

RESEARCH PAPER

INTERMEDIUM-C mediates the shade-induced bud growth arrest in barley

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Abstract

Tiller formation is a key agronomic determinant for grain yield in cereal crops. The modulation of this trait is controlled by transcriptional regulators and plant hormones, tightly regulated by external environmental conditions. While endogenous (genetic) and exogenous (environmental factors) triggers for tiller formation have mostly been investigated separately, it has remained elusive how they are integrated into the developmental program of this trait. The transcription factor gene *INTERMEDIUM-C* (*INT-C*), which is the barley ortholog of the maize domestication gene *TEOSINTE BRANCHED1* (*TB1*), has a prominent role in regulating tiller bud outgrowth. Here we show that *INT-C* is expressed in tiller buds, required for bud growth arrest in response to shade. In contrast to wild-type plants, *int-c* mutant plants are impaired in their shade response and do not stop tiller production after shading. Gene expression levels of *INT-C* are up-regulated under light-limiting growth conditions, and down-regulated after decapitation. Transcriptome analysis of wild-type and *int-c* buds under control and shading conditions identified target genes of *INT-C* that belong to auxin and gibberellin biosynthesis and signaling pathways. Our study identifies *INT-C* as an integrator of the shade response into tiller formation, which is prerequisite for implementing shading responses in the breeding of cereal crops.

Keywords: Abscisic acid, barley, bud growth arrest, decapitation, *INTERMEDIUM-C*, shade avoidance, yield.

Introduction

Ensuring yield stability of cereal crops is a major requirement for plant breeding in the face of climate change (Kang *et al.*, 2009). In particular, breeding of new elite varieties with optimized shoot architecture, such as tiller number, will allow the maintenance of high yield potential in unfavorable environments.

The effect of genetic factors on this trait was described recently (Haaning *et al.*, 2020). In many countries, particularly at very high latitudes or on shaded slopes, light is a limiting factor affecting crop growth and yield. Moreover, during the past decades, intensive crop management has increased sowing

and plant stand densities to improve homogeneity among individual plants that now produce fewer tillers. To increase light capture, plants have evolved refined mechanisms to maximize light harvesting and/or to prevent shade, namely low light intensity and/or a low ratio of red (R) to far-red (FR) light. Suboptimal light triggers a suite of phenotypic changes, defined as the shade avoidance response (SAR), that includes hypocotyl and petiole elongation, an upward orientation of leaves, and early flowering. Additionally, a common characteristic of SAR is the suppression of shoot branching in a wide variety of species (Smith and Jordan, 1994; Tucic *et al.*, 2006; Aguilar-Martinez *et al.*, 2007; González-Grandio *et al.*, 2013).

The extensive work carried out in Arabidopsis has provided a detailed understanding of the SAR (Wang and Wang, 2015). By sensing and responding to R and FR light, the five photoreceptors of the phytochrome family (phyA–phyE) regulate a variety of developmental processes (Franklin and Quail, 2010). Among these, phytochrome B (phyB) appears to be the most important photoreceptor involved in shade detection and avoidance (Reed *et al.*, 1993; Ballaré, 1999), functioning redundantly with other members of its clade (Stamm and Kumar, 2010). Phytochrome proteins act as dimers and exist in two photoconvertible forms: ‘Pr’ (the red-light-absorbing, inactive form) and ‘Pfr’ (the far-red-light-absorbing, active form), with the Pfr:Pr ratio reflecting the R:FR ratio of the environment (Smith, 2000). Upon photoconversion into active Pfr, phytochromes migrate to the nucleus, where they regulate gene expression by interacting with several basic helix–loop–helix (bHLH) transcription factors, including phytochrome-interacting factor (PIF) and PIF-like (PIL) proteins (Chen *et al.*, 2004; Duek and Fankhauser, 2005). In many plant species, genome-wide transcriptional dynamics of the SAR have been studied in petioles or leaf blades at the seedling stage, while SAR is less defined in economically important monocots (Devlin *et al.*, 2003; Tao *et al.*, 2008; Hornitschek *et al.*, 2012; Wang *et al.*, 2016).

In the agricultural production of graminaceous crop species, plant density is a major determinant for crop yield. Shading caused by elevated plant densities reduces not only photosynthetic active radiation (PAR) flux density but also the R/FR ratio of the light reaching the lower strata of the canopy. Generally, increasing plant density results in progressively stronger suppression of tillering due to accelerated apical shoot development and stem elongation. This pattern continues until the beginning of the stem elongation phase (Zadoks *et al.*, 1974). Moreover, early-emerging tillers contribute more to grain yield than do tillers that emerge later. The regulation of barley tiller outgrowth by shade has also been supported by the observation that supplemental FR illumination of elongating leaves or of the main stem base reduced the total number of tillers per plant (Skinner and Simmons, 1993). In spite of its great ecological and economic impact, little is known about the underlying molecular mechanisms linking shade-initiated transcriptional changes with the suppression of tiller outgrowth in barley plants, especially regarding the tiller bud that is one of the most important sites of shade action.

Lateral shoot growth is coordinately controlled by conserved interactions that regulate the biosynthesis and signaling of the hormones auxin, abscisic acid (ABA), strigolactones (SLs), and cytokinins (CKs). Auxin and SLs, synthesized mainly in the shoot apex and root, respectively, inhibit branching, while CKs, synthesized mostly in the root and stem, promote branching (Kebrom *et al.*, 2013). The class II TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) transcription factors TEOSINTE BRANCHED 1 (TB1)-like, in monocots, and BRANCHED1 (BRC1)-like, in dicots, act locally inside the axillary bud where they are subject to transcriptional regulation by hormonal crosstalk (auxin–SL–CK) and cause bud growth arrest (Doebley *et al.*, 1997; Aguilar-Martinez *et al.*, 2007; Minakuchi *et al.*, 2010; Martin-Trillo *et al.*, 2011; Braun *et al.*, 2012). Moreover, recent studies have implicated the involvement of TB1 orthologs in the shade-induced response of branch suppression. In *Sorghum bicolor* and maize, active phyB (Pfr) suppresses the expression of the TB1 gene and induces bud outgrowth. On the other hand, light signals that inactivate phyB allow increased expression of TB1 and suppression of bud outgrowth (Kebrom *et al.*, 2006, 2010; Whipple *et al.*, 2011). In Arabidopsis, BRC1 is up-regulated in axillary buds of plants grown at high density and is required for complete branch suppression in these conditions (Aguilar-Martinez *et al.*, 2007). These results suggest that the phytochrome pathway is involved in the control of TB1 and axillary meristem outgrowth, and provides a link between environmental variation and gene action controlling branching (González-Grandio *et al.*, 2013; Reddy *et al.*, 2013). In barley, INT-C is an ortholog of the maize domestication gene TB1, and its mutants exhibit higher tiller numbers and enlarged lateral spikelets (Ramsay *et al.*, 2011).

Here, we investigated the SAR of early formed tiller buds in barley plants before the rapid stem elongation stage, and studied the role of INT-C during the SAR by comparable transcriptome analysis. The analysis revealed that the dynamics of INT-C expression during the SAR are critical for genome-wide reprogramming of gene expression and that the gene categories affected support a central role for INT-C in tiller bud arrest. A comparison of genes responding to shade-induced bud arrest and decapitation-triggered bud activation allowed us to identify key regulators influencing bud dormancy and bud activation genes. Thus, these findings would enable us to better understand the genetic mechanisms controlling the reversible transition of growth to dormancy in barley tiller buds.

