoretical and applied genetics, 118(7), 1351-1360.*

Deng W, Nickle DC, Learn GH, Maust B, and Mullins JI. (2007). ViroBLAST: A stand-alone BLAST web server for flexible queries of multiple databases and user's datasets. *Bioinformatics 23(17):2334-2336.*

Druka, Franckowiak, Lundqvist, Bonar, Alexander, Houston, Radovic, Shahinnia, Vendramin, Morgante, Stein, Waugh (2011). Genetic Dissection of Barley Morphology and Development. *Plant Physiology, Vol. 155: 617–627*

FAOSTAT (2013) http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QC/E

Franckowiak, Lundqvist, Konishi (1997). New revised names for barley genes. *Barley Genetics Newsletter 26: 4*

Fukuyama, T., J. Hayashi, I. Moriya, and R. Takahashi (1972). A test for allelism of 32 induced six-rowed mutants. *BGN 2:25-27.*

Gomez-Macpherson, H., Richards, R. A., & Masle, J. (1998). Growth of near-isogenic wheat lines differing in development—plants in a simulated canopy. *Annals of Botany*, *82*(3), 323-330.

Greb, Clarenz, Schäfer, Müller, Herrero, Schmitz, Theres (2003). Molecular analysis of the

LATERAL SUPPRESSOR gene in Arabidopsis reveals a conserved control mechanism for axillary meristem formation. *Genes Dev 17: 1175-1187*

Gu J, Marshall C (1988). The effect of tiller removal and tiller defoliation on competition between the main shoot and tillers of spring barley. *Annals of Applied Biology 112, 597-608.*

Gustafsson and Lundqvist (1980). Hexastichon and intermedium mutants in barley. *Hereditas 92: 229-236*

Hussien, Tavakol, Horner, Muñoz-Amatriaín, Muehlbauer, Rossini (2014). Genetics of Tillering in Rice and Barley. *The Plant Genome, Vol. 7, No. 1*

Hubbard, L., McSteen, P., Doebley, J., & Hake, S. (2002). Expression patterns and mutant phenotype of teosinte branched1 correlate with growth suppression in maize and teosinte. *Genetics, 162(4), 1927-1935.*

Kebrom, T. H., Burson, B. L., & Finlayson, S. A. (2006). Phytochrome B represses Teosinte Branched1 expression and induces sorghum axillary bud outgrowth in response to light signals. *Plant Physiology, 140(3), 1109-1117.*

Kebrom, Spielmeyer, Finnegan (2013). Grasses provide new insights into regulation of shoot branching. *Trends in Plant Science, Vol. 18, No. 1: 41 – 48.*

Keller, T., Abbott, J., Moritz, T., & Doerner, P. (2006). Arabidopsis REGULATOR OF AXILLARY MERISTEMS1 controls a leaf axil stem cell niche and modulates vegetative development. *The Plant Cell Online, 18(3), 598-611.*

Kirby, E. J. M., & Riggs, T. J. (1978). Developmental consequences of two-row and six-row ear type in spring barley: 2. Shoot apex, leaf and tiller development. *The Journal of Agricultural Science*, *91*(01), 207-216.

Kirby and Appleyard (1987). Cereal development guide. *2nd ed. Arable Unit, Stoneleigh, Warwickshire.*

Komatsuda, Pourkheirandish, He, Azhaguvel, Kanamori, Perovic, Stein,Graner, Wicker, Tagiri, Lundqvist, Fujimura, Matsuoka, Matsumoto, Yano (2007). Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. *PNAS vol. 104 no. 4: 1424–1429*

Koppolua, Anwarb, Sakumab, Tagirib, Lundqvistc, Pourkheirandishb, Ruttend, Seilere, Himmelbacha, Ariyadasaa, Youssefa, Stein, Sreenivasulue, Komatsudab, Schnurbusch (2013). Six-rowed spike4 (Vrs4) controls spikelet determinacy and row- type in barley. *PNAS vol. 110, no. 3: 213198–13203*

Li, X., Qian, Q., Fu, Z., Wang, Y., Xiong, G., Zeng, D., ... & Li, J. (2003). Control of tillering in rice. *Nature, 422(6932), 618-621.*