Materials and methods

Plant material and growth conditions

Hordeum vulgare cv. Bowman, a two-rowed spring barley cultivar, was used as the wild type for comparison with its near isogenic mutant BW421 (*int-c.5*) (Ramsay *et al.*, 2011). For the experimental analyses except the shading experiment, wild-type and *int-c* mutant plants were cultivated in a greenhouse. Seeds were sown in either 54- or 96-well trays and germinated in a climate chamber for 10 d at 11 °C day and 7 °C night temperature under 16 h light. After that, seedlings were transferred to pots (diameter 16 cm) filled with two parts of compost, two parts of ‘Substrat

2' (Klasmann), and one part of quartz sand, and allowed to grow in the greenhouse at 20 °C day/14 °C night under 16 h light. For the density experiments, three barley planting densities (one, five, and 10 plants per pot) were established in pots (size, 21 liter; top diameter, 310 cm; base diameter, 250 cm; height, 214 cm), and the planting density test was repeated four times. For the decapitation experiments, when plants were grown until the early stem elongation stage, the shoot apices were removed, and the apical dominance test was repeated three times. For the shading experiments, seedlings were grown in a climate-controlled growth chamber at a temperature of 12 °C, 70% humidity, and a 12/12 h day/night cycle. Green shading was achieved by using a green plastic filter (122 Fern Green; LEE Filters, Andover, UK) (Kegge *et al.*, 2013).

Sequence retrieval for TCP proteins

In order to identify barley genes putatively encoding TCP transcription factors, the latest barley annotation and genome sequence at <http://webblast.ipk-gatersleben.de/barley/viroblast.php> was searched using the TBLASTN algorithm with Arabidopsis TCP proteins or TCP domains as query sequence. All redundant sequences were discarded from further analysis based on ClusterW (Thompson *et al.*, 1994). Furthermore, to verify the reliability of the initial results, all non-redundant candidate TCP sequences were analyzed to confirm the presence of the conserved TCP domain using the InterProScan program (Quevillon *et al.*, 2005). The sequences of TCP family members in the genome of Arabidopsis were retrieved from the PlantTFDB plant transcription factor database (<http://plantfdb.gao-lab.org/>, v3.0). *Antirrhinum majus* and *Oryza sativa* TCP sequences, were obtained from NCBI.

Phylogenetic analysis

Multiple sequence alignments were conducted on the amino acid sequences of TCP proteins in Arabidopsis and barley genomes using Cluster X (Thompson *et al.*, 1997) with default settings. Subsequently, MEGA 6.0 software (Tamura *et al.*, 2013) was employed to construct an unrooted phylogenetic tree based on alignments using the Neighbor-joining (NJ) method with the following parameters: JTT model, pairwise gap deletion, and 1000 bootstraps.

Extraction and quantification of ABA

ABA was extracted from fresh plant materials using ethyl acetate (100%). Isotopically labeled D6-ABA was used as an internal standard and added to each sample during the extraction procedure. Extraction was carried out twice with 1 ml of ethyl acetate at 4 °C. The supernatant collected after centrifugation (13 000 g, 10 min, and 4 °C) was evaporated to dryness at room temperature using a vacuum concentrator. The dried samples were dissolved in acetonitrile:methanol (1:1) and filtered using a 0.8 µm filter (Vivaclear). The filtrate (10 µl) was used for subsequent quantification by LC-MS/MS (Dionex Summit coupled to Varian 1200 L). Chromatographic separation was carried out on a C18 column (4 µm, 100 mm; GENESIS; Vydac/USA). Multiple reaction monitoring (MRM) and quantification were done using the mass traces 263/153 for ABA and 269/159 for D6-ABA. The validity of the extraction and measurement procedure was verified in recovery experiments (~82–95%). Quantification was based on calibration with known ABA standards and individual recovery rates for the samples, as described in Kong *et al.* (2008).

qRT-PCR and microarray hybridization, and data analyses

RNA was extracted from fresh plant tissues from independent or pooled biological replicates with the same treatment using a plant mini RNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, and its quality and quantity were assessed with a Nano drop device (Peqlab, Erlangen, Germany). A 500 ng aliquot was taken as template for the

synthesis of the first cDNA strand, primed by oligo(dT), using a RevertAid cDNA kit (ThermoFisher Scientific, Waltham, MA, USA). The subsequent quantitative reverse transcription-PCR (qRT-PCR) was based on the Power SYBR® Green PCR Master Mix (ThermoFisher Scientific) and conducted in an Applied Biosystems 7900HT Fast Real-Time PCR system (ThermoFisher Scientific) following the manufacturer's protocol. Evaluation of the data was performed according the MIQE guidelines (Bustin *et al.*, 2009). Relative transcript abundances were obtained using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) and were normalized against the abundance of the serine/threonine phosphatase PP2A transcript which was selected as reference earlier (Seiler *et al.*, 2011; Sreenivasulu *et al.*, 2012). The primer sequences employed are given in Supplementary Table S1. The presence of a unique PCR product was verified by dissociation analysis, and each qRT-PCR was repeated at least three times. Each biological replicate was represented by three technical replicates.

For the microarray procedure, the same RNA samples extracted from three biological replicates were used and the quality of the RNA was verified with a Bioanalyzer 2100 device (Agilent Technologies, Santa Clara, CA, USA). The RNA was labeled by using the Low input QuickAmp Labeling kit (Agilent Technologies) and hybridized, following the manufacturer's protocol, to a custom-synthesized 60 k Barley Microarray (Agilent Technologies) (Koppolu *et al.*, 2013). The resulting data were analyzed using GeneSpring 13.0 GX software (Agilent Technologies). After quantile normalization and baseline transformation to the median of all samples, the probesets (genes) were filtered by coefficient of variation <50%, followed by moderated *t*-test and Bonferroni-Holm multiple testing corrections. Probesets passing the *P*-value cut-off of <0.05 with a fold change of ≥ 2.0 were selected as differentially expressed genes (DEGs). Analyses of functional categories with *INT-C*-dependent up-regulated and down-regulated genes were performed using MapMan. The fold enrichment was calculated as follows: (number in class input_set/number of total input_set)/(number in class reference_set/ number of total reference_set). The *P*-value was determined by a hypergeometric distribution test (R Core Team, 2013). The data were sorted by fold enrichment with a cut-off of *P*<0.05. The specific pathway enrichment analysis by Wilcoxon Rank Sum test was implemented in MapMan. The resulting enrichments for the functional classes (*P*<0.05, determined by a hypergeometric test; R Core Team, 2013) provided a map of gene modules regulated dependent on *INT-C* during SAR. To confirm that the common genes of the two identified groups are not random, the test of statistical significance was applied by a web-based tool at http://nemates.org/MA/progs/overlap_stats.html. The co-regulated genes were retrieved from Genevestigator (Zimmermann *et al.*, 2004), and Gene Ontology analysis was performed in agriGO v2.0 with default parameters (Tian *et al.*, 2017).

Statistical analysis

To test the statistical significance of the data, Student's *t*-test and one-way ANOVA with Tukey test for significance were used. In the figures, asterisks denote significant differences in Student's *t*-tests and different letters denote significant differences in Tukey's test.

Results

INT-C is a member of the barley TCP gene family

INTERMEDIUM-C (INT-C) is the barley orthologue of *TEOSINTE BRANCHED1* (Ramsay *et al.*, 2011), one of the name-giving members of the well-studied *TEOSINTE BRANCHED1*, *CYCLOIDEA*, *PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTOR (TCP)* gene family (Li, 2015). TCP transcription factors are defined by the

TCP domain, which is a 59 amino acid long bHLH structure which provides the ability to bind GC-rich DNA sequence motifs (Martin-Trillo and Cubas, 2010). In barley, *INT-C* is encoded by *HORVU4Hr1G007040* and was identified as the corresponding gene of the *vrs5* locus on chromosome 4 (Ramsay *et al.*, 2011) which is involved in the fertility of lateral spikelets.

To identify the genes closest to *INT-C*, the barley TCP gene family was analyzed using the latest barley annotation and genome sequence at <http://webblast.ipk-gatersleben.de/barley/viroblast.php> and the TBLASTN algorithm with Arabidopsis TCP proteins or TCP domains as query sequence (Fig. 1A; Supplementary Table S2). This family consists of 19 genes which contain a putative TCP-HLH-type domain at the N-terminus (Fig. 1B). The phylogenetic tree which was built based on multiple alignments of the TCP domain in TCP proteins showed that barley TCP proteins could be divided into two groups, as for all species so far (Fig. 1A, B). The class I group was formed by nine predicted proteins related to the PCF rice factors (Kosugi and Ohashi, 1997), while class II was comprised of 10 predicted proteins related to the *Antirrhinum* *CYC* and *CIN* genes and to *OsTB1* (Luo *et al.*, 1996; Doebley *et al.*, 1997; Nath *et al.*, 2003; Takeda *et al.*, 2003). In addition, the class II group could be further divided into two subclades: the *CIN* group formed by seven members and the *CYC/TB1* group formed by three members. *HvTC16* and *HvTC17* from the class II *CYC/TB1* contain an R domain that is also found in *HvTC12* from the class II *CIN* group (Fig. 1C), as previously described in Arabidopsis (Yao *et al.*, 2007). Although in eudicots several *CYC/TB1* sequences are found, and phylogenetic analyses have suggested that duplications within this clade occurred at the base of eudicots, in monocots only one type of *CYC/TB1* has been identified (e.g. *OsTB1*) (Howarth and Donoghue, 2006). Our phylogenetic analysis revealed that, based on the absence of the R domain, none of the newly identified barley TCP genes can be considered as a paralog of *INT-C*.

As described for the model plant Arabidopsis, five of the *CIN* subclade members are post-transcriptionally regulated by *miRNA319* (*AtTCP2*, 3, 4, 10, and 24) (Palatnik *et al.*, 2003, 2007; Ori *et al.*, 2007). In barley, *hvu-miR319a* (UUGGACUGAAGGGAGCUGCC) is encoded by *CL16998_Contig1* (Ozhuner *et al.*, 2013). The closest barley homologs of these Arabidopsis genes are the five genes, *HvTCP3*, *HvTCP6*, *HvTCP9*, *HvTCP12*, and *HvTCP15*. These barley *CIN* subclade members contain sequences with putative binding sites for *hvu-miR319* (Supplementary Table S2). Figure 1D shows the alignment of the target sites of these genes with the *miR319* sequence. This suggests that regulation of leaf development by a redundant set of miRNA-regulated homologous TCP genes could occur in barley, while *INT-C* does not represent a *miR319* target.

INT-C loss of function leads to an early promotion of tiller bud outgrowth in barley

Previous studies noticed that *int-c* mutants in various cultivars produce more tillers than the respective wild-type plants during early vegetative stages (Ramsay *et al.*, 2011; Liller *et al.*, 2015). However, this increased tiller formation did not translate into more productive tillers. In contrast, the number of tillers was significantly lower at the point of harvest. The growth experiment described here performed with the near isogenic mutant BW421 (*int-c.5*) and the corresponding wild type confirmed these earlier observations. An increase in tiller number in *int-c* was only detectable at the early developmental stages (Fig. 2A) between 2 and 5 weeks after germination. At later developmental stages (6–8 weeks after germination), the pattern of tiller number was reversed. This observation correlated with an earlier anthesis of *int-c* compared with the wild type cv. Bowman (Supplementary Fig. S1), leading to an earlier arrest of tiller bud production in *int-c*. The tillers of barley are formed in a sequential order, starting with the first tiller bud under the coleoptile. The development of the tiller buds in the axils of successive leaves was studied by dissecting the plants at different developmental stages (Fig. 2B). To investigate the involvement of *INT-C* in bud initiation and bud outgrowth, the primary tiller buds were classified (Fig. 2C, D) as dormant bud (800–1200 μm), outgrowing bud (1.5–100 mm), or tiller emergence (10–35 cm) in each leaf axil at an early developmental stage (2–3 weeks after germination). Figure 2D indicates the enhanced bud outgrowth in *int-c* mutants compared with the wild type. This result suggests that the outgrowth of tiller buds is accelerated in *int-c* mutants at early developmental stages but is slowed down at later stages (>5 weeks).

INT-C mRNA levels were analyzed by real-time qRT-PCR in different tissues and during spike development (Fig. 2E, F). *INT-C* mRNA was detectable at the highest levels in tiller buds, supporting its role in the control of tiller bud development. It was expressed at lower levels in other tissues such as root, stem, and leaf. During spike meristem development, mRNA levels of *INT-C* peaked at the glume primordium stage. In the later stages (stamen primordium and awn primordium), relatively high levels of *INT-C* mRNA persisted. This peak of expression correlated with the observation that after the awn primordium stage, profound differences in the development of the lateral spikelet in *int-c* occurred compared with the wild type (Ramsay *et al.*, 2011).

INT-C mRNA abundance decreased after decapitation

Apical dominance is the inhibitory control exerted by the shoot apex over the outgrowth of the lateral buds (Cline, 1997). Decapitation stimulates bud reactivation after breaking the apical dominance (Hall and Hillman, 1975; Napoli *et al.*, 1999; Cline, 2000; Tatematsu *et al.*, 2005; Aguilar-Martinez *et al.*, 2007). To analyze the involvement of *INT-C* in integrating the

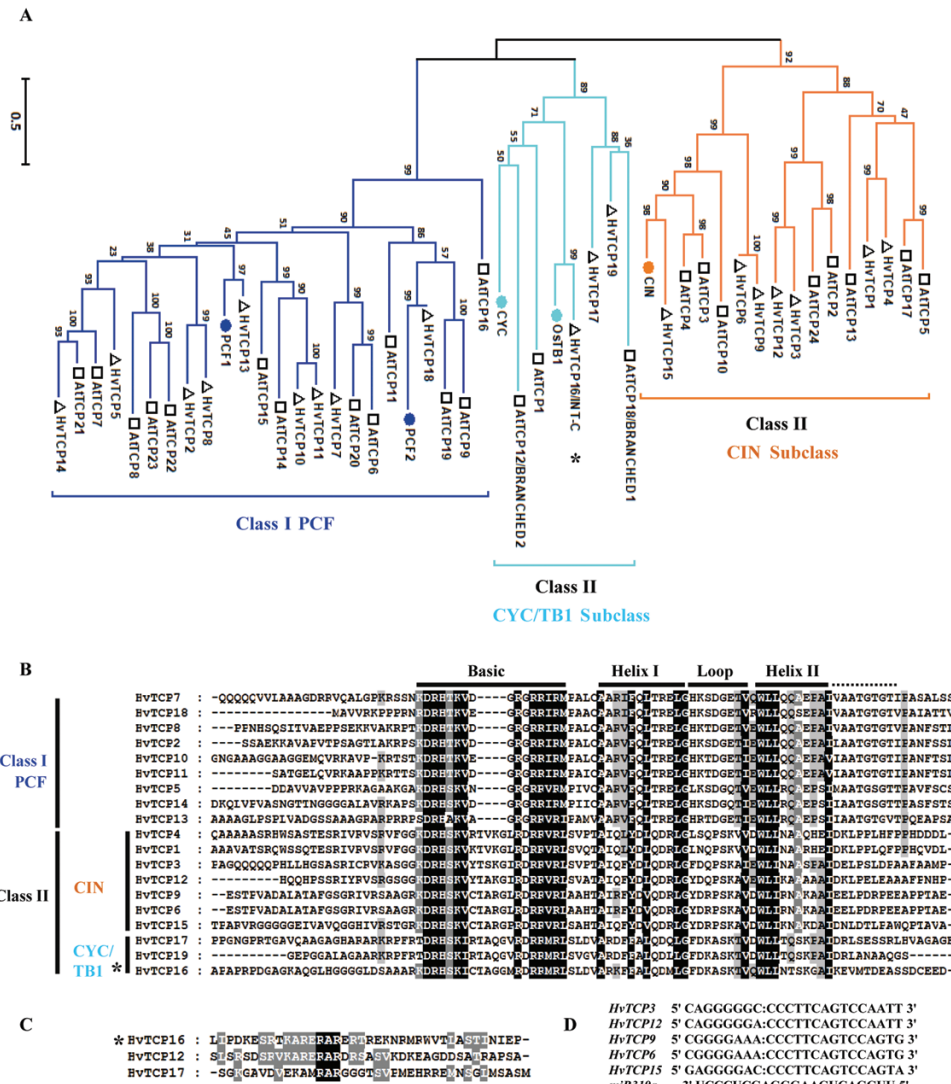


Fig. 1. Phylogenetic tree and alignments of Arabidopsis and barley TCP proteins. (A) The phylogenetic tree was built based on multiple alignment of the TCP domain in the TCP proteins using the Neighbor-Joining method with 1000 bootstrap replicates. Blue, light blue, and orange lines indicate the PCF, CYC/TB1, and CIN clades, respectively. Each Arabidopsis protein is indicated by a square, each barley protein is indicated by a triangle. (B) Alignment of the TCP domain and adjoining sequence for the predicted barley TCP proteins. Overall conserved amino acids are shaded in black. Amino acids 80% or 100% conserved in Class II or Class I are shaded in light gray and dark gray, respectively. The basic, helix I, loop, and helix II regions are indicated. (C) Alignment of the R-domain of Class II subfamily members. Amino acids are expressed in the standard single letter code. Sequences were aligned with ClustalW and represented with Genedoc. (D) Alignment of putative target areas for *miR319a* (aligned in reverse). Asterisks indicate *INT-C* (*HvTCP16*).