Lundqvist, Franckowiak, Konishi (1997). New and revised descriptions of barley genes. *Barley Genet Newslett 26: 22-43*

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

Lundqvist, U. (2014). Scandinavian mutation research in barley–a historical review. *Hereditas.*

Mascher, M., & Stein, N. (2014). Genetic anchoring of whole-genome shotgun assemblies. *Frontiers in genetics, 5.*

Mason, M. G., Ross, J. J., Babst, B. A., Wienclaw, B. N., & Beveridge, C. A. (2014). Sugar demand, not auxin, is the initial regulator of apical dominance. *Proceedings of the National Academy of Sciences, 111(16), 6092-6097.*

McConnell, J. R., & Barton, M. K. (1995). Effect of mutations in the PINHEAD gene of Arabidopsis on the formation of shoot apical meristems. *Developmental Genetics, 16(4), 358-366.*

Müller, J., Wang, Y., Franzen, R., Santi, L., Salamini, F., & Rohde, W. (2001). In vitro interactions between barley TALE homeodomain proteins suggest a role for protein–protein associations in the regulation of Knox gene function. *The Plant Journal, 27(1), 13-23.*

Müller, D., Schmitz, G., & Theres, K. (2006). Blind homologous R2R3 Myb genes control the pattern of lateral meristem initiation in Arabidopsis. *The Plant Cell Online*, *18*(3), 586-597.

Ogilvie, H. A., Imin, N., & Djordjevic, M. A. (2014). Diversification of the C-TERMINALLY ENCODED PEPTIDE (CEP) gene family in angiosperms, and evolution of plant-family specific CEP genes*. BMC genomics, 15(1), 870*.

Oikawa, T., & Kyozuka, J. (2009). Two-step regulation of LAX PANICLE1 protein accumulation in axillary meristem formation in rice. *The Plant Cell Online, 21(4), 1095-1108.*

Pandey, D. K., & Chaudhary, B. (2014). Oxidative Stress Responsive SERK1 Gene Directs the Progression of Somatic Embryogenesis in Cotton (Gossypium hirsutum L. cv. Coker 310). *American Journal of Plant Sciences, 2014.*

Péret, B., De Rybel, B., Casimiro, I., Benková, E., Swarup, R., Laplaze, L., ... & Bennett, M. J. (2009). Arabidopsis lateral root development: an emerging story. *Trends in plant science*, *14*(7), 399-408.

Pourkheirandish, Wicker, Stein, Fujimura, Komatsuda (2007). Analysis of the barley chromosome 2 region containing the six-rowed spike gene vrs1 reveals a breakdown of rice–barley micro collinearity by a transposition. *Theor Appl Genet 114: 1357–1365.*

Raman, S., Greb, T., Peaucelle, A., Blein, T., Laufs, P., & Theres, K. (2008). Interplay of miR164, CUP‐SHAPED COTYLEDON genes and LATERAL SUPPRESSOR controls axillary meristem formation in Arabidopsis thaliana. *The Plant Journal, 55(1), 65-76.*

Ramsay, Comadran, Druka, Marshall, Thomas, Macaulay, MacKenzie, Simpson, Fuller, Bonar, Hayes, Lundqvist, Franckowiak, Close, Muehlbauer, Waugh (2011). INTERMEDIUM-C, a modifier of lateral spikelet fertility in barley, is an ortholog of the maize domestication gene TEOSINTE BRANCHED 1. *Nature Genetics 43: 169–172*

Sakamoto and Matsuoka (2004) Generating high-yielding varieties by genetic manipulation of plant architecture. *Curr. Opin. Biotechnol. 15(2): 144-147*

Schmitz and Theres (2005) Shoot and inflorescence branching. *Curr. Opin. Plant Biol. 8(5): 506-511.*

Schmitz, G., Tillmann, E., Carriero, F., Fiore, C., Cellini, F. and Theres, K. (2002). The tomato Blind gene encodes a MYB transcription factor that controls the formation of lateral meristems. *Proc. Natl. Acad. Sci. USA, 99, 1064–1069.*

Schumacher, Schmitt, Rossberg, Schmitz Theres (1999) The Lateral suppressor (Ls) gene of tomato encodes a newmember of the VHIID protein family. *Proc Natl Acad Sci USA 96: 290-295.*