decapitation response into bud outgrowth, barley *int-c* mutant plants were compared with the respective wild-type plants. For this approach, 3-week-old plants that had undergone early stem elongation were decapitated. Two weeks later, two tiller buds of decapitated Bowman plants had elongated prematurely. Thus, total tiller number in decapitated wild-type plants reached the same level as in *int-c* mutants, in which decapitation had no effect (Fig. 3A). To investigate whether this response was related to a transcriptional down-regulation of *INT-C*, mRNA levels in axillary buds were analyzed by real-time qRT-PCR after decapitation, before any visible sign of bud outgrowth (Fig. 3B). *INT-C* mRNA decreased significantly 6 h after

decapitation. *DRM1/ARP* (*DORMANCY-ASSOCIATED GENE/AUXIN-REPPRESSED PROTEIN*) mRNA, an early marker for bud dormancy (Stafstrom *et al.*, 1998; Tatematsu *et al.*, 2005), also showed reduction and reached its minimum 24 h after decapitation. These results support the idea that *INT-C* is involved in the early response to bud release from apical dominance and required for bud activation.

Transcriptome analysis of buds after decapitation

To investigate the transcriptome response of dormant versus activated buds, Agilent 8 × 60K customized barley microarray

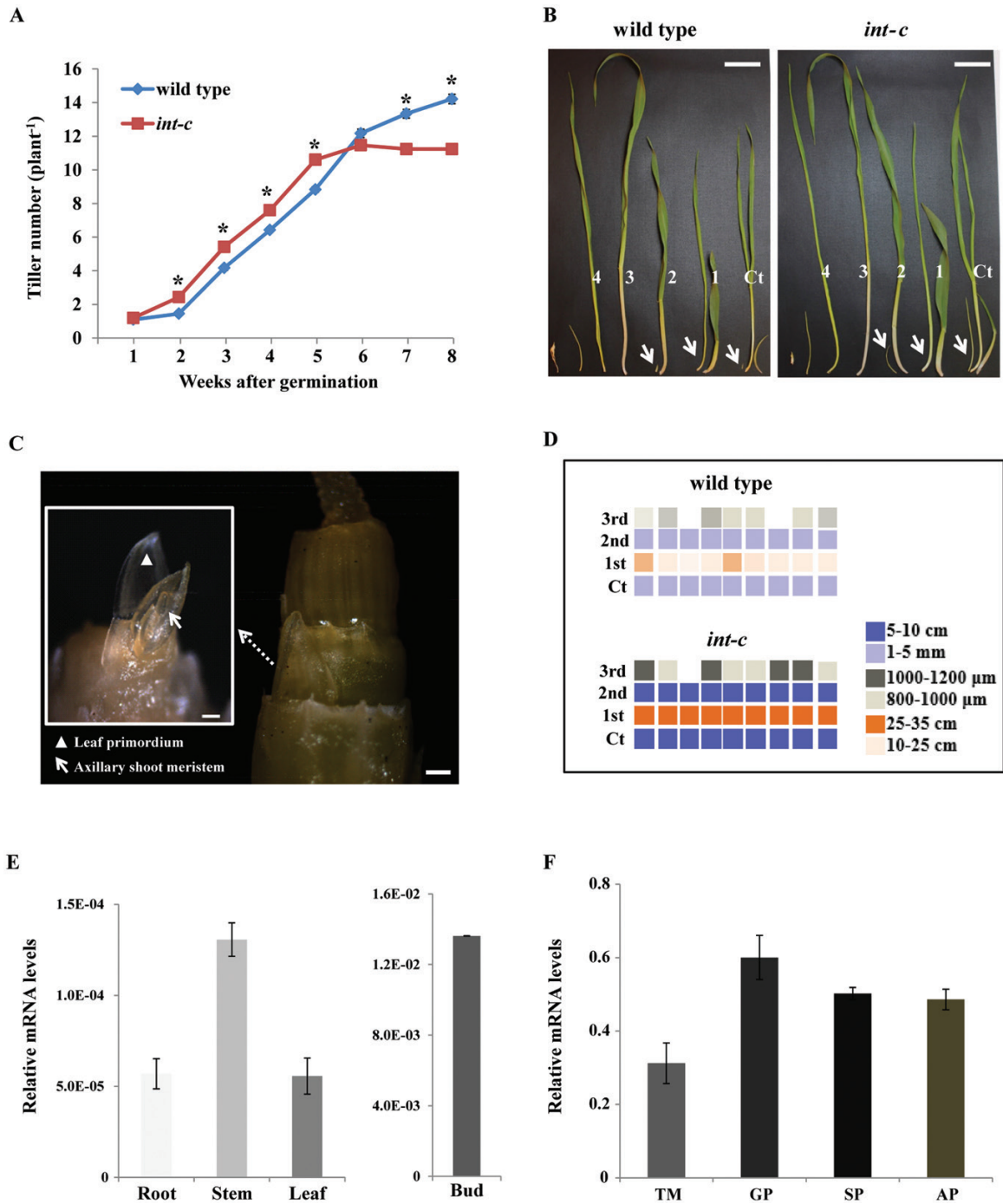


Fig. 2. *INT-C* is involved in barley plant architecture by tiller bud outgrowth. Analysis of tiller development in wild-type and *int-c* plants. (A) Tiller number of *int-c* and wild-type plants 1–8 weeks after germination ($n=25-30$ plants). Asterisks indicate significant differences (Student's *t*-test, $P<0.001$) between wild-type and *int-c* mutant plants. (B) Dissected tillers from successive leaf axils in ~2- to 3-week-old seedlings. Ct, coleoptile tiller; 1–4, order of leaves; scale bars=5 cm; arrows, tiller buds. (C) Exemplary tiller bud formation stage in the third leaf axil. The area of the close-up view is outlined with a white box in the left image. Dissection of a tiller bud at this stage will reveal a shoot apex with leaf primordia and a meristematic dome. Scale bars represent 200 μm. (D) Schematic representations of tiller bud production in each leaf axil of the wild type and *int-c* in 2- to 3-week-old seedlings. Each column stands for a single plant, and each row stands for a leaf axil in order from bottom to top, starting with the coleoptile tiller. Different colored squares denote different tiller bud lengths. (E) *INT-C* (HvTCP16) mRNA levels in different tissues and (F) during spike development as analyzed by real-time qRT-PCR. Bars represent means \pm SD; $n=3$ biological replicates. Serine/threonine protein phosphatase HvPP2A-4 mRNA was used as a reference. TM, triple mound; GP, glume primordium; SP, stamen primordium; AP, awn primordium.

expression analysis (Thirulogachandar *et al.*, 2017) using total mRNA prepared from tiller buds at the early stem elongation stage was performed. The time point 24 h after decapitation was chosen for the following reasons: (i) down-regulation of *DRM1* expression was evident at this time point (Fig. 3B); (ii) this time point was 1 d before the first visible effects of decapitation on the growth were detectable—2 d later, the vegetative buds of decapitated plants had activated and begun to elongate; and (iii) the time point enabled us to exclude genes affected by circadian rhythm.