Stirnberg P, van De Sande K, Leyser (2002) . MAX1 and MAX2 control shoot lateral branching in Arabidopsis. *Development 129:1131-1141.*

Takeda, Suwa, Suzuki, Kitano, Ueguchi-Tanaka, Ashikari, Matsuoka Ueguchi (2003) The OsTB1 gene negatively regulates lateral branching in rice. Plant J 33: 513-520

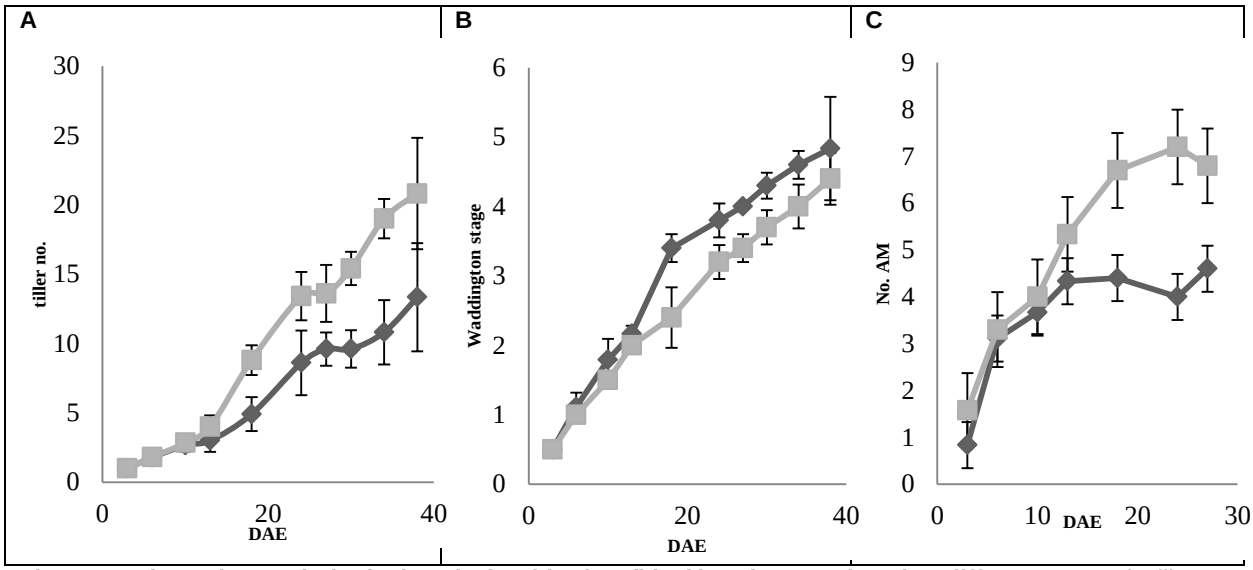
Varshney, R. K., Marcel, T. C., Ramsay, L., Russell, J., Röder, M. S., Stein, N., ... & Graner, A. (2007). A high density barley microsatellite consensus map with 775 SSR loci. *Theoretical and Applied Genetics, 114(6), 1091-1103.*

Whipple, C. J., Kebrom, T. H., Weber, A. L., Yang, F., Hall, D., Meeley, R., ... & Jackson, D. P. (2011). grassy tillers1 promotes apical dominance in maize and responds to shade signals in the grasses. *Proceedings of the National Academy of Sciences, 108(33), E506-E512.*

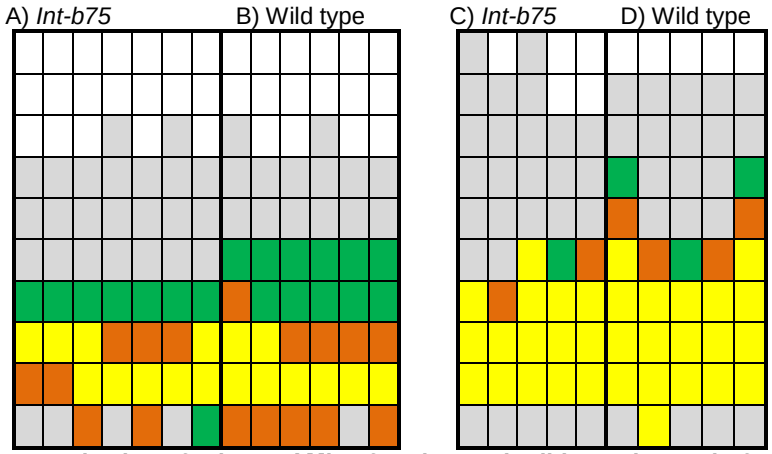
Zhu, Q. H., Ramm, K., Shivakkumar, R., Dennis, E. S., & Upadhyaya, N. M. (2004). The ANTHER INDEHISCENCE1 gene encoding a single MYB domain protein is involved in anther development in rice. *Plant physiology, 135(3), 1514-1525.*