A total of 1704 DEGs were detected in tiller buds of decapitated versus non-decapitated plants (Supplementary Table S3), among those 1011 were down-regulated and 693 up-regulated. Microarray results were confirmed by qRT-PCR for selected genes (Supplementary Fig. S2). Besides *DRM1*, which was found among the down-regulated genes, several genes encoding transcription regulators were detected. A total of 81 out of 494 DEGs regulated in the opposite direction under shading conditions (see below) could be mapped to the term ‘transcription regulator associated with ABA’. Among them are the auxin-binding protein ABP44 and a putative ripening-related bZIP protein (CAB85632). The up-regulated genes included a large number of ribosomal proteins, cell organization, and cell cycle-related genes.

Plant density affects *INT-C* expression

Planting density affects shoot branching in many plant species. Low plant density results in more branches, compared with growth in dense plant stands as a result of a neighbor-sensing response (Casal *et al.*, 1986). To test the impact of plant density on *INT-C*, wild-type and *int-c* plants were grown in three

different densities under greenhouse conditions (one, five, and 10 plants per pot). Tiller numbers were counted until the early stem elongation stage (Fig. 4).

Wild-type plants responded to increased planting density with reduced tillering. At a density of 10 plants per pot, tiller bud suppression was more than half, as tiller number was 56% lower than in plants grown at one plant per pot. However, *int-c* mutants showed reduced sensitivity to this condition (33% reduction compared with plants at one plant per pot). The mRNA levels of *INT-C* were then analyzed by qRT-PCR in wild-type plants grown at low (one plant per pot) and high (10 plants per pot) density. At high density, *INT-C* mRNA levels showed a 2-fold increase compared with those at low density (Fig. 4B). These results support the involvement of transcriptional regulation of *INT-C* in bud dormancy.

INT-C is up-regulated as part of the shade avoidance response

To further examine whether *INT-C* is participating in the SAR, plants were grown under two different light conditions: control light (PAR=840 $\mu\text{mol m}^{-2} \text{s}^{-1}$; R:FR ratio=2.2) and low R:FR light mimicking shade imposition by neighboring plants (green shade, PAR=260 $\mu\text{mol m}^{-2} \text{s}^{-1}$; R:FR ratio=0.2) (Kegge *et al.*, 2013). To minimize a putative bias resulting from variations in leaf number and *int-c*-mediated earlier flowering, plants were grown under conditions attenuating the *int-c* early flowering phenotype (Fig. 5A).

The shading treatment was started at the two-leaf stage. Four weeks later, primary tiller number was quantified. Bowman plants grown under shade conditions responded strongly and had four times fewer tillers than plants grown in control

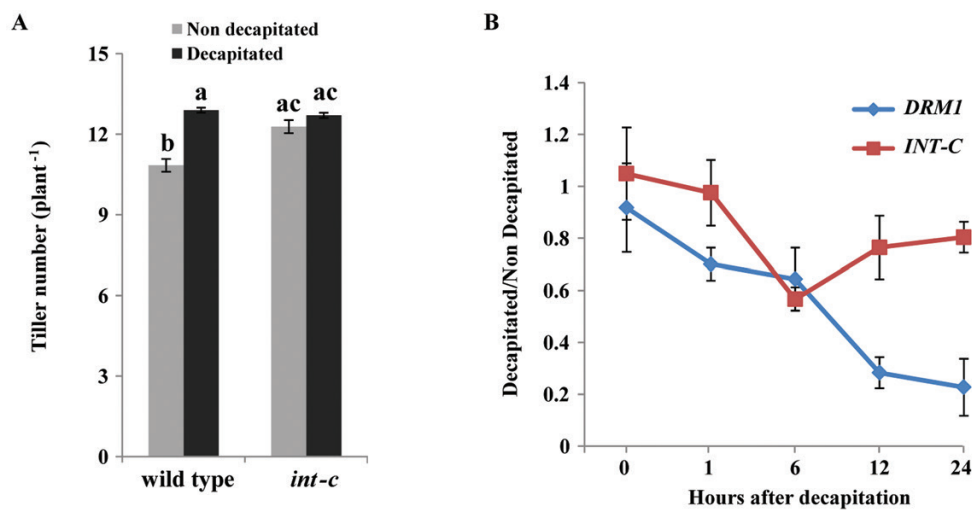


Fig. 3. *INT-C* expression in response to decapitation. (A) Tiller number of cv. Bowman (wild-type) and *int-c* plants 2 weeks after decapitation. Bars represent means \pm SD; $n=3$ replicates with ≥ 16 plants. Different letters indicate significant differences according to Tukey's test ($P < 0.05$). (B) Ratio of mRNA levels of *INT-C* and *DRM1* in tiller buds between decapitated and non-decapitated plants. Relative mRNA abundance of *INT-C* mRNA was analyzed by real-time qRT-PCR. Bars represent means \pm SD; $n=4$ biological replicates. *Serine/threonine protein phosphatase HvPP2A-4* was used as a reference gene. Analyzed is the early transcriptional response within 24 h after decapitation.

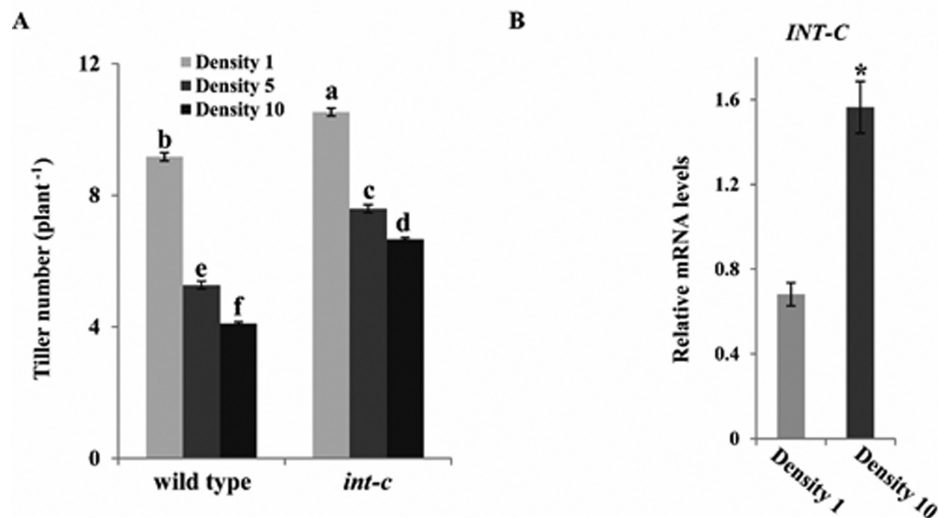


Fig. 4. *INT-C* expression responds to planting density. (A) Tiller number of wild-type and *int-c* plants grown at planting densities of one, five, or 10 plants per pot. Plants were analyzed 5 weeks after sowing. Bars represent means \pm SD; $n=3$ replicates with ≥ 20 plants. Different letters indicate significant differences according to Tukey's test ($P < 0.01$). (B) Transcript levels of *INT-C* in the tiller bud tissue analyzed by real-time PCR at a density of one or 10 plants per pot. Bars represent means \pm SD; $n=3$ biological replicates. *Serine/threonine protein phosphatase HvPP2A-4* was used as a reference gene. The asterisk indicates a significant difference according to Student's *t*-test at $*P < 0.001$.

conditions (Fig. 5B), indicating that exposure of plants with young vegetative buds to a low R:FR ratio promotes bud arrest in barley. In contrast, the response of *int-c* mutants to shading was much weaker. *int-c* plants grown under shade had only 1.4 times fewer tillers than plants grown under normal light conditions (Fig. 5A, B). Other phenotypic SARs, such as hyponasty and stem elongation, were indistinguishable between the wild-type and mutant plants (Fig. 5A). The short-term response of *INT-C* to shade treatment was analyzed by transferring plants at the five-leaf stage (when plants had small vegetative buds) to low R:FR light. The mRNA levels of *INT-C* in tiller buds increased after 4, 6, or 8 h exposure to shade, indicating a transcriptional activation of *INT-C* in response to shade (Fig. 5C). This result is in agreement with the increase of tillers in plants grown in dense stands. The higher tiller number in *int-c* coincided with a lower concentration of ABA in tiller buds (Fig. 5B, D), supporting the role of ABA as a mediator of *INT-C*-dependent tiller bud suppression in the shade.

Transcriptome analysis of tiller buds after shading identifies *INT-C*-dependent genes

To obtain further insight into the molecular mechanisms of *INT-C*-mediated growth responses, the transcriptome of tiller buds in wild-type and *int-c* plants was analyzed. The plants were exposed to control conditions or shade (Fig. 5) and the transcriptome was analyzed 6 h after exposure to shade using an Agilent $8 \times 60K$ customized barley microarray. As *INT-C* expression was highest in tiller buds (Fig. 2), we selected this tissue for detailed analysis of RNA expression levels.

While under control conditions a minor influence of *int-c*-dependent transcript changes was found, 305 DEGs were identified between the wild type and *int-c*, out of which 185 were up-regulated and 120 were down-regulated. Under shading, a stronger effect of *int-c* on the transcriptome was noted. In wild-type buds, 2726 shade-responsive DEGs (1803 DEG up-regulated and 923 DEGs down-regulated) were detected. In the *int-c* mutant, a total of 906 DEGs were identified in response to shade, with 226 up-regulated genes and 680 down-regulated genes (Fig. 6; Supplementary Table S3). DEGs detected by microarray were validated and confirmed for five up-regulated and five down-regulated genes by qRT-PCR (Supplementary Fig. S3). The number of DEGs in response to shading decreased by $\sim 67\%$ in *int-c* (Fig. 6; Supplementary Table S3). This drop supports the hypothesis of the involvement of *INT-C* in the shade response. The overlapping DEGs detectable in the wild type and *int-c* are related to a common SAR independent of *INT-C*, while DEGs detectable in the wild type could directly or indirectly depend on *INT-C* function. These 2154 DEGs were termed *INT-C*-dependent genes of the shade response (Fig. 6; Supplementary Table S3).

In Bowman buds, the up-regulation of *INT-C* after shading [2.56-fold increase at a false discovery rate (FDR) of $4.78E-04$] could be confirmed. In the BW421 (*int-c.5*) deletion mutant, a similar induction of *INT-C* (2.10-fold increase, FDR=0.0086) was detectable (Supplementary Table S3). However, in the *int-c.5* mutant, the deletion in the *INT-C* gene leads to a frameshift in the C-terminus downstream of the R-domain. This mutation results in a non-functional gene product. The inspection of the respective marker genes *HAT4/ATHB2*, *PIF3*,

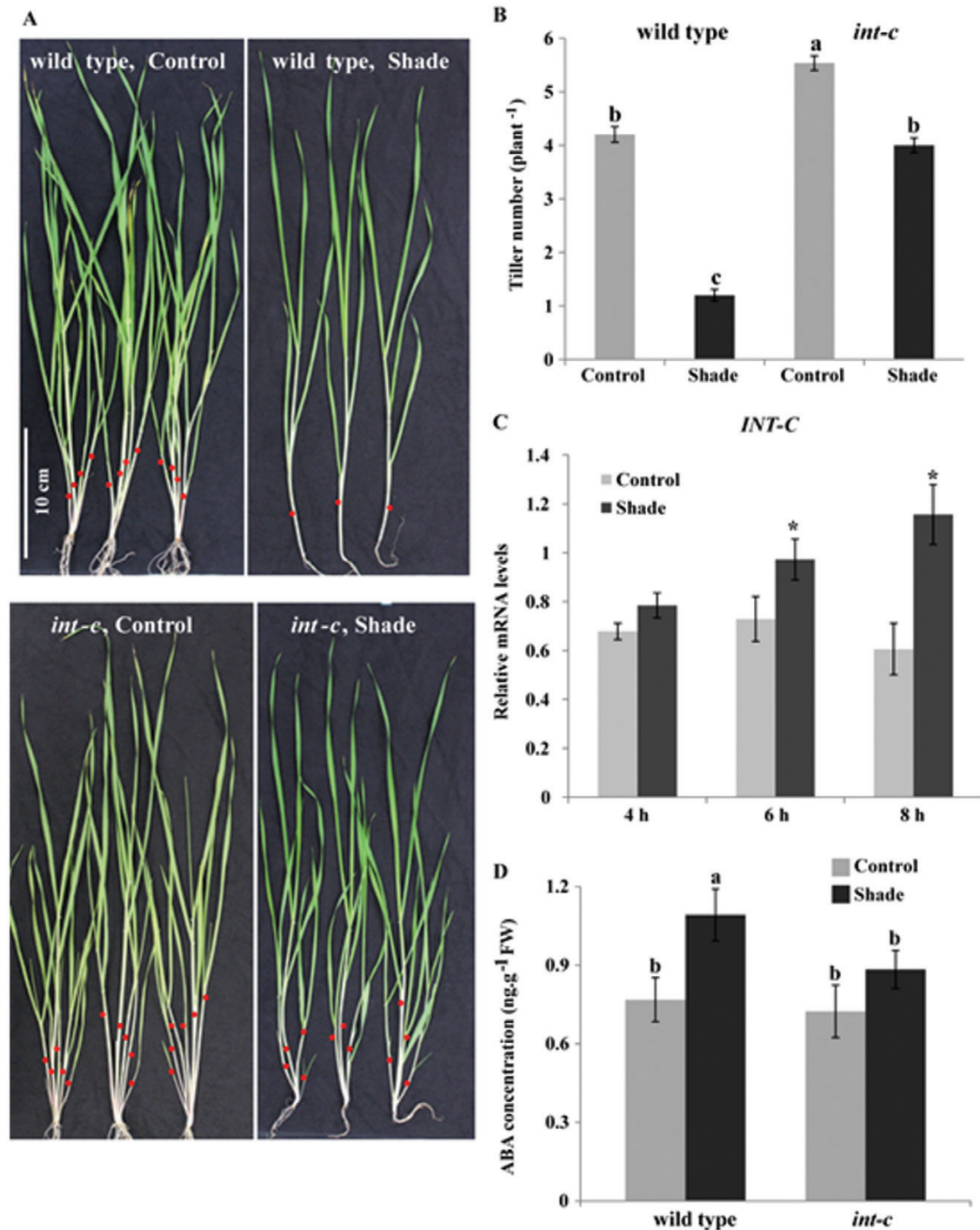


Fig. 5. Effect of shading on tiller bud outgrowth and *INT-C* expression. (A) Tillering phenotype of wild-type and *int-c* plants grown under control conditions or shading. Red dots indicate the primary tillers. (B) Tiller number of wild-type and *int-c* plants grown under control or shade conditions. Bars represent means \pm SD; three independent experiments with $n \geq 35$ plants each. Different letters indicate significant differences according to Tukey's test ($P < 0.01$). (C) Transcript levels of *INT-C* analyzed by qPCR, in buds of shaded plants, relative to levels in control plants. Bars represent means \pm SD; $n = 3$ biological replicates. *Serine/threonine protein phosphatase PP2A-4* was used as a reference gene. Asterisks indicate significant differences according to Student's *t*-test at $*P < 0.05$. (D) ABA concentrations in tiller buds of wild-type and *int-c* plants 6 h after exposure to shade. Bars represent means \pm SD of six independent biological replicates. Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

and *PIF4* (Leivar and Monte, 2014) by their transcriptional activation validated the applied experimental shade conditions (Supplementary Table S3). Among the significantly up-regulated genes after shade in the wild type, transcript levels of the marker gene *DRM1* that is associated with tiller bud dormancy (Stafstrom et al., 1998; Tatematsu et al., 2005) were found to be >2-fold higher (Supplementary Table S3).

As shown in Fig. 5A, the shade response led to a transcriptional activation of *INT-C* and a repression of tiller outgrowth. In contrast, the decapitation of barley plants resulted in a temporal reduced expression of *INT-C* and the opposite phenotype. As *INT-C* expression was reduced under this simulated condition, the generated dataset was used to validate the list of *INT-C*-dependent shade response genes (Supplementary Table S3).