Zou, J., Zhang, S., Zhang, W., Li, G., Chen, Z., Zhai, W., ... & Zhu, L. (2006). The rice HIGH‐TILLERING DWARF1 encoding an ortholog of Arabidopsis MAX3 is required for negative regulation of the outgrowth of axillary buds. *The Plant Journal, 48(5), 687-698.*

Supplementary



Figures 10 (A,B,C): Morphological analysis of *int-b75* (black) and Bonus (grey) at different DAE. A) Tiller number, B) SAM development, staged according to the Waddington scale (Waddington *et al.,* 1983), C) Number of primary apical meristems. Each time-point 5 plants for each genotype was used. Errors bars represent standard deviation.



Figures 11 (A-D): Characterization of primary AM's of *Int-b75* and Wild type (Bonus) after 16 DAE (A,B) and after 30 DAE (C,D). Each column represents a single individual, with each square within a column

representing an individual leaf axil, starting with the coleoptile. Green denotes an AB, brown an outgrowing bud and yellow a tiller. Grey denotes the absence of an AM.

Table 3: Averages of primary, secondary and tertiary meristems counted on Waddington stage 3.5 in *Int-b75* and wild type (Bonus). Average values with standard deviation are shown (n>5 plants).

	Primary AM	Secondary AM	Tertairy AM
Wild type	6.8 ±1.2	15.2±2.3	9.2±3.5
Int-b75	3.6±0.8	5.2±2.2	4.4±2.1

Table 4. Significantly differentially regulated genes in all three *int-b* mutants.

Function	MLOC	Chromosome	Location	Expression
FAD-binding Berberine family protein	MLOC_77132.1	0	0	down
Glucan endo-1,3-beta-glucosidase, putative	MLOC_10669.1	1	129.7	up
Protein kinase	AK363102	3	14.9	up
Protein kinase family protein	MLOC_11098.1	5	75.9	up
Beta-1,3-glucanase	AK248896.1	3	135.5	up
Beta-1,3-glucanase	AK359184	#N/B	#N/B	up
Receptor protein kinase-like protein	MLOC_13737.2	1	3.2	up
Protein of unknown function	MLOC_14009.1	3	52.0	up
Glucan endo-1,3-beta-glucosidase, putative	MLOC_81871.1	1	128.3	up

Table 5: List of used molecular markers with their position on the Popseq map in cM (with use of the IPK Barley Blast Service).

Marker	Fw	Sequence	Rv	Sequence	Position
A2122	A21	TGAGGGCCGGTATATGACAC	A22	CAGAAGCCAAGCCGAATACG	88.5
R2324	R23	ATCTCTGGCCTGATCTCACG	R24	ATAGCTCTCCGAACCACAGC	95.9
GBM1227	Fw	GGTCATCATACATACGCTGCTG	Rv	GGGTGGTGTAGGAGGAGGAT	99.9
Scssr10148	Fw	AAGCAGCAAAGCAAAGTACC	Rv	TCATCAGCATCTGATCATCC	130.7

Table 6: Unmapped genes with a bulk frequency ratio higher as 5 in the BSA-RNAseq.

Annotation	position	location	SNP/del/noreads Proc	function
MLOC_16969.1	0	0	SNP	26S protease regulatory subunit 7
MLOC_58564.1	0	0	SNP	Armadillo repeat only 1 protein
AK359352	0	0	SNP	DAG protein
AK355067	0	0	SNP	hydroxyproline-rich glycoprotein family protein LENGTH=623
AK362112	0	0	SNP	Pyruvate dehydrogenase E1 component subunit beta