The Venn diagram (Fig. 6) illustrates 753 overlapping DEGs after decapitation and after shading (27% of 2726 DEGs after shading and 44% of 1704 DEGs after decapitation). A total of 495 (29%) of the decapitation-induced DEGs were also detectable in response to shading. Almost all of these DEGs were oppositely regulated after decapitation and shading, respectively (Supplementary Table S4). This list was defined as *INT-C*-dependent genes.

MapMan software was used to define gene functional categories (Thimm et al., 2004; Usadel et al., 2005). For the *INT-C*-dependent genes, we identified hormone- and stress-related genes among the up-regulated genes, and cell division- and protein synthesis-related genes among the down-regulated genes as the most prominent functional categories.

As the majority of *INT-C*-dependent genes showed opposite responses after decapitation and under shade, these gene sets infer the causal mechanisms associated with bud activation and bud arrest, respectively. A total of 123 genes (25%) found to be up-regulated after decapitation were down-regulated under shade, while 372 (75%) genes which were down-regulated

after decapitation were up-regulated under shade (Fig. 6; Supplementary Table S4). During decapitation-induced bud activation, *INT-C* was rapidly down-regulated (Fig. 3B). Further, a strong overlap of DEGs responding to decapitation and shading was observed. Theoretically, the 372 down-regulated (after decapitation) genes might be directly involved in the promotion of axillary bud arrest. This group included a number of genes related to ethylene, auxin, and gibberellin signaling (*AP2/ERBP*, *ACC*, *ERF1*, *IAA17*, and *GID1L2*), as well as protein degradation (*SKIP1*, *SKIP5*, *SKP2A*, *UBQ3*, and *UBQ4*). Moreover, genes related to sugar metabolism and transport were also identified within that group (*TPS6*, sucrose transporter). Trehalose-6-phosphate is known to be involved in sugar signaling (Figuera and Lunn, 2016). On the other hand, the 123 up-regulated genes (after decapitation) could be involved in promoting axillary bud growth. This group included many genes associated with chloroplast function and chlorophyll synthesis (chlorophyll-binding protein, ATP synthase), protein synthesis (EF-Ts, ribosomal protein), and chromatin structure (*HISTONE H3.2*, *HISTONE 2B.3*). This set of genes was co-regulated with a subset of genes related to functional categories involving the thylakoid and photosynthesis (Supplementary Fig. S4).

Discussion

The present study shows that *INT-C* is transcriptionally regulated by apical dominance and by light perception, and that *INT-C* expression in tiller buds regulates tillering in response to these signals. This highlights the role of *INT-C* as a major transcriptional regulator integrating endogenous with environmental signals to determine the outgrowth of tiller buds. Based on the microarray hybridization experiment, *INT-C*-dependent genes are defined.

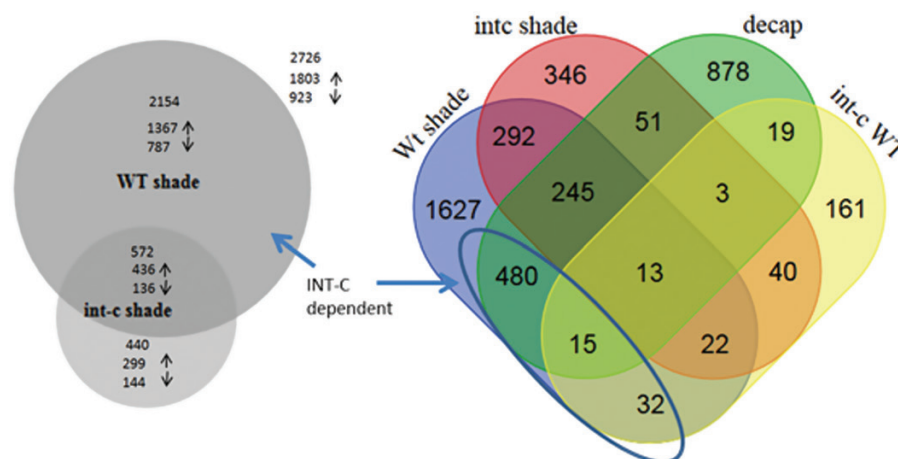


Fig. 6. Venn diagram of differentially expressed genes (DEGs) detected after shade treatment in cv. Bowman wild type (Wt shade) and *int-c* (*int-c* shade). These blocks were compared with DEGs detectable after decapitation (decap) in the *int-c* mutant and wild type (*int-c* versus WT). Numbers indicate transcript fold changes ≥ 2 at an FDR of $P < 0.05$. (shade, *INT-C* mRNA induced; *int-c*, no functional *INT-C*; decap, *INT-C* mRNA reduced).

INT-C integrates environmental signals to regulate tiller bud outgrowth

In crop plants, tillering is an important agronomic trait for yield formation. However, in barley, little is known about the genetic mechanisms acting inside tiller buds to cause growth arrest. Although a quantitative trait locus (QTL) study revealed several genes associated with tiller number (Haaning *et al.*, 2020), the fact that some tillering genes do not overlap with identified QTLs point toward the complex involvement of environmental influence. Here, we show that the bHLH transcription factor gene *INT-C* is involved in the integration of different branching signals mediating a suppressive effect on bud outgrowth (Fig. 3). *INT-C* itself is regulated on the transcriptional level as a response of the investigated environmental conditions. This modulation of *INT-C* transcription appears to be under tight regulation of environmental and developmental stimuli that are correlated with bud outgrowth and activation of tillering. *INT-C* up-regulation was observed after shading or under high planting densities (Figs 3, 4), linked to reduced tiller numbers; in contrast, suppression of *INT-C* expression (i.e. after decapitation) triggered bud outgrowth (Fig. 5). This emphasizes a role for *INT-C* as a regulator integrating signals within the axillary bud to determine tiller number. This finding agrees with the results in rice where TB1 was also reported to mediate a negative function in tillering (Takeda *et al.*, 2003; Choi *et al.*, 2012). A comparable approach using transgenic overexpression and antisense-mediated repression of TB1 resulted in the modulation of tiller and panicle development in rice. In rice, the duplication of Tb1 and neofunctionalization of Tb2 resulted in a novel regulatory hub for tiller development (Lyu *et al.*, 2020). Such a hub was not detectable in barley.

The influence and integration of environmental stimuli in rice were only addressed in response to greenhouse and paddy field conditions. In maize, the involvement of the SPL gene family as direct regulators of TB1 in plant architectural traits was described, and optimized plants were engineered for high-density planting (Wei *et al.*, 2018). Whether directed modification of *INT-C* can help to improve the yield of barley plants is very difficult to predict. As the association of tiller formation and yield or fertile tillers is also dependent on the developmental stage and environmental condition, only a modulation of *INT-C* function might be helpful. As *INT-C* was not detected among the genes in tiller QTLs (Haaning *et al.*, 2020), an easy association of the presence or absence of the gene with yield or fertile tillers is not possible.

As the shade-induced reduction of tiller number was observed in both wild-type and mutant plants (Fig. 5), it can be concluded that the underlying regulation is not solely mediated by *INT-C*. Also, other factors might contribute to the integration of the environmental signals.

Definition of INT-C-dependent genes

The analysis of the shading response at the transcriptome level led to the identification of important DEGs that were directly or indirectly dependent on *INT-C*. Our data suggest that the number of *INT-C*-dependent genes (372 up-regulated and 123 down-regulated) was closely associated with tiller bud activation, confirmed through *int-c* mutant study and *INT-C* function inferred from the stimulus experiments (either shade or decapitation). These target genes are promising candidates to play an important role in tiller bud transition between repression and promotion of growth. The MAPMAN-based categorization of DEGs resulted in a significant over-representation of the term thylakoid and genes associated with photosynthesis. In addition to this, several target genes of sink/source properties of a tissue are found (e.g. TPS6, sucrose transporters). This supports the idea of bud activation, accompanied by generation of a new initial sink tissue. Also, photosynthesis will be activated in this new tiller. The scheme in Fig. 7 shows the integration of the respective categories and important genes involved in *INT-C*-mediated bud growth.

ABA signaling and the promotion of bud dormancy

ABA has been related to the maintenance and promotion of bud dormancy in many plant species: elevated ABA levels in buds are associated with the inhibition of branching during plant development (Tamas *et al.*, 1979; Knox and Wareing, 1984; Gocal *et al.*, 1991; Mader *et al.*, 2003; Destefano-Beltran *et al.*, 2006; Ruttink *et al.*, 2007), as well as in the context of responses to low R:FR ratios (Tucker and Mansfield, 1971; Reddy *et al.*, 2013; González-Grandio *et al.*, 2017). Moreover, a correlation has been found between the up-regulation of ABA response genes in axillary buds and bud dormancy (Ruttink *et al.*, 2007; González-Grandio *et al.*, 2013; Kebrom and Mullet, 2016). So far, ABA measurements have not been reported in tiller buds, and neither have ABA-inducible genes been studied in the tiller bud development of barley (Hussien *et al.*, 2014). Nonetheless, it has been presumed that in barley a correlation exists between ABA signaling and bud arrest. Although the precise role of ABA in the promotion of bud dormancy in Arabidopsis is still not clear, a strong overlap of common regulated genes can be found (González-Grandio *et al.*, 2013). Recently, Luo *et al.* (2019) have shown that application of ABA to hydroponic cultures of rice SL mutants and wild-type plants suppressed axillary bud outgrowth. SL and ABA biosynthesis share the same precursor and are closely related in regulating the tiller number in barley (Wang *et al.*, 2018). In the present study, we demonstrate that *INT-C* as a regulatory hub controls various ABA hormone pathway genes as well decapitation and shade responses. The large number of transcription factors found in the list of defined *INT-C*-dependent genes also points toward a function of *INT-C* as a regulatory hub controlling a complex downstream network. Our transcript

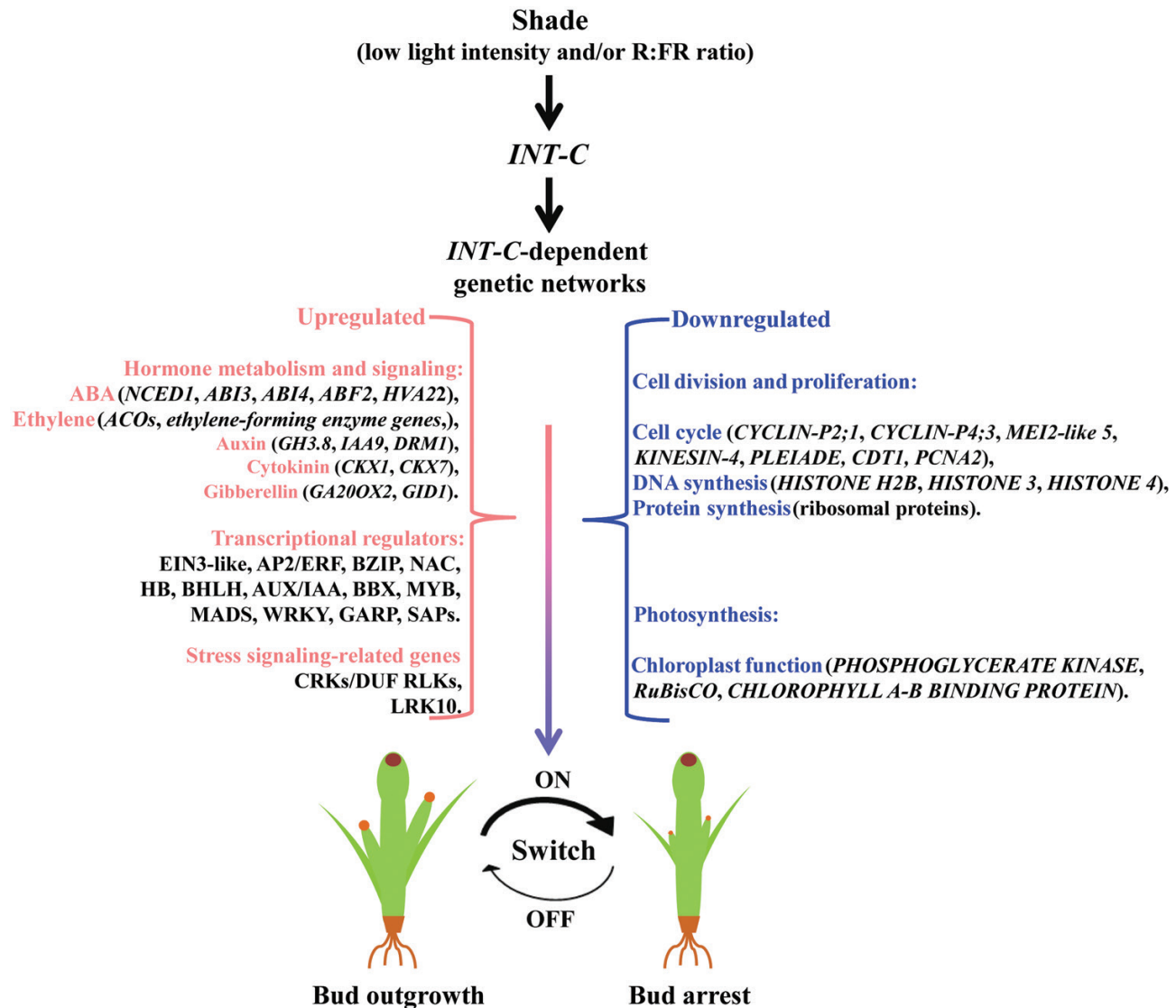


Fig. 7. Working model for the dynamic balance of INT-C-dependent transcriptional programming to regulate tiller bud outgrowth in barley. Shade perception occurring in tiller buds activates the ‘master-switch’ transcription factor INT-C, thus altering INT-C-dependent target genes; among them is a set of up-regulated hormone and stress response genes and down-regulated cell division- and ribosome-related genes. The output of this transcriptional regulation mediates bud transition from outgrowth to arrest.

data provide evidence that wild-type tiller buds display a strong increase in the global response of ABA-related genes, while *int-c* buds, which exhibit less dormancy and continue to grow, show reduced ABA-related responses. Consistently, the number of differentially expressed ABA-related genes in the tiller buds was higher after shading. These findings are subsumed in the model (Fig. 7), building the hypothesis that *INT-C* employs the ABA signaling pathway in conjunction with other hormones to mediate bud growth arrest.

Supplementary data

The following supplementary data are available at [JXB online](#).

Fig. S1. Flowering in wild-type and *int-c* mutant plants at 15 weeks after germination.

Fig. S2. Validation of microarray data by qRT-PCR. mRNA levels of a subset of genes identified as responding to the decapitation treatment, and Pearson’s correlations between gene expression levels determined by qRT-PCR and microarray expression profiling for the same genes.

Fig. S3. Validation of microarray data by qRT-PCR. mRNA levels of a subset of genes identified as responding to the shade treatment in both wild-type and *int-c* samples, and Pearson’s correlations between gene expression levels determined by qRT-PCR and microarray expression profiling for the same genes.

Fig. S4. MAPMAN analysis of DEGs for regulation overview, biotic stress, chloroplast, and metabolism overview.

Table S1. List of the primer sequences used in this study.

Table S2. List of TCP genes and associated gene expression derived from public databases.

Table S3. List of differentially expressed genes (DEGs) for the wild-type versus *int-c* in control, shading, and decapitation condition.

Table S4. List of INT-C-dependent genes, including comparison of direction after shading and decapitation.

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Author contributions

HW: planning and design; HW, CS, and KE: performing experiments and data analysis; HW, MK, and NvW: conceptualization; HW and MK: writing.

Conflict of interest

The authors declare no competing interests.

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Data availability

The datasets generated during and/or analysed during the current study are available in the eDAL repository: <https://doi.ipk-gatersleben.de/DOI/73ddd8d1-046c-4934-80fa-189a9cf0bdc8/dc282984-1535-465a-a7e0-9b61fa831fcf/2/1847940088>

